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Precision Medicine in Oncology / Re, Angela; Nardella, C; Quattrone, A; Lunardi, A. - ELETTRONICO. - (2019), pp. 1-163. [10.3389/978-2-88945-705-2]

Availability: This version is available at: 11583/2970517 since: 2023-05-30T14:46:59Z

Publisher: Frontiers media

Published DOI:10.3389/978-2-88945-705-2

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PRECISION MEDICINE IN ONCOLOGY

EDITED BY: Angela Re, Caterina Nardella, Alessandro Quattrone and Andrea Lunardi PUBLISHED IN: Frontiers in Cell and Developmental Biology and Frontiers in Oncology







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ISBN 978-2-88945-705-2 DOI 10.3389/978-2-88945-705-2

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PRECISION MEDICINE IN ONCOLOGY

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Citation: Re, A., Nardella, C., Quattrone, A., Lunardi, A., eds. (2019). Precision Medicine in Oncology. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-705-2

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Editorial: Precision Medicine in Oncology

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Keywords: precision medicine, oncology, pathology, *in vivo* preclinical platform, computational biology, noncoding RNA

Editorial on the Research Topic

Precision Medicine in Oncology

Recent advances in technology have unveiled a tremendous heterogeneity in cancer dysfunctional mechanisms. This gain of knowledge has opened a new era in oncology, which relays on the concept that each tumor is different and should be treated in a specific way depending on its distinctive molecular dysfunctions. Fundamental achievements in cancer biology paralleled by unprecedented improvements in disease modeling from all *in silico, in vitro* and *in vivo* perspectives, have converged to offer nowadays the compelling opportunity to design therapeutic approaches tailored on individual patients, namely precision medicine.

This Research Topic embodies 13 multidisciplinary manuscripts focused on multifaceted aspects related to "Precision Medicine in Oncology." Overall, each investigator discusses some of the numerous pending issues associated with this ground-breaking field, ranging from basic research findings, novel technologies, and computational approaches to potential innovative translational venues and widely needed new platforms for precision medicine implementation. Specifically, this issue includes: (i) original research reports on novel biological findings and an innovative technology for immunotherapy; (ii) comprehensive reviews on key cancer biomarkers, signaling, and metabolic pathways as well as on theoretical and preclinical models, and analytical integrative methodologies; (iii) insightful perspectives on advanced computational platforms as well as on a novel integrated murine/human clinical infrastructure.

A key aspect for accelerating the development of new effective targeted therapies is represented by a deeper, faster and broader genomic characterization of patient samples. The National Cancer Institute is currently leading numerous multi-disciplinary projects aiming at facilitating the development of precision oncology diagnostics and therapeutic treatments. In a timely review hosted in this Research Topic, Hinkson et al. introduce the Genomic Data Commons (GDC) initiative, which redistributes high quality data and metadata and three Cloud Resources, thus supporting cloud-based access to data, computational scalability and collaboration. Additionally, the review from Davis' group provides an insightful overview on catalogs, software and tools useful for the interpretation of single nucleotide variants and short insertions and deletions in point-of-care high throughput sequencing applications (Tsang et al).

Deep genome and transcriptome sequencing are having two major roles in: (i) facilitating the discovery of new pathways and molecular players involved in cancer onset, progression and drug resistance, thereby offering the opportunity to identify more reliable biomarkers and novel druggable targets; (ii) revolutionizing the clinical approach to human diseases as a result of the unprecedented characterization of the non-coding space of our genome.

OPEN ACCESS

Edited and reviewed by: Paolo Pinton, University of Ferrara, Italy

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Specialty section:

This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: 21 September 2018 Accepted: 08 October 2018 Published: 26 October 2018

Citation:

Re A, Nardella C, Quattrone A and Lunardi A (2018) Editorial: Precision Medicine in Oncology. Front. Oncol. 8:479. doi: 10.3389/fonc.2018.00479

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While the non-coding dark matter still remains a challenging target *in vivo*, pharmacological tuning of the coding space has been shown to yield promising results *in vitro* and, to a certain extent, in preclinical and clinical trials. Comunanza and Bussolino describe the insights gained on the vascularendothelial growth factor (VEGF) over the last 40 years and relevant challenges raised by VEGF-targeted anti-angiogenic therapies (Comunanza and Bussolino). They deeply review the emergence of approaches combining anti-angiogenic regimens with compounds targeting angiogenic mechanisms, oncogenic drivers, and immunotherapy (Comunanza and Bussolino).

In an original research article, Astrologo and colleagues provide evidence that the Bone Morphogenetic Protein 9 (BMP9) might represent a novel therapeutic target in prostate cancer (Astrologo et al). They nicely demonstrate that preventing BMP9 binding to its cell surface receptors, and thus blocking BMP9 signaling, efficiently diminishes prostate cancer cell proliferation and substantially attenuates tumor growth in both an orthotopic model of human prostate cancer and a xenograft derived from an androgen-dependent bone metastatic prostate tumor patient (Astrologo et al).

In respect of non-coding elements, Montironi's group mini review focused on in vitro and in vivo gain-of-function and loss-of-function experiments showing that long non-coding RNAs play a crucial role in cancer cell invasiveness and metastasis through antagonizing the genome-wide localization and regulatory functions of the SWI/SNF chromatin-modifying complex (Cimadamore et al.) In addition to long noncoding RNAs, another class of regulatory RNAs, namely microRNAs, have been implicated in nearly every signaling pathway. Specifically, microRNA-mediated altered signaling pathway regulation appears to affect a heterogeneous spectrum of cancer behaviors. In this respect, Denti's group provide a comprehensive overview of the tight connection between microRNA misfunction and cancer hallmarks (Detassis et al). They also thoroughly discuss benefits and hurdles of microRNAs as biomarkers to move personalized cancer biogenesis, evolution, diagnosis, and treatment a step forward. Additionally, Gabra and Salmena's review contributes to the debate on the role of microRNAs in personalized cancer therapy focusing on drug resistance and the mechanisms of action that lead to poor overall survival. They also discuss the potential clinical use of miRNA mimic- or antagomir-based approaches in drug resistance overcome (Gabra and Salmena).

Interestingly, two contributors pointed out to the relevance of approaches encompassing metabolism to develop suitable cancer-specific treatments. The extensive crosstalk within and between reactive oxygen species (ROS) detoxification, redox signaling transduction, energy metabolism and central metabolism has been finely reviewed in this Research Topic by Benfeitas et al. The outlined reconstruction of redox metabolism has been connected to the heterogeneity in redox responses displayed by different types of cancer, between individuals affected by the same form of tumor, as well as within different cancer stages. They also highlighted the utility of systemlevel approaches to capture the role of redox systems in cancer and to design redox-targeting drugs producing synergistic responses for cancer treatment or prevention (Benfeitas et al). On another review, Martín-Martín and colleagues accurately depict the complex interrelationship between metabolism and gene expression regulation in cancer (Martín-Martín et al). The authors report recent advances highlighting how the tight and dynamic coordination between gene expression programs and metabolism dictates cellular adaptations during cancer progression and might lead to new therapeutic opportunities (Martín-Martín et al).

Although counteracting pro-tumorigenic stimuli has always been a major goal in oncology, alternative innovative therapeutic strategies are currently emerging impetuously. Among the most promising ones, we highlight here the synthetic lethality approach and cancer vaccines.

As reviewed by Caffo's group, impairment of DNA damage repair pathways is a common event in cancer, resulting in genomic instability which is crucial for the tumorigenic process (Caffo et al). Exacerbation of such a condition through the administration of DNA damage agents in combination with molecules further affecting DNA repair pathways has been shown to effectively result in cancer cell death (Caffo et al). Importantly, Caffo and colleagues discuss the relevance of applying DNA sequencing approaches for the screening of genomic aberrations affecting DNA repair pathways in prostate cancer with the ultimate goal of stratifying prostate cancer patients for personalized synthetic lethal therapeutic approaches (Caffo et al).

In an original research article, Grandi's group explore the applicability of the Outer Membrane Vesicles (OMVs) platform technology in cancer vaccination (Grandi et al). Technological promising aspects of OMVs, such as the rapidity they can be decorated with foreign epitopes, the high yield production from bacterial fermentation and the easy purification process, inspired the authors to test OMVs amenability for cancer vaccines. Immunization with OMVs engineered with the B cell cancer-specific epitope strongly protected mice from tumor development once injected with a syngeneic cancer cell line expressing the epitope on its surface (Grandi et al). Finally, the synergistic protective activity of multiple epitopes administered with OMVs was found to potentiate the overall efficacy of the OMV cancer vaccine (Grandi et al).

From a clinical perspective, our deeper understanding of oncogenic mechanisms has recently begun to have a crucial impact on clinical decisions at several steps, from cancer prevention and diagnosis to therapeutic intervention. Nowadays, the development of innovative investigational *in silico*, *in vitro*, and *in vivo* platforms fostering the clinical translational potential of basic research findings is of primary relevance.

In this Research Topic, Re reviews significant advancements in our capabilities to tailor synthetic genetic circuits to specific applications in tumor diagnosis, tumor cell- and gene-based therapy, and drug delivery (Re). From a different perspective, Clohessy and Pandolfi present the Mouse Hospital and the Co-Clinical Trial Project focused on the integration of data collected from cancer patients and faithful cancer mouse models enrolled in concomitant trials (co-clinical trials) with identical treatment protocols. They discuss how co-clinical studies can quickly lead to effective clinical decisions by predicting patients' drug response on genetic and molecular bases as well as by anticipating effective second line treatments for drug resistance-driven cancer relapse (Clohessy et al).

Altogether, the original articles, reviews and perspectives collected in this Research Topic represent an invaluable resource of insights on important achievements attained so far in identifying altered molecular events that lead to the development of cancer and therapy resistance as well as novel therapeutic strategies for the successful delivery of precision medicine approaches in oncology.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A Comprehensive Infrastructure for Big Data in Cancer Research: Accelerating Cancer Research and Precision Medicine

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OPEN ACCESS

Edited by: Angela Re, University of Trento, Italy

Reviewed by:

Fahd Al-Mulla, Genatak, Kuwait Lawrence Schook, University of Illinois at Chicago, United States

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Specialty section:

This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: 30 June 2017 Accepted: 05 September 2017 Published: 21 September 2017

Citation:

Hinkson IV, Davidsen TM, Klemm JD, Chandramouliswaran I, Kerlavage AR and Kibbe WA (2017) A Comprehensive Infrastructure for Big Data in Cancer Research: Accelerating Cancer Research and Precision Medicine. Front. Cell Dev. Biol. 5:83. doi: 10.3389/fcell.2017.00083 Advancements in next-generation sequencing and other -omics technologies are accelerating the detailed molecular characterization of individual patient tumors, and driving the evolution of precision medicine. Cancer is no longer considered a single disease, but rather, a diverse array of diseases wherein each patient has a unique collection of germline variants and somatic mutations. Molecular profiling of patient-derived samples has led to a data explosion that could help us understand the contributions of environment and germline to risk, therapeutic response, and outcome. To maximize the value of these data, an interdisciplinary approach is paramount. The National Cancer Institute (NCI) has initiated multiple projects to characterize tumor samples using multi-omic approaches. These projects harness the expertise of clinicians, biologists, computer scientists, and software engineers to investigate cancer biology and therapeutic response in multidisciplinary teams. Petabytes of cancer genomic, transcriptomic, epigenomic, proteomic, and imaging data have been generated by these projects. To address the data analysis challenges associated with these large datasets, the NCI has sponsored the development of the Genomic Data Commons (GDC) and three Cloud Resources. The GDC ensures data and metadata quality, ingests and harmonizes genomic data, and securely redistributes the data. During its pilot phase, the Cloud Resources tested multiple cloud-based approaches for enhancing data access, collaboration, computational scalability, resource democratization, and reproducibility. These NCI-led efforts are continuously being refined to better support open data practices and precision oncology, and to serve as building blocks of the NCI Cancer Research Data Commons.

Keywords: genomics, proteomics, imaging, big data, cancer, precision medicine, cloud infrastructure

INTRODUCTION

Precision medicine has evolved out of the seminal work of the Human Genome Project, advancements in DNA sequencing technology, developments in high throughput and large-scale molecular biology technologies, improvements in the speed and scale of computation, and innovations in biomedical informatics. This progress has resulted in the molecular characterization

of individual patient tumors, the identification of actionable genetic alterations, and the development of evidence-based molecular cancer diagnostics and targeted therapies. Although, cancer types have been traditionally classified by organ or cell type, with the aid of genomics, cancer patients are increasingly being treated according to their cancer's unique molecular signature. Cancer is a diverse array of genetically-driven diseases. The identification and validation of actionable genetic alterations including amplifications, rearrangements, and gain-of-function mutations, has spurred the use of genomic data in oncology practices. Targeted gene sequencing panels, for example, offer insight into the genetic drivers of an individual's tumor and inform the diagnosis, prognosis, and targeted treatment of cancer patients. A number of targets for drug development have been outlined previously (Hyman et al., 2017). Imantiniba BCR-ABL inhibitor for chronic myelogenous leukemia, trastuzumab-a monoclonal antibody-based treatment for HER-2 positive breast cancer, vermurafenib—a mutated BRAF V600E inhibitor for metastatic melanoma, and many others serve as precision oncology success stories. Other candidate genes are currently under pre-clinical and clinical investigation for the development of targeted cancer therapies. Increasing our understanding of how molecular signatures are associated with treatment outcomes in patient populations, and translating these discoveries into the clinic, will improve treatment decisions for the individual.

In support of NCI's Precision Medicine in Oncology Initiative and the Beau Biden Cancer Moonshot, NCI is leading numerous multi-disciplinary efforts to accelerate the development of precision oncology diagnostics and treatments. Here, we describe a subset of ongoing NCI programs that combine biomedical big data, biotechnology, informatics, clinical research, and computer science to create new ways to more precisely study, predict, diagnose, and treat cancers.

NCI PROGRAMS PROVIDE BIG DATA RESOURCES TO SERVE THE CANCER RESEARCH COMMUNITY

The goal of precision oncology is to use each patient's unique collection of germline variants and somatic mutations to inform their diagnosis, prognosis, and therapy; working toward this goal, there has been a push toward large-scale, high throughput studies of patient-derived biospecimens.

Molecular profiling of patient-derived samples, including whole genome sequencing, has led to a data explosion that is contributing to our increased understanding of cancer driver genes, cancer molecular subtyping, cancer risk, therapeutic response, and treatment outcomes. NCI-supported programs such as The Cancer Genome Atlas (TCGA), Therapeutically Applicable Research to Generate Effective Treatments (TARGET), and Clinical Proteomic Tumor Analysis Consortium (CPTAC) have generated large datasets amassing petabytes of data. These, along with other datasets and resources described in this paper are available to researchers both in the US and internationally (**Table 1**).

In December 2005, TCGA was announced as a new collaboration between the NCI and the National Human Genome Research Institute (NIH, 2005). Building upon the pioneering work of the Human Genome Project, the two institutes embarked on a mission to explore the genomic changes that occur in human cancers. The overarching goal of TCGA was to increase our understanding of different cancer types to improve screening and treatments, and to build on this data to create new prevention strategies. TCGA includes the genomic analysis of 33 different tumor types and matched normal tissue from over 11,000 patients and has resulted in thousands of publications (Cancer Genome Atlas Research Network, 2008, 2011, 2012, 2017; Cancer Genome Atlas, 2012). Data types collected include DNA copy number arrays, DNA methylation, exome, and whole genome sequencing, mRNA arrays, microRNA sequencing, and reverse phase protein arrays, totaling \sim 2.5 petabytes of data.

TARGET was launched in 2006. TARGET's goal is to characterize the genome and transcriptome of hundreds of pediatric acute lymphoblastic leukemia, acute myeloid leukemia, Wilms tumor, clear cell sarcoma of the kidney, rhabdoid tumor, neuroblastoma, and osteosarcoma samples. Through genomic and transcriptomic analyses, researchers are studying the relationships among alterations at the DNA and RNA levels, cancer growth, cancer progression, and pediatric patient survival (Mullighan et al., 2009; Pugh et al., 2013; Eleveld et al., 2015). The TARGET project has performed whole genome sequencing on most samples collected and the entire dataset is in the petabytes range.

NCI's CPTAC aims to interrogate cancers at the protein level to link genotype to proteotype, with the goal of understanding the basis of cancer phenotypes. CPTAC's objectives are fourfold: (1) characterize the proteomes of tumor and normal tissues; (2) perform proteogenomic analyses of cancer biospecimens; (3) identify potential biomarker candidates through discovery proteomics and develop targeted assays against those candidates; and (4) perform verification tests on those targeted assays. Phase I of CPTAC consisted of technical quality assurance studies (Paulovich et al., 2010). Complementary to TCGA studies, CPTAC Phase II consisted of mass spectrometry-based proteomic analyses of TCGA breast, ovarian, and colorectal samples (Zhang et al., 2014, 2016; Mertins et al., 2016). The recently launched CPTAC Phase III is a proteogenomic analysis of prospectively collected tissues from additional cancer types. Furthermore, to support precision oncology, CPTAC Phase III has established Proteogenomic Translational Research Centers that will study the efficacy of cancer therapies on individual tumor samples to generate predictive models. CPTAC data currently totals ~16 TB of data, and upon completion of CPTAC III, this number is expected to increase four-fold to \sim 66 TB of data.

With the announcement of the Beau Biden Cancer Moonshot, the Applied Proteogenomics Organizational Learning and Outcomes (APOLLO) Network has emerged as a tri-agency collaboration to enable oncologists to use their patients' unique proteogenomic profiles to inform precision oncology treatments (Moonshot, 2016; OCCPR, 2016). Together with the Department of Veterans Affairs (VA) and the Department of Defense (DoD),

TABLE 1 | Selected NCI-supported projects.

Project name	Lead institution(s)	Project URL
The Cancer Genome Atlas (TCGA)	National Cancer Institute National Human Genome Research Institute	cancergenome.nih.gov
Therapeutically Applicable Research to Generate Effective Treatments (TARGET)	NCI Office of Cancer Genomics	ocg.cancer.gov/programs/target
Clinical Proteomic Tumor Analysis Consortium (CPTAC)	NCI Office of Cancer Clinical Proteomics Research	proteomics.cancer.gov/programs/cptac
Applied Proteogenomics Organizational Learning and Outcomes (APOLLO) Network	Department of Defense Department of Veterans Affairs National Cancer Institute	proteomics.cancer.gov/programs/apollo-network
The Cancer Imaging Archive (TCIA)	University of Arkansas for Medical Sciences NCI Division of Cancer Treatment and Diagnosis	www.cancerimagingarchive.net
Genomic Data Commons (GDC)	NCI Center for Cancer Genomics	gdc.cancer.gov
Database of Genotypes and Phenotypes (dbGaP)	National Center for Biotechnology Information	www.ncbi.nlm.nih.gov/gap
NCI Cloud Resources	National Cancer Institute	cbiit.cancer.gov/cloudresources
Broad FireCloud	Broad Institute	firecloud.org
Institute for Systems Biology Cancer Genomics Cloud (ISB-CGC)	Institute for Systems Biology	isb-cgc.org
Seven Bridges Cancer Genomics Cloud (SB-CGC)	Seven Bridges	www.cancergenomicscloud.org
NCI Cancer Research Data Commons	National Cancer Institute	cbiit.cancer.gov/cancerdatacommons

NCI-supported projects annotated with lead institutions and URLs.

NCI aims to perform proteogenomic analyses of a cohort of 8,000 cancer patients within the VA and DoD healthcare systems. These analyses will provide insight into the mutations and pathways that drive cancer progression and support the development of targeted and combination therapies. In the next 5 years, APOLLO is expected to amass petabytes of genomic, proteomic, imaging, and clinical data.

As the -omics sciences increase the volume of data collection, the need for big data solutions intensifies. To address this need, biomedical research has been moving toward data curation and data sharing models established by other big data fields such as astrophysics. Through major technological advancements, the Hubble Deep Field image marked a turning point in astrophysics where researchers led a concerted effort in data quality assessment, annotation, and curation. This work led to the development open source data resources, and user interfaces that obviated the resource intensive download of large datasets (Andersen, 2012). Biomedical informatics has reached a similar a turning point where key innovations in data storage and distribution such as compression algorithms, indexing systems, and cloud platforms must be leveraged.

NCI GENOMIC DATA COMMONS AND CLOUD RESOURCES

In addition to the data curation and storage needs of modern biomedical research, other challenges include the development of robust analytical tools, as well as infrastructure and funding models to support these efforts. As data generation expands, local storage, and computational solutions become less feasible. Thus, NCI has set out to build the NCI Cancer Research Data Commons (NCRDC), a cloud-based infrastructure in support of data sharing, tool development, and compute capacity to democratize big data analysis and to increase collaboration among researchers. NCI has sponsored two recent initiatives that serve as the foundation for the Cancer Research Data Commons—the Genomics Data Commons (GDC), and three Cloud Resources.

The GDC, built and managed by the University of Chicago Center for Data Intensive Science, in collaboration with Ontario Institute for Cancer Research, all under an NCI contract with Leidos Biomedical Research, is a unified genomic data repository that hosts authoritative NCI reference datasets such as TCGA and TARGET (Grossman et al., 2016; NIH, 2016). The primary goals of the GDC are to ensure data and metadata quality, ingest and harmonize genomic data, support data dissemination practices in alignment with Findable Accessible Interoperable Reusable (FAIR) principles (Mons et al., 2017), and securely redistribute data to researchers. In addition, the GDC takes part in collaborative efforts such as the Global Alliance for Genomics and Health (Knoppers, 2014). Through the GDC, researchers can download harmonized genomic data for analysis on their local servers. To bolster data sharing practices and streamline genomic data analysis, much of the genomic data stored at the GDC have been made available through the NCI Cloud Resources.

The NCI Cloud Resources were initially launched in 2016 as the Cancer Genomics Cloud (CGC) Pilots. The purpose of the CGC Pilots was to explore multiple cloud-based approaches for enhancing secure data access, collaboration, computational scalability, resource democratization, and reproducibility. Through this program, the Broad Institute, the Institute for Systems Biology, and Seven Bridges have each developed what are now known as Cloud Resources. Each platform is deployed in a commercial cloud, and has applied a distinct approach to providing access to TCGA and TARGET genomic data in a cloud environment, and integrating proteomic data from CPTAC as well as radiology images and associated metadata from The Cancer Imaging Archive (TCIA). In addition to providing access to these datasets through rich Application Programming Interfaces (APIs) and graphical user interfaces, the Cloud Resources each provide a platform to enable the deployment of analysis, visualization, and other computational tools in the cloud, bypassing the need to bring data to a local infrastructure. The Cloud Resources support tool deployment through the use of Docker containers, which allow users to package their tools along with all associated dependencies. These "containerized" tools can be connected and executed as workflows in these cloud environments. End user documentation provides users with guidance on how to query data, install tools, as well as create and run workflows in each environment. All three platforms conform to strict federal information system security requirements and manage access to controlled data through Database of Genotype and Phenotype (dbGaP) authorization. In addition to their fundamental charter of providing secure cancer genomic data access co-localized with analysis pipelines and visualization tools, the Cloud Resources each offer unique capabilities suitable for a range of research needs.

Broad Firecloud

The Broad Institute's FireCloud, was built as the next generation of Broad Institute's Firehose data analysis infrastructure developed for the TCGA program (Ulrich, 2016). FireCloud harnesses the elastic compute capacity of Google Cloud Platform for large-scale genomic analyses akin to those available through Firehose. Key advantages offered by FireCloud include running Broad's best practice tools and pipelines such as ContEst, MuTect, and Oncotator. FireCloud users can also access curated open and controlled-access TCGA workspaces, upload their own data, and share workspaces with collaborators. FireCloud also allows users to leverage the rich query interface of the GDC to create cohorts of interest and download data "just-in-time" to a FireCloud-based workspace for follow on analyses. Similar approaches are under development to support the analysis of CPTAC data and TCIA images. Researchers at the Broad Institute, in collaboration with IBM Watson, are using FireCloud to tackle one of precision oncology's toughest questions-which genomic signatures are linked to drug-resistant cancers (Park, 2016)? While targeted therapies are currently being applied in the clinic, oncologists have been unable to predict when a patient will no longer respond to a given line of therapy. The data analysis infrastructure provided by FireCloud directly supports researchers investigating problems such as this one to increase the efficacy of precision medicine for cancer patients.

ISB-CGC

The Institute for Systems Biology Cancer Genomics Cloud (ISB-CGC) runs on the Google Cloud Platform and offers an interactive web-based application and hosts Application Programming Interfaces (APIs) such as the Global Alliance for Global Health API. ISB-CGC takes advantage of Google Cloud Platform's built-in resources such as BigQuery, Compute Engine, App Engine, Cloud Datalab, and Google Genomics. Researchers can use BigQuery to explore clinical, biospecimen, level-3 open access TCGA, and CPTAC II data. ISB-CGC hosts numerous genomics tools and has recently added the Trans Proteomic Pipeline analysis suite. Researchers can now access complementary genomic and proteomic data, run multiomic analyses, and perform BigQuery searches to investigate genetic alterations, copy number, transcript expression, protein expression, and molecular pathways that are involved in cancer biology. ISB-CGC has also made radiology and tissue images from TCIA and the GDC available through Google Cloud Storage. Additional datasets available at ISB-CGC include the Catalog of Somatic Mutations in Cancer¹ and the Cancer Cell Line Encyclopedia (CCLE)². A recent publication in Nature Scientific Reports showcased a project which used the ISB-CGC to perform fast, cheap, and robust RNA-sequencing analyses of 12,307 samples from CCLE and TCGA (Tatlow and Piccolo, 2016). Authors, P. J. Tatlow and Dr. Stephen Piccolo, used preemptible virtual machines to analyze over 64 terabytes of TCGA data for only \$0.09 per sample. The scalable, cost effective compute capabilities of ISB-CGC have enabled researchers to perform robust analyses of big data that will ultimately lead to the enhanced understanding of individual cancers.

SB-CGC

Currently, over 1,600 researches from over 40 countries are using the Seven Bridges Cancer Genomics Cloud (SB-CGC) to analyze hosted genomic data, and/or their own data³. Dr. Julia Salzman's lab at Stanford University has deployed Mismatched Alignment CHimEra Tracking Engine (MACHETE) (Hsieh et al., 2017), a statistical algorithm for the detection of gene fusions, on the SB-CGC (Salzman, 2017). Using RNA-seq data from hundreds of TCGA samples, MACHETE was used to perform statistical modeling of fusion artifacts to precisely detect novel gene fusions including rare potential drivers of cancer. This research, fueled by cloud computing, is enabling precision oncology through the discovery of novel, potentially druggable gene fusions. In addition to the TCGA and TARGET data, SB-CGC hosts TCGA radiology images, CCLE data, as well as Simons Genome Diversity Project data⁴. Leveraging its Cancer Genomic Cloud work, Seven Bridges has partnered with the Blood Profiling Atlas in Cancer Consortium⁵, to develop the Blood Profiling

¹cancer.sanger.ac.uk/cosmic

²portals.broadinstitute.org/ccle

³http://www.cancergenomicscloud.org/usage

⁴docs.cancergenomicscloud.org/docs

⁵http://www.bloodpac.org

Atlas Analysis Cloud and provide the research community with analysis algorithms for liquid biopsy.

CGC Pilot Beta-Testing and Evaluation

The initial versions of The NCI Cloud Resources, the CGC Pilots, were created to maximize the value of cancer -omics data through harnessing multi-disciplinary expertise. Synergizing technologies from the fields of medicine, molecular biology, informatics, and cloud computing, the CGC Pilots have begun to transform how cancer data analysis is conducted. Researchers, both in the US and internationally, have been able to advantage of these cloud-based resources. To ensure the success of this project and to identify areas for improvement, the CGC Pilot and NCI teams established mechanisms to support early adopters' use of these platforms and to collect their feedback.

The CGC Pilots teams provided technical support to new users who sought to implement new tools, access data, or create collaborative workspaces. Through the three CGC Pilots, NCI provided cloud compute and storage "credits" to offset the costs of evaluation of these platforms by cancer researchers. These funds directly impacted the work of researchers such as post-doctoral scholar Dr. Brittany Lasseigne, at the HudsonAlpha Institute for Biotechnology in Huntsville, AL. Dr. Lasseigne used the SB-CGC, to study dosage effects, context dependency, and tissue specificity of tumor suppressors across human cancers in TCGA. These Cloud credits supported the use of the large-scale genomic datasets co-located with computational resources and analysis tools, and increased research efficiency for many early stage researchers like Dr. Lasseigne (Lasseigne, Personal Communication).

To evaluate the CGC Pilots and support on-going NCIfunded cancer research, NCI funded administrative supplements to the active grants of investigators performing genomicsbased research. The Funding Opportunity Announcement, Supplements to Support Evaluation of the NCI Cancer Genomics Cloud Pilots (PA-15-305), funded projects to use one or more of the NCI CGC Pilots for ongoing research activities. Funds were awarded to investigators whose projects aimed to install and test the performance of new analysis tools on a CGC Pilot, upload locally-generated genomic data and



perform analyses on a CGC Pilot, and/or perform analyses of hosted TCGA data. The researchers reported having a generally positive experience working on the CGC Pilots; however, as expected, some encountered technical hurdles. When those technical issues arose, the vast majority of the groups were able to resolve their problems by working directly with the CGC Pilot support teams. Each research group provided extensive feedback to the CGC Pilots and NCI teams on what elements of the CGC Pilots could be improved. The majority of the administrative supplement awardees reported that they plan to continue to use the CGC Pilots to accelerate their research and that the CGC Pilots have the potential to be a vital resource for the cancer research community. The activities and outcomes of projects funded through these supplements have helped inform NCI's decision to continue supporting this project beyond the pilot phase and to develop a more comprehensive computational infrastructure for -omics and other big data types.

Future Vision: NCI Cancer Research Data Commons

Cancer research in the era of big data presents major challenges: computing on large datasets, combining expertise from various disciplines, and developing the infrastructure needed to enhance research efficiency. Recognizing the importance and urgency of these needs, the Beau Biden Cancer Moonshot Blue Ribbon Panel has recommended that the cancer research community aim to, "collect, share, and interconnect a broad array of large datasets so that researchers, clinicians, and patients will be able to both contribute and analyze data, facilitating discovery that will ultimately improve patient care and outcomes (BRP, 2016)." In line with this recommendation, the NCI is taking steps toward establishing the NCRDC, with the GDC and the Cloud Resources serving as the foundation for this vision.

The GDC and Cloud Resources currently support basic and translational research, primarily using genomic and clinical data. These activities serve as the building blocks of the cloud-based NCRDC (Figure 1). The NCRDC will consist of multiple "nodes," or digital knowledge bases with functionalities like those of the GDC and Cloud Resources. NCRDC nodes will each be centered on different research and clinical data types such as genomics, proteomics, imaging, cancer models, and epidemiology. Each node will house annotated datasets, raw data files, metadata, analysis, and visualization tools, as well as individual and collaborative workspaces. NCRDC users will be able to access authoritative datasets generated by NCI funded programs such as TCGA, TARGET, CPTAC, APOLLO, and TCIA. Each node will also employ a standardized process for data submission and quality control that will allow for the harmonization of new data, including user-generated data. Containerized tool deployment will also be supported by each Data Commons node. Each node will provide consistent, well-defined identifiers and semantics for access to data housed in that node and provide broadly-available computational support critical to the demands of modern cancer research and precision oncology. The Data Commons will thus support cancer research across multiple domains and platforms, allow for these data to be queried and analyzed in an integrated, secure, cross-domain manner, and provide the mechanisms for new data sources to be incorporated as they are generated. Through fostering community-driven, open-development informatics initiatives, the Cancer Research Data Commons will create, maintain, and extend informatics infrastructure and standards to improve connectivity among disparate information systems. Combining innovation, cloud computing, big data, and FAIR principles, this robust infrastructure will provide significant support for NCI's Precision Medicine in Oncology Initiative and the Beau Biden Cancer Moonshot by accelerating the discovery of novel therapeutic targets and disease biomarkers for individual cancer patients.

The era of big data in biomedical research and precision oncology calls for creative strategies borrowed from multiple scientific and technological disciplines. The GDC and Cloud Resources are important steps in supporting the next generation of data-driven cancer research. Looking ahead, the NCRDC represents an interdisciplinary solution to the challenges of big data in cancer research. NCI will continue to lead open science efforts toward the goals of improving prevention strategies, developing targeted diagnostics and therapeutics, and reducing the burden of cancer on patients, their families, and society.

AUTHOR CONTRIBUTIONS

IH: Manuscript writing, figure design. TD, JK, AK, and WK: Manuscript and figure revision, approval of final manuscript. IH, TD, JK, IC, AK, and WK: Management and project support for Cancer Genomics Cloud Pilots.

FUNDING

This work has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contracts HHSN261200800001E, HHSN261201400006C, HHSN261201400007C, and HHSN261201400008C. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

ACKNOWLEDGMENTS

We thank NCI Center for Biomedical Informatics and Information Technology (CBIIT), Center for Cancer Genomics, Office of Clinical Cancer Proteomics Research, GDC, and the CGC Pilot teams for advice on the manuscript. IH is supported by an AAAS Science & Technology Policy Fellowship served at NIH NCI CBIIT.

REFERENCES

- Andersen, R. (2012 April 19). *How Big Data is Changing Astronomy (Again)*. The Atlantic.
- BRP (2016). Cancer Moonshot Blue Ribbon Panel Report 2016. Available online at: https://www.cancer.gov/research/key-initiatives/moonshot-cancer-initiative
- Cancer Genome Atlas Research Network (2008). Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 455, 1061–1068. doi: 10.1038/nature07385
- Cancer Genome Atlas Research Network (2011). Integrated genomic analyses of ovarian carcinoma. *Nature* 474, 609–615. doi: 10.1038/nature10166
- Cancer Genome Atlas Research Network (2012). Comprehensive genomic characterization of squamous cell lung cancers. *Nature* 489, 519–525. doi: 10.1038/nature11404
- Cancer Genome Atlas Research Network, Albert Einstein College of Medicine, Analytical Biological Services, Barretos Cancer Hospital, Baylor College of Medicine, Beckman Research Institute of City of Hope, et al. (2017). Integrated genomic and molecular characterization of cervical cancer. *Nature* 543, 378–384. doi: 10.1038/nature21386
- Cancer Genome Atlas (2012). Comprehensive molecular portraits of human breast tumours. *Nature* 490, 61–70. doi: 10.1038/nature11412
- Eleveld, T. F., Oldridge, D. A., Bernard, V., Koster, J., Daage, L. C., Diskin, S. J., et al. (2015). Relapsed neuroblastomas show frequent RAS-MAPK pathway mutations. *Nat. Genet.* 47, 864–871. doi: 10.1038/ ng.3333
- Grossman, R. L., Heath, A. P., Ferretti, V., Varmus, H. E., Lowy, D. R., Kibbe, W. A., et al. (2016). Toward a shared vision for cancer genomic data. *N. Engl. J. Med.* 375, 1109–1112. doi: 10.1056/NEJMp1607591
- Hsieh, G., Bierman, R., Szabo, L., Lee, A. G., Freeman, D. E., Watson, N., et al. (2017). Statistical algorithms improve accuracy of gene fusion detection. *Nucleic Acids Res.* 45:e126. doi: 10.1093/nar/gkx453
- Hyman, D. M., Taylor, B. S., and Baselga, J. (2017). Implementing genome-driven oncology. *Cell* 168, 584–599. doi: 10.1016/j.cell.2016.12.015
- Knoppers, B. M. (2014). Framework for responsible sharing of genomic and health-related data. *Hugo J.* 8:3. doi: 10.1186/s11568-014-0003-1
- Mertins, P., Mani, D. R., Ruggles, K. V., Gillette, M. A., Clauser, K. R., Wang, P., et al. (2016). Proteogenomics connects somatic mutations to signalling in breast cancer. *Nature* 534, 55–62. doi: 10.1038/nature18003
- Mons, B., Neylon, C., Velterop, J., Dumontier, M., da Silva Santos, L., and Wilkinson, M. (2017). Cloudy, increasingly FAIR; revisiting the FAIR Data guiding principles for the European Open Science Cloud. *Inf. Serv. Use* 37, 49–56. doi: 10.3233/ISU-170824
- Moonshot, C. (2016). APOLLO Network #canserve Cancer Patients through Collaboration. Cancer Moonshot. Available online at: https://medium. com/cancer-moonshot/apollo-network-canserve-cancer-patients-throughcollaboration-15a3530ed57e
- Mullighan, C. G., Su, X., Zhang, J., Radtke, I., Phillips, L. A., Miller, C. B., et al. (2009). Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. N. Engl. J. Med. 360, 470–480. doi: 10.1056/NEJMoa0808253

- NCI (2017). The NCI Cancer Research Data Commons: An Expandable Infrastructure. This figure is in the public domain and can be freely reused. Please credit the National Cancer Institute.
- NIH (2005). NIH launches comprehensive effort to explore cancer genomics. News Release. Available online at: https://cancergenome.nih.gov/newsevents/ newsannouncements/news_12_13_2005
- NIH (2016). Newly Launched Genomic Data Commons to Facilitate Data and Clinical Information Sharing. News Releases. Available online at: https:// www.nih.gov/news-events/news-releases/newly-launched-genomic-datacommons-facilitate-data-clinical-information-sharing
- OCCPR (2016). Applied Proteogenomics Organizational Learning and Outcomes (APOLLO) Network. News and Events. Available online at: https://proteomics. cancer.gov/newsevents/newsannouncements/archive/2016/APOLLONetwork
- Park, A. (2016). How IBM Watson May Help Solve Cancer Drug Resistance. Available online at: http://time.com/4565526/ibm-watson-cancer-drugresistance/
- Paulovich, A. G., Billheimer, D., Ham, A. J., Vega-Montoto, L., Rudnick, P. A., Tabb, D. L., et al. (2010). Interlaboratory study characterizing a yeast performance standard for benchmarking LC-MS platform performance. *Mol. Cell. Proteomics* 9, 242–254. doi: 10.1074/mcp.M900222-MCP200
- Pugh, T. J., Morozova, O., Attiyeh, E. F., Asgharzadeh, S., Wei, J. S., Auclair, D., et al. (2013). The genetic landscape of high-risk neuroblastoma. *Nat. Genet.* 45, 279–284. doi: 10.1038/ng.2529
- Salzman, J. (2017). "Cancer genomics cloud pilot user talks: sensitive and specific gene fusion detection in the, NCI seven bridges cloud pilot," in *Proceedings of the American Association for Cancer Research Annual, Meeting* (Washington, DC).
- Tatlow, P. J., and Piccolo, S. R. (2016). A cloud-based workflow to quantify transcript-expression levels in public cancer compendia. *Sci. Rep.* 6:39259. doi: 10.1038/srep39259
- Ulrich, T. (2016). Time to Light the Firecloud. Broadminded Blog: Broad Institute.
- Zhang, B., Wang, J., Wang, X., Zhu, J., Liu, Q., Shi, Z., et al. (2014). Proteogenomic characterization of human colon and rectal cancer. *Nature* 513, 382–387. doi: 10.1038/nature13438
- Zhang, H., Liu, T., Zhang, Z., Payne, S. H., Zhang, B., McDermott, J. E., et al. (2016). Integrated proteogenomic characterization of human high-grade serous ovarian cancer. *Cell* 166, 755–765. doi: 10.1016/j.cell.2016.05.069

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Resources for Interpreting Variants in Precision Genomic Oncology Applications

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Precision genomic oncology—applying high throughput sequencing (HTS) at the pointof-care to inform clinical decisions—is a developing precision medicine paradigm that is seeing increasing adoption. Simultaneously, new developments in targeted agents and immunotherapy, when informed by rich genomic characterization, offer potential benefit to a growing subset of patients. Multiple previous studies have commented on methods for identifying both germline and somatic variants. However, interpreting individual variants remains a significant challenge, relying in large part on the integration of observed variants with biological knowledge. A number of data and software resources have been developed to assist in interpreting observed variants, determining their potential clinical actionability, and augmenting them with ancillary information that can inform clinical decisions and even generate new hypotheses for exploration in the laboratory. Here, we review available variant catalogs, variant and functional annotation software and tools, and databases of clinically actionable variants that can be used in an *ad hoc* approach with research samples or incorporated into a data platform for interpreting and formally reporting clinical results.

Keywords: precision oncology, high throughput sequencing, genomic variation, cancer variants, precision medicine, databases, genetic

1. INTRODUCTION

Genomic technologies and approaches have transformed cancer research and have led to the production of large-scale cancer genomics compendia (1, 2). The resulting molecular characterization and categorization of individual samples from such compendia has driven development of molecular subtypes cancers as well as enhanced understanding of the molecular etiologies of carcinogenesis (3–5). The development of novel and effective targeted therapies has proceeded in parallel with and been accelerated by deeper, faster, and broader genomic characterization (6), enabling early application of molecular characterization at the point of care to inform clinical decision-making (7–10) and to address resistance to primary therapy (11). Genomic characterization also has applications in immune approaches to cancer. For example, chimeric antigen receptor T-cell (CARt) therapy has shown great success in diseases with well-characterized antigens that are relatively tumor-specific (12) as identified by genomic profiling. Variously referred to as precision oncology (13), genomicsdriven oncology (14), genomic oncology, and even simply as precision medicine, the paradigm

OPEN ACCESS

Edited by:

Angela Re, University of Trento, Italy

Reviewed by:

Lawrence Schook, University of Illinois at Chicago, United States Myriam Alcalay, Istituto Europeo di Oncologia, Italy

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Specialty section:

This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: 30 June 2017 Accepted: 29 August 2017 Published: 19 September 2017

Citation:

Tsang H, Addepalli K and Davis SR (2017) Resources for Interpreting Variants in Precision Genomic Oncology Applications. Front. Oncol. 7:214. doi: 10.3389/fonc.2017.00214

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of applying high-throughput genomic approaches to patient samples is rapidly changing the landscape of oncology care and clinical oncology research.

Conventional approaches to clinical trials design may be inadequate due to molecular heterogeneity of tumors derived from a single primary tissue (15), leading to the adoption of basket, umbrella, and hybrid trials designs. A number of studies are ongoing to determine feasibility and potential impact of precision genomic oncology at the point-of-care (16–18). In addition to studies focused on identifying targetable mutations, immunebased therapeutic approaches are also being informed by HTS applied to patient samples (19–21).

One of the most recent developments in the field of precision oncology is the approval of Pembrolizumab (Keytruda), an anti-PD-1 antibody that functions as a checkpoint inhibitor, by the US Food and Drug Administration for treatment of solid tumors that show genetic evidence of mismatch repair and, therefore, carry very high mutational burdens (22). Pembrolizumab was previously approved for use in melanoma, but the most recent approval is the first that is targeting allows a drug to be used in a non-tissue-specific context in patients showing a specific genomic marker in any solid tumor (23).

As with any clinical testing modality, whether in a research setting or at the point-of-care, a clear understanding of the goals of applying the test is necessary when first designing the test and its validation. However, the flexibility and number of potential data items that arise from even a limited application of HTS has lead the US Food and Drug Administration (FDA) to begin to define its regulatory role (24) and, critically, how existing knowledge bases can be applied in real time to address findings from clinical HTS testing (25).

This review aims to provide an organized set of biological knowledge bases with relevance to the interpretation of small variants, defined as single nucleotide variants or short (on the order of 20 base pairs or fewer) insertions and deletions. The catalogs of observed variants section list large-scale catalogs of variants, useful for filtering known common polymorphisms and identifying previously identified cancer variants. When a variant observed in a clinical sample has not been seen but appears to affect the protein coding sequence, the functional annotation resources section presents a sampling of some of the most common software and databases for predicting the impact on protein function. Finally, we catalog several data products and knowledgebases have been developed to provide decision support (with strong disclaimers and caveats) directly linking observed variants to clinical intervention in point-of-care HTS applications. Integrating the various data sources described in this review with variants observed in individual patients can be accomplished with combinations of software tools for the manipulation of variant datasets.

1.1. Catalogs of Observed Germline and Somatic Variants

Databases of observed variation in normal populations, diseased individuals, and cancer compendia form the map onto which observed variants in patients are projected. Because of the vast quantities of genomic data and, specifically, DNA variants, there is a tension between providing rich, highly curated information about individual variants and producing the largest possible catalog of variants with manageable levels of curation. This section reviews some of the available catalogs (**Table 1**) of genomic variation observed in the germline as well as those that appear in tumors as somatic mutations. Note that many of the databases mentioned below overlap in data sources (some nearly completely), but they may differ in the amount and depth of curation, additional metadata added to each variant, speed of updates, and methods or formats for access.

1.2. Germline

Comprehensive catalogs of germline variants inform decisions about the frequency of variants as seen in the general population as well as to identify variants that are annotated as cancer associated. In the context of tumor sequencing, common variants are unlikely to be genomic drivers of carcinogenesis and are often filtered from a report of potential somatic variants. This filtering process is particularly important when tumor sequencing is not accompanied by matched normal sequencing. Additional germline databases that catalog disease-associated variants can be useful to begin to address familial risk and potentially pharmacogenomic loci (38, 39).

Perhaps the oldest of the variant catalogs, dbSNP contains 325,658,303 individual variant records (build 150, accessed

TABLE 1 Catalogs of germline and somatic variants.				
Resource	Variant Type	URL	Reference	
dbSNPª	Germline and somatic	https://www.ncbi.nlm.nih.gov/projects/SNP/	(26)	
COSMIC ^a	Somatic	http://cancer.sanger.ac.uk/cosmic	(27)	
ClinVarª	Germline predisposition and somatic	https://www.ncbi.nlm.nih.gov/clinvar/intro/	(28)	
gnomAD⁵	Germline	http://gnomad.broadinstitute.org/	(29)	
69 genomes from CGI°	Germline	http://www.completegenomics.com/public-data/69-genomes/	(30)	
Personalized Genome Project	Germline	http://www.personalgenomes.org/	(31)	
NCI Genomic Data Commons	Germline and somatic	https://portal.gdc.cancer.gov/	(32)	
cBioPortal	Somatic	http://www.cbioportal.org	(33, 34)	
Intogen (Partial TCGA dataset)	Somatic	https://www.intogen.org/search	(35, 36)	
Pediatric Cancer Genome Project	Somatic	http://explorepcgp.org	(37)	

The most commonly used catalogs include dbSNP, COSMIC, ClinVar, and gnomAD. ^aPrimary resources useful for all studies.

^bParticularly useful for exome sequencing projects.

^cUseful if the Complete Genomics platform was used.

May 30, 2017) and is available in multiple formats, searchable, and linked to records in literature and other data resources and databases. While the vast majority of variants in dbSNP have been observed in individuals without cancer, somatic variants are included and annotated in the database. Because dbSNP is driven by community submission of variants, levels of evidence vary among individual variants. The genome Aggregation Database, or gnomAD (29, 40), contains information from 123,136 exomes and 15,496 whole-genomes from unrelated individuals sequenced as part of various disease-specific and population genetic studies (accessed May 30, 2017). These data were collected by numerous collaborations, underwent standard processing, and unified quality control and results area accessible as a searchable online database and as a downloadable VCF-format text file. ClinVar (28), maintained by the NIH National Center for Biotechnology Information (NCBI), is a freely available archive for interpretations of clinical significance of variants for reported conditions. Entries in ClinVar are taken directly from submitters and represent the relationship between variants and clinical significance. When multiple submissions concerning a single variant are available, ClinVar supplies high-level summaries of agreement or disagreement across submitters. Importantly, though, clinical significance in ClinVar is reported as supplied by the submitter. The Personalized Genome Project (31) provides a limited number of fully open-access genome sequencing results provided by volunteers with trait surveys and even some microbiome surveys of participants. A catalog of germline variants derived from 69 genomes sequenced using the Complete Genomics sequencing platform (30) may be useful for groups who have data generated from the same platform, particularly for identifying sequencingplatform-specific false positive results.

1.3. Somatic

Whereas databases of germline variants are useful to filter out variants unlikely to be directly involved in carcinogenesis, databases of somatic variants are useful to identify variants and their frequencies as observed in tumors. In some cases, identified variants may be associated with specific tumor types, offering mechanistic clues, particularly in the rare cancer setting where biological understanding may be limited.

Several catalogs of somatic variants have, at their core, variants derived from The Cancer Genome Atlas (TCGA). These databases vary in the pipelines used to define the variants, the level of annotation associated with individual variants, the proportion of TCGA included, and methods for accessing or querying. Recently, National Cancer Institute (NCI) has established the Genomic Data Commons (GDC) to harmonize clinical information and genomic results across enterprise cancer datasets (32), particularly those funded by NCI, such as TCGA. In addition to the adult tumors profiled as part of the TCGA, the NCI GDC also contains data from several pediatric tumors profiled as part of the Therapeutically Applicable Research To Generate Effective Treatments (TARGET) project (41). Cancer cell line data from the Cancer Cell Line Encyclopedia (CCLE) are also included (42) in the GDC data collection. The GDC is a modern data platform that provides multiple access methods, including a programmatic application programming interface (API), data file download, and web browser-based text and graphical queries and visualization. The International Cancer Genome Consortium (ICGC) is a large, international collaboration with a collection of 76 studies (including TCGA studies) encompassing 21 tissue primary sites. Like the NCI GDC, the ICGC data portal provides modern data platform approaches to data access, visualization, and query (43). The Catalog of Somatic Mutations in Cancer (COSMIC) database is perhaps the largest and best-known cancer variant database. It presents a unified dataset consisting of curated cancer variants for specific genes as well as genomic screens from projects, such as TCGA. Several other cancer variant data resources are listed in **Table 1**.

2. FUNCTIONAL ANNOTATION RESOURCES

When faced with variants with little or no literature or database support, differentiating those that variants that are likely to be deleterious, perhaps contributing to carcinogenesis, versus those that likely are tolerated by the cell is a critical task, particularly in the setting of clinical precision genomic oncology. Note that determing that a variant is deleterious is not likely to result in a change in diagnosis, prognosis, or therapy. However, prioritizing variants for further study, research interest, and for discussion in forums such as a molecular tumor board is a valuable and necessary aspect of applying genomic technologies in the clinical arena.

A number of algorithms and methods have been developed to predict the effect of observed variants on protein structure and function as well as the potential for clinical impact. These prediction methods utilize features of the variant and its context, such as sequence identity, sequence conservation, evolutionary relationship, protein primary and secondary structure, entropybased protein stability, and approaches such as clustering based on sequence alignments and machine learning. Some of them are specific to the type of variant or mutation, some to a disease type, and some more general. Therefore, applying these functional annotational tools and interpreting the results in a clinical or research setting may require significant human curation before being recognized as clinically actionable. Here, we present a review of a representative set of approaches for predicting pathogenicity of different variants. For a comprehensive list of prediction tools and their details, see Table 2. For more detailed scientific and technical explanations of these methods, we refer the reader to a comprehensive review (44).

2.1. SIFT

Sorting Intolerant From Tolerant, or SIFT, that predicts functional impacts of amino acid substitutions (48) is one of the earliest variant effect prediction tools and represents the class of prediction algorithms that utilizes protein conservation. It has since been updated and an online version of the tool is available (67). SIFT uses sequence homology, as measured by protein-level conservation, to classify variants based as tolerated or deleterious based on the associated protein coding changes. SIFT has served as a benchmark against which other methods are compared because

Resource	URL	Reference	Notes
Integrated predictive me	ethods and aggregated databases		
dbNSFP ^{a,b,c,d}	https://sites.google.com/site/jpopgen/dbNSFP	(45)	Aggregated database of variant information
myvariant.info ^a	http://myvariant.info/	(46)	Aggregated database of variant information
Functional effect predict	tion software and algorithms		
PolyPhen-2 ^b	http://genetics.bwh.harvard.edu/pph2	(47)	Bayesian classification
SIFT⁰	http://sift.jcvi.org	(48)	Alignment scores
MutationAssessor	http://mutationassessor.org	(27)	Conservation, naive Bayes classifier
MutationTaster	http://www.mutationtaster.org	(49)	
PROVEAN	http://provean.jcvi.org/index.php	(50)	
CADD ^{b,c}	http://cadd.gs.washington.edu	(51)	
GERP++°	http://mendel.stanford.edu/SidowLab/downloads/gerp/index.html	(52)	
PhyloP and PhastCons	http://compgen.cshl.edu/phast/index.php	(53, 54)	
nsSNPAnalyzer	http://snpanalyzer.uthsc.edu/	(55)	Random Forest
SNPs&GO	http://snps-and-go.biocomp.unibo.it/snps-and-go/	(56)	SVM
SNAP2	https://rostlab.org/services/snap2web/	(57)	Neural Networks
SNPs3D	http://www.snps3d.org/	(58)	Structure and sequence analysis
MutPred2	http://mutpred.mutdb.org/	(59)	Random Forest
AUTO-MUTE	http://binf2.gmu.edu/automute/	(60)	Topology and statistical contact potential
Panther	http://www.pantherdb.org/tools/csnpScoreForm.jsp	(61)	Hidden Markov Model
stSNP	http://ilyinlab.org/StSNP/	(62)	Comparative modeling of protein structure
Condel⁵	http://bg.upf.edu/fannsdb/	(63)	A weighted average of multiple methods
CoVEC	https://sourceforge.net/projects/covec/files		
CAROL ^b	http://www.sanger.ac.uk/science/tools/carol	(64)	Combines PolyPhen-2 and SIFT
Cancer-specific predicti	on tools		
CHASM	http://wiki.chasmsoftware.org/index.php/Main_Page	(65)	Random Forest
CanDrA	http://bioinformatics.mdanderson.org/main/CanDrA#CanDrA	(66)	96 structural, evolutionary and gene features

^aAggregated databases combine outputs of other databases and algorithms are, therefore, efficient resources to use in annotation pipelines. Adding these resources to observed variants is supported software in **Table 4** including Ensembl VEP software (noted[®]) in this table), Annovar (noted[®]), and snpEff (noted[#]).

of its relative simplicity. SIFT considers the type of amino acid change induced by a genomic variant and the position at which the change/mutation occurs. SIFT relies on the presence of sequences from which conservation can be determined; variants for which such databases are limited will potentially lack robust predictions.

2.2. PolyPhen-2

Polymorphism Phenotyping v2, or PolyPhen2, predicts the effecting of coding non-synonymous SNPs on protein structure and function and annotates them (47). This algorithm uses a naive Bayes approach to combine information across a panel of 3D structural, sequence-based, and conservation-based features. Trained on two datasets, HumDiv and HumVar, and associated non-deleterious controls, the PolyPhen2 algorithm represents a class of multivariate prediction algorithms that employ machine learning and multiple features of variant impact.

2.3. Mutation Assessor

Mutation Assessor is an algorithm and tool that, such as SIFT, uses a conservation-based approach. However, Mutation Assessor also incorporates evolutionary information in an attempt to account for shifts in function between subfamilies of proteins (27), potentially extending the functional annotation of variants to "switch of function" as well as loss or gain of function. By quantifying the impact to conserved residues both globally and within subfamilies (residues that distinguish subfamilies from each other are thought to be less tolerant to change), Mutation Assessor defines a functional impact score to predict which variants are likely to be deleterious.

2.4. CONDEL

The CONsensus DELeteriousness, or CONDEL score, is an integrated prediction method for missense mutations that is relatively easy to extend with additional prediction resources (63). Originally implemented as a weighted average of the normalized scores from the output of two computational tools, Mutation Assessor and FATHMM, CONDEL can be extended or adapted to data at hand and represents an "aggregator" approach to variant effect prediction. Condel scores can be derived for a limited set of specified mutations via an online web application. The Ensembl database provides a variation of position-specific CONDEL predictions that combine SIFT and Polyphen-2 for every possible amino acid substitution in all human proteins.

2.5. CHASM

Cancer-specific High-throughput Annotation of Somatic Mutations, or CHASM, is a computational method that identifies and prioritizes the missense mutations likely to enhance tumor cell proliferation (65). CHASM uses machine learning to classify putative "driver" cancer mutations as distinct from "passenger" mutations. Training the CHASM model employed *in silico* simulation to generate realistic "passenger" mutations, specifically modeled to represent variant context and genes that are observed in cancer settings. Multiple features of the variants, including their DNA and protein contexts, were then used to build a machine learning approach that attempted to maximize the specificity of separating driver mutations from passenger mutations. CHASM represents a relatively specific algorithm focused not on "deleteriousness" but, rather, on the likelihood that an observed variant is a cancer "driver."

2.6. dbNSFP

Recognizing that applying all of the effect prediction tools available is potentially challenging (45), developed a database that aggregates predictions for *all* possible SNVs associated with coding changes (in Gencode gene models). With more than ten different prediction algorithms and extensive additional annotation, this database can be a useful one-stop-shop for adding annotations to variant datasets. The snpEff suite (described below) can be used in conjunction with dbNSFP to efficiently annotate SNPs with the potential to effect coding genes.

3. CLINICAL ACTIONABILITY

The ultimate goal for many of the abovementioned resources is to develop an individualized approach to the diagnosis, prevention, and treatment of cancer, or precision oncology. However, despite recent advances in HTS, determining the clinical relevance of experimentally observed cancer variants remains a challenge in the application of HTS in clinical practice. Difficulties in differentiating driver and passenger mutations, lack of standards and guidelines in reporting and interpretation of genomic variants, lack of clinical evidence in associating genomic variants to clinical outcome, lack of resources to disseminate clinical knowledge to the cancer community, and the precise definition of actionability have been reported to contribute to the bottleneck (68-71). Comprehensive resources linking experimentally determined cancer variants and clinical actionability have been developed to address some of these challenges and address various aspects of translating research results into clinical valuable information to support clinical decisions in precision oncology (see Table 3). In recognition of the fact that central curation of information regarding actionability is extremely challenging, several of the resources below use crowdsourcing as a means of gathering updates and enhancing curation efforts. In addition to a web

interface, some tools provide additional access via API, mobile app, and/or social media tagging to facilitate dissemination of information and enhance accessibility. While some of these tools share similar functions, in the section below, we highlight distinct features and capabilities for a representative set of resources that might be used as a "starter" set for clinical annotation of variants.

The myvariant.info database is one of the newest and attempts to provide a "one-stop-shop" for variants. It is included in this section because it has recently incorporated the CIViC and Cancer Genome Interpreter databases. In addition, it provides annotations for SNVs from multiple other data sources (a growing list, so see the site for updates) and aggregates functional annotations for variants present in its database, making it a good all-around tool for cancer variant annotation. It is available as a performant web API only at this time.

Clinical Interpretation of Variants in Cancer (CIViC) is an open access and open source platform for community-driven curation and interpretation of cancer variants. It is based on a crowdsourcing model where individuals in the community can contribute to produce a centralized knowledge base with the goal of disseminating knowledge and encouraging active discussion. Users, including patients, patient advocates, clinicians, and researchers, can participate, along with community editors, in various stages of interpreting the clinical significance of cancer variants using standards and guidelines developed by community experts (68, 72).

The Drug Gene Interaction Database (DGIdb) is an open source and open access platform for gene and drug annotation for known interaction and potential druggability. Users can crossreference genes of interest and drugs against up to 15 sources and in functionally classified gene categories (73, 74). Cancer Genome Interpreter (CGI) identifies mutational events that are biomarkers of drug response or interact with known chemical compounds (75). PharmGKB is a pharmacogenomic resource for building clinical implementation and interpretation based on annotating, integrating, and aggregating knowledge extracted from research-level publications. It provides scored clinical annotation, prescription annotation (drug dosing, prescribing information), as well as pharmacokinetics/pharmacodynamics (PK/PD) annotation, with primary literature reference.

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Resource	URL	Reference	Crowd-sourcing used	Bulk access
myvariant.infoª	http://myvariant.info/	(46)	Yes	APIª
CIViC ^a	https://civic.genome.wustl.edu/home	(72)	Yes	API, Download
DGIdba	http://dgidb.genome.wustl.edu/	(73, 74)	Yes	API, Download
Cancer Genome Interpreter ^a	https://www.cancergenomeinterpreter.org/home	(75)	Yes	API
OncoKbª	http://oncokb.org/	(76)		API
Cancer Driver Log	https://candl.osu.edu/	(77)	Yes	Download
Clinical Knowledge Base	https://www.jax.org/clinical-genomics/clinical-offerings/ckb			
My Cancer Genome	http://www.mycancergenome.org	(78)	Yes	(licensed) API
Personalized Cancer Therapy	https://pct.mdanderson.org		Account required	
PharmGKB	https://www.pharmgkb.org/	(79)	Yes	Download
Precision Medicine Knowledge Base (Beta)	https://pmkb.weill.cornell.edu/	(80)	Yes	

TABLE 3 | In a clinical setting, these databases are the most relevant, as they are maintained to provide clinically actionable and curated content.

While evalutation of each database by both clinical and informatics team members, databases marked with "*" are maintained, recently (or continuously) updated, and curated. The myvariant.info database includes both CiVIC and Cancer Genome Interpreter data. The last column in the table notes bulk access approaches as these are relevant when including databases in an annotation pipeline or automated report.

OncoKb contains information on the clinical implication of specific genetic alterations in cancer. Each variant is annotation from multiple sources and scored using Levels of Evidence ranging from Level 1, which includes FDA-approved biomarker predictive of response to an FDA-approved drug, to Level 2, which includes variants for which an FDA-approved or standard of care treatment is available, Level 3 and Level 4 contain variants with investigational and hypothetical therapeutic implications, respectively. A similarly structured scoring system is available for indicating therapeutic implications for variants associated with resistance (76). Cancer Driver Log (CanDL), an expert-curated database for potential driver mutations in cancer, employs a similar four-level scoring system based on FDA approval, clinical, pre-clinical, and experimental functional evidence (77).

MyCancerGenome (MCG) is a knowledge resource highlighting the implication of tumor mutation on cancer care. It allows users to access its content via a mobile app and provide patientfocused information. Patients can access a database entitled DNAmutation Inventory to Refine and Enhance Cancer Treatment (DIRECT) for Epidermal Growth Factor Receptor (EGFR) mutation for non-small cell lung cancer (NSCLC). Personalized Cancer Therapy (PCT) at the MD Anderson Cancer Center is a resource for clinical response associated with cancer variants and aims to facilitate patient involvement in biomarker-related clinical trials. Drug effectiveness is associated with a specific biomarker and scored based on prospective clinical study as well as Food and Drug Administration (FDA) approval.

4. TOOLS FOR MANIPULATING VARIANT DATASETS

Processing sequence data with the goal of determining variants (somatic or germline) often end with a file in Variant Call Format (VCF format), a loose, self-describing data standard describing variants along a genome, associated statistical and numeric metrics for each variant, and information integrated from data resources such as those described in the preceding sections (81). An ecosystem of tools, listed in **Table 4**, has been developed for basic transformations, manipulations, merge operations, and for adding transcript, protein, and higher-level functional annotations to variants in a VCF file. The vt and bcftools software suites

 $\ensuremath{\mathsf{TABLE}}\xspace 4 \mid$ Software tools for manipulating and adding annotations to variant datasets.

Software	URL	Reference
vt	http://genome.sph.umich.edu/wiki/Vt	(87)
bcftools	http://www.htslib.org/download/	(88)
ANNOVAR	http://annovar.openbioinformatics.org/en/latest/	(83)
Ensembl Variant	http://www.ensembl.org/vep	(85)
Effect Predictor (VEI	⊃)	
SnpEff	http://snpeff.sourceforge.net/	(84)
Oncotator	https://portals.broadinstitute.org/oncotator/	(89)
vcfanno	https://github.com/brentp/vcfanno	(86)

Variant calling produces a list of observed variants. The tools in this table are useful for adding biological interpretation and for annotating the variants with information from resources in **Tables 1–3**.

perform operations such as slicing by genomic coordinate, data compression, and, importantly variant normalization, rendering variants more readily comparable across resources. Annovar (82, 83) and the SnpEff suite (84) add annotations relative to gene annotations, including information about transcript and protein-coding changes. The Ensembl Variant Effect Predictor (VEP) utilizes Ensembl gene models to annotate variants in gene context and offers an interesting plugin architecture that supports adding variant information from resources in (Table 1) (85). Recently, several software developers of variant annotation tools have developed a standard for reporting gene-centric annotations that has simplified post-processing of variants after annotation. Finally, tools such as Vcfanno (86) have been developed that can flexibly add fields to variants in a VCF file based on relatively sophisticated logic and data transformations, reducing the number of tools required to bring a new data resource into the annotation pipeline.

5. DISCUSSION

5.1. Pragmatic Details

Despite advanced toolsets for manipulating variant files and increasing adoption available standard formats, practical pitfalls and challenges remain to the basic manipulation of variant datasets. Some data resources are available in multiple formats and not all formats contain identical information. Matching variants between resources and observed variants can be challenging, as some variants can be represented validly in multiple forms. Ideally, variants are cataloged with clarity with respect to a reference genome and, whenever possible, using HGVS nomenclature (90). In spite of increasing awareness and uptake of HGVS standard nomenclature, the critical step of matching variants across tools and databases in assessing clinical significance is still hampered by inconsistencies across tools and databases (91). Particularly, when handling clinical samples, an information system that provides results from multiple resources when assessing novel variants, incorporates in silico controls when adding or updating data resources (to avoid introducing errors), and adheres to HGVS nomenclature wherever possible in data processing pipelines can increase the likelihood of discovering potentially relevant variants.

5.2. Where to Start?

This review is meant to be comprehensive, so the reader might wonder "Where do we start?." While it is difficult to make hardand-fast recommendations about what resources, tools, and databases are "the best" given the lack of gold-standard datasets on which to base such evalutations, annotations in **Tables 1–3** are meant to provide context for prioritization. The context for sequencing (clinical or not, targeted mutations, trial setting, or novel variant and biomarker discovery) will also drive annotation pipeline development. Not all data resources need to be added simultaneously if developing a pipeline for annotating cancer variants for precision oncology applications. In a clinical setting, targeting the reporting workflow and working with clinicians to understand the most relevant annotations is the most efficient approach to determining relevant resources for annotation. Developing a modular informatics pipeline, perhaps using a computational workflow framework (https://github.com/pditommaso/ awesome-pipeline) that can be easily extended and re-run on previously annotated data is helpful to keep pace with the rapidly changing and growing collection of annotation resources. Newer aggregation resources such as myvariant.info offer a wholistic solution (annotation, catalog, and clinical actionability), but with some risk of "lossiness" with respect to the primary resources contained within.

Finally, given the rapid pace of new development in this space, we have established a crowd-sourced list of cancer variant resources for precision medicine available at https://github.com/ seandavi/awesome-cancer-variant-databases.

5.3. Conclusion

Robust sequencing technologies and increasingly reliable bioinformatics pipelines, combined with parallel development of therapeutics and diagnostics has bolstered the field of precision genomic oncology. However, the sheer number of resources available that can inform the interpretation of small variants is staggering, except for the very few variants with well-established clinical relevance or an associated targeted therapy. This review has highlighted a number of important data resources individually. For other variants, data integration remains a significant hurdle to the rapid turnaround required to apply HTS in a clinical context. Expert panel review (the molecular tumor board) has been effective for some groups (13, 92, 93) while other groups have adopted a protocol-based approach (94). Even when molecularly targetable lesions are identified, barriers to delivering therapy have been observed, limiting the impact of precision genomic oncology in some settings (95). Not covered in this review is the increasing utility of HTS in the burgeoning field of immunotherapy, where early efforts to predict response based on HTS results have been promising (19, 96, 97).

Some interesting trends are evident in the databases and resources presented in this review that highlight the overarching trends in delivering precision medicine. First is the sheer volume and rapid growth of numbers of observations to learn about the spectrum of variation cancer and normal genomes. Projects such as GnomAD, COSMIC, and other data sharing efforts enhance precision by cataloging rare variants as well as precise estimates of the frequencies of common variants. Second is the use of crowd-sourcing to produce rich clinical annotation (e.g., CiVIC) in response to the need for intensive human

REFERENCES

- 1. International cancer genome consortium. (2017). Available from: http://icgc.org
- Cancer Genome Atlas Research Network, Weinstein JN, Collisson EA, Mills GB, Shaw KRM, Ozenberger BA, et al. The cancer genome atlas pancancer analysis project. *Nat Genet* (2013) 45(10):1113–20. doi:10.1038/ ng.2764
- Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature* (2012) 490(7418):61–70. doi:10.1038/nature11412
- Cancer Genome Atlas Research Network. The molecular taxonomy of primary prostate cancer. *Cell* (2015) 163(4):1011–25. doi:10.1016/j.cell. 2015.10.025

interaction to interpret the clinical impact of a variant or its relationship to potential medical intervention. On the other hand, with volumes of data ever-increasing, machine learning techniques drive many of the most commonly used approaches for assigning scores for impact of observed variants. As wellannotated datasets and variant catalogs grow, application of machine learning will become both more common and more powerful.

While significant progress has been made in applying technology to precision oncology, cancer arises in an individual after a typically complex and incompletely understood set of oncogenic events that are increasingly observable at the molecular level. Progress in cancer prevention, early detection, diagnosis, prognosis, and treatment is increasingly driven by insight gained through the analysis and interpretation of large genomic, proteomic, and pharmacological knowledge bases. Reductionist approaches to cancer biology can achieve only limited success in understanding cancer biology and improving therapy. Cancer is a disease associated with disruption of normal cellular circuitry and processes that leads to abnormal or uncontrolled proliferative growth, characterized by a complex spectrum of biochemical alterations that affects biological processes at multiple scales from the molecular activity and cellular homeostasis to intercellular and inter-tissue signaling. The cancer research community has made great strides in measuring the oncogenic events that lead to the development of cancer and therapy resistance. Because of the complexity inherent in protein networks, intercellular signaling, cellular heterogeneity, and the dynamic nature of cancer, future progress will require a more wholistic approach to precision oncology, including multiscale systems and modeling approaches that address the interrelatedness of the biological processes underlying cancer.

AUTHOR CONTRIBUTIONS

SD initiated the manuscript. SD, KA, and HT all contributed to the writing and editing of the manuscript.

FUNDING

This work was supported by the National Cancer Institute Center for Biomedical Informatics and Information Technology and the National Cancer Institute Center for Cancer Research in the Intramural Research Program at the National Institutes of Health.

- Network TCGAR. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* (2008) 455(7216):1061–8. doi:10.1038/nature07385
- Blumenthal GM, Mansfield E, Pazdur R. Next-generation sequencing in oncology in the era of precision medicine. JAMA Oncol (2016) 2(1):13–4. doi:10.1001/jamaoncol.2015.4503
- Flaherty KT, Infante JR, Daud A, Gonzalez R, Kefford RF, Sosman J, et al. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. N Engl J Med (2012) 367(18):1694–703. doi:10.1056/NEJMoa1210093
- Shaw AT, Kim D-W, Nakagawa K, Seto T, Crinó L, Ahn M-J, et al. Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. N Engl J Med (2013) 368(25):2385–94. doi:10.1056/NEJMoa1214886

- Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med* (2010) 362(25):2380–8. doi:10.1056/NEJMoa 0909530
- Mughal TI, Schrieber A. Principal long-term adverse effects of imatinib in patients with chronic myeloid leukemia in chronic phase. *Biologics* (2010) 4:315–23. doi:10.2147/BTT.S5775
- Ai J, Tiu RV. Practical management of patients with chronic myeloid leukemia who develop tyrosine kinase inhibitor-resistant BCR-ABL1 mutations. *Ther Adv Hematol* (2014) 5(4):107–20. doi:10.1177/2040620714537865
- Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, et al. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. N Engl J Med (2013) 368(16):1509–18. doi:10.1056/NEJMoa1215134
- Sohal DPS, Rini BI, Khorana AA, Dreicer R, Abraham J, Procop GW, et al. Prospective clinical study of precision oncology in solid tumors. *J Natl Cancer Inst* (2016) 108(3):djv332. doi:10.1093/jnci/djv332
- Garraway LA. Genomics-driven oncology: framework for an emerging paradigm. J Clin Orthod (2013) 31(15):1806–14. doi:10.1200/JCO.2012.46.8934
- Simon R. Genomic alteration-driven clinical trial designs in oncology. Ann Intern Med (2016) 165(4):270–8. doi:10.7326/M15-2413
- Cheng DT, Mitchell TN, Zehir A, Shah RH, Benayed R, Syed A, et al. Memorial sloan kettering-integrated mutation profiling of actionable cancer targets (MSK-IMPACT): a hybridization capture-based next-generation sequencing clinical assay for solid tumor molecular oncology. *J Mol Diagn* (2015) 17(3):251–64. doi:10.1016/j.jmoldx.2014.12.006
- NCI-MATCH trial (molecular analysis for therapy choice). (2017). Available from: https://www.cancer.gov/about-cancer/treatment/clinical-trials/ncisupported/nci-match
- Lopez-Chavez A, Thomas A, Rajan A, Raffeld M, Morrow B, Kelly R, et al. Molecular profiling and targeted therapy for advanced thoracic malignancies: a biomarker-derived, multiarm, multihistology phase II basket trial. *J Clin Oncol* (2015) 33(9):1000–7. doi:10.1200/JCO.2014.58.2007
- Bethune MT, Joglekar AV. Personalized T cell-mediated cancer immunotherapy: progress and challenges. *Curr Opin Biotechnol* (2017) 48:142–52. doi:10.1016/j.copbio.2017.03.024
- Chalmers ZR, Connelly CF, Fabrizio D, Gay L, Ali SM, Ennis R, et al. Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. *Genome Med* (2017) 9(1):34. doi:10.1186/s13073-017-0424-2
- Faltas B, Bhinder B, Beltran H, Tagawa ST, Molina AM, Nanus DM, et al. Generating a neoantigen map of advanced urothelial carcinoma by whole exome sequencing. *J Clin Oncol* (2016) 34(2_suppl):354. doi:10.1200/jco.2016. 34.2_suppl.354
- Le DT, Durham JN, Smith KN, Wang H, Bartlett BR, Aulakh LK, et al. Mismatch-repair deficiency predicts response of solid tumors to PD-1 blockade. *Science* (2017) 357:409–13. doi:10.1126/science.aan6733
- Garber K. In a major shift, cancer drugs go 'tissue-agnostic'. Science (2017) 356(6343):1111–2. doi:10.1126/science.356.6343.1111
- 24. FDA. Optimizing FDA's Regulatory Oversight of Next Generation Sequencing Diagnostic Tests—Preliminary Discussion Paper. (2015). Available from: https://www.fda.gov/downloads/medicaldevices/newsevents/workshopsconferences/ucm427869.pdf
- FDA. Draft Guidance for Stakeholders and Food and Drug Administration Staff. (2016). Available from: https://www.fda.gov/downloads/MedicalDevices/ DeviceRegulationandGuidance/GuidanceDocuments/UCM509838.pdf
- Sherry ST, Ward MH, Kholodov M, Baker J, Phan L, Smigielski EM, et al. dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res* (2001) 29(1):308–11. doi:10.1093/nar/29.1.308
- Forbes SA, Beare D, Boutselakis H, Bamford S, Bindal N, Tate J, et al. COSMIC: somatic cancer genetics at high-resolution. *Nucleic Acids Res* (2016) 45:D777–83. doi:10.1093/nar/gkw1121
- Landrum MJ, Lee JM, Benson M, Brown G, Chao C, Chitipiralla S, et al. ClinVar: public archive of interpretations of clinically relevant variants. *Nucleic Acids Res* (2016) 44(D1):D862–8. doi:10.1093/nar/gkv1222
- Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, et al. Analysis of protein-coding genetic variation in 60,706 humans. *Nature* (2016) 536(7616):285–91. doi:10.1038/nature19057
- 30. Drmanac R, Sparks AB, Callow MJ, Halpern AL, Burns NL, Kermani BG, et al. Human genome sequencing using unchained base reads on self-

assembling DNA nanoarrays. *Science* (2010) 327(5961):78–81. doi:10.1126/science.1181498

- Church GM. The personal genome project. *Mol Syst Biol* (2005) 1:2005.0030. doi:10.1038/msb4100040
- Grossman RL, Heath AP, Ferretti V, Varmus HE, Lowy DR, Kibbe WA, et al. Toward a shared vision for cancer genomic data. N Engl J Med (2016) 375(12): 1109–12. doi:10.1056/NEJMp1607591
- Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cbio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* (2012) 2(5):401–4. doi:10.1158/2159-8290.CD-12-0095
- Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* (2013) 6(269):11. doi:10.1126/scisignal.2004088
- Rubio-Perez C, Tamborero D, Schroeder MP, Antolin AA, Deu-Pons J, Perez-Llamas C, et al. In silico prescription of anticancer drugs to cohorts of 28 tumor types reveals novel targeting opportunities. *Cancer Res* (2015) 75(15 Suppl):2983. doi:10.1158/1538-7445.AM2015-2983
- Gonzalez-Perez A, Perez-Llamas C, Deu-Pons J, Tamborero D, Schroeder MP, Jene-Sanz A, et al. IntOGen-mutations identifies cancer drivers across tumor types. *Nat Methods* (2013) 10(11):1081–2. doi:10.1038/nmeth.2642
- Downing JR, Wilson RK, Zhang J, Mardis ER, Pui C-H, Ding L, et al. The pediatric cancer genome project. *Nat Genet* (2012) 44(6):619–22. doi:10.1038/ ng.2287
- Wheeler HE, Maitland ML, Dolan ME, Cox NJ, Ratain MJ. Cancer pharmacogenomics: strategies and challenges. *Nat Rev Genet* (2013) 14(1):23–34. doi:10.1038/nrg3352
- Relling MV, Evans WE. Pharmacogenomics in the clinic. Nature (2015) 526(7573):343–50. doi:10.1038/nature15817
- 40. gnomAD browser. (2017). Available from: http://gnomad.broadinstitute.org/
- 41. Therapeutically Applicable Research to Generate Effective Treatments (TARGET). (2017). Available from: https://ocg.cancer.gov/programs/target
- Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, et al. The cancer cell line encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* (2012) 483(7391):603–7. doi:10.1038/ nature11003
- Zhang J, Baran J, Cros A, Guberman JM, Haider S, Hsu J, et al. International cancer genome consortium data portal – a one-stop shop for cancer genomics data. *Database (Oxford)* (2011) 2011:bar026. doi:10.1093/database/bar026
- Addepalli K. Models Predicting Effects of Missense Mutations in Oncogenesis. Ph.D. thesis. George Mason University (2014).
- Liu X, Wu C, Li C, Boerwinkle E. dbNSFP v3.0: a one-stop database of functional predictions and annotations for human nonsynonymous and splicesite SNVs. *Hum Mutat* (2016) 37(3):235–41. doi:10.1002/humu.22932
- Xin J, Mark A, Afrasiabi C, Tsueng G, Juchler M, Gopal N, et al. Highperformance web services for querying gene and variant annotation. *Genome Biol* (2016) 17(1):91. doi:10.1186/s13059-016-0953-9
- Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. *Curr Protoc Hum Genet* (2013) Chapter 7: Unit 7.20. doi:10.1002/0471142905.hg0720s76
- Ng PC, Henikoff S. SIFT: predicting amino acid changes that affect protein function. *Nucleic Acids Res* (2003) 31(13):3812–4. doi:10.1093/nar/ gkg509
- Schwarz JM, Cooper DN, Schuelke M, Seelow D. MutationTaster2: mutation prediction for the deep-sequencing age. *Nat Methods* (2014) 11(4):361–2. doi:10.1038/nmeth.2890
- Choi Y, Sims GE, Murphy S, Miller JR, Chan AP. Predicting the functional effect of amino acid substitutions and indels. *PLoS One* (2012) 7(10):e46688. doi:10.1371/journal.pone.0046688
- Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet* (2014) 46(3):310–5. doi:10.1038/ng.2892
- Davydov EV, Goode DL, Sirota M, Cooper GM, Sidow A, Batzoglou S. Identifying a high fraction of the human genome to be under selective constraint using GERP++. *PLoS Comput Biol* (2010) 6(12):e1001025. doi:10.1371/ journal.pcbi.1001025
- Siepel A, Bejerano G, Pedersen JS, Hinrichs AS, Hou M, Rosenbloom K, et al. Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes. *Genome Res* (2005) 15(8):1034–50. doi:10.1101/gr.3715005

- Pollard KS, Hubisz MJ, Rosenbloom KR, Siepel A. Detection of nonneutral substitution rates on mammalian phylogenies. *Genome Res* (2010) 20(1): 110–21. doi:10.1101/gr.097857.109
- Bao L, Zhou M, Cui Y. nsSNPAnalyzer: identifying disease-associated nonsynonymous single nucleotide polymorphisms. *Nucleic Acids Res* (2005) 33(Web Server issue):W480–2. doi:10.1093/nar/gki372
- Calabrese R, Capriotti E, Fariselli P, Martelli PL, Casadio R. Functional annotations improve the predictive score of human disease-related mutations in proteins. *Hum Mutat* (2009) 30(8):1237–44. doi:10.1002/humu.21047
- Hecht M, Bromberg Y, Rost B. Better prediction of functional effects for sequence variants. *BMC Genomics* (2015) 16(Suppl 8):S1. doi:10.1186/1471-2164-16-S8-S1
- Yue P, Melamud E, Moult J. SNPs3D: candidate gene and SNP selection for association studies. *BMC Bioinformatics* (2006) 7(1):166. doi:10.1186/ 1471-2105-7-166
- Pejaver V, Urresti J, Lugo-Martinez J, Pagel KA, Lin GN, Nam H-J, et al. MutPred2: inferring the molecular and phenotypic impact of amino acid variants. *bioRxiv* (2017) 134981. doi:10.1101/134981
- Masso M, Vaisman II. AUTO-MUTE: web-based tools for predicting stability changes in proteins due to single amino acid replacements. *Protein Eng Des Sel* (2010) 23(8):683–7. doi:10.1093/protein/gzq042
- Thomas PD, Campbell MJ, Kejariwal A, Mi H, Karlak B, Daverman R, et al. PANTHER: a library of protein families and subfamilies indexed by function. *Genome Res* (2003) 13(9):2129–41. doi:10.1101/gr.772403
- Uzun A, Leslin CM, Abyzov A, Ilyin V. Structure SNP (StSNP): a web server for mapping and modeling nsSNPs on protein structures with linkage to metabolic pathways. *Nucleic Acids Res* (2007) 35(Web Server issue):W384–92. doi:10.1093/nar/gkm232
- González-Pérez A, López-Bigas N. Improving the assessment of the outcome of nonsynonymous SNVs with a consensus deleteriousness score, condel. Am J Hum Genet (2011) 88(4):440–9. doi:10.1016/j.ajhg.2011. 03.004
- Lopes MC, Joyce C, Ritchie GRS, John SL, Cunningham F, Asimit J, et al. A combined functional annotation score for non-synonymous variants. *Hum Hered* (2012) 73(1):47–51. doi:10.1159/000334984
- Carter H, Chen S, Isik L, Tyekucheva S, Velculescu VE, Kinzler KW, et al. Cancer-specific high-throughput annotation of somatic mutations: computational prediction of driver missense mutations. *Cancer Res* (2009) 69(16): 6660–7. doi:10.1158/0008-5472.CAN-09-1133
- Mao Y, Chen H, Liang H, Meric-Bernstam F, Mills GB, Chen K. CanDrA: cancer-specific driver missense mutation annotation with optimized features. *PLoS One* (2013) 8(10):e77945. doi:10.1371/journal.pone.0077945
- Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc* (2009) 4(7):1073–81. doi:10.1038/nprot.2009.86
- Li MM, Datto M, Duncavage EJ, Kulkarni S, Lindeman NI, Roy S, et al. Standards and guidelines for the interpretation and reporting of sequence variants in cancer. J Mol Diagn (2017) 19(1):4–23. doi:10.1016/j.jmoldx.2016.10.002
- Prawira A, Pugh TJ, Stockley TL, Siu LL. Data resources for the identification and interpretation of actionable mutations by clinicians. *Ann Oncol* (2017) 28(5):946–57. doi:10.1093/annonc/mdx023
- Uzilov AV, Ding W, Fink MY, Antipin Y, Brohl AS, Davis C, et al. Development and clinical application of an integrative genomic approach to personalized cancer therapy. *Genome Med* (2016) 8(1):62. doi:10.1186/s13073-016-0313-0
- Hedley Carr T, McEwen R, Dougherty B, Johnson JH, Dry JR, Lai Z, et al. Defining actionable mutations for oncology therapeutic development. *Nat Rev Cancer* (2016) 16(5):319–29. doi:10.1038/nrc.2016.35
- Griffith M, Spies NC, Krysiak K, Coffman AC, McMichael JF, Ainscough BJ, et al. CIViC is a community knowledgebase for expert crowdsourcing the clinical interpretation of variants in cancer. *Nat Genet* (2017) 49(2):170–4. doi:10.1038/ng.3774
- Wagner AH, Coffman AC, Ainscough BJ, Spies NC, Skidmore ZL, Campbell KM, et al. DGIdb 2.0: mining clinically relevant drug-gene interactions. *Nucleic Acids Res* (2016) 44(D1):D1036–44. doi:10.1093/nar/gkv1165
- Griffith M, Griffith OL, Coffman AC, Weible JV, McMichael JF, Spies NC, et al. DGIdb: mining the druggable genome. *Nat Methods* (2013) 10(12): 1209–10. doi:10.1038/nmeth.2689

- Tamborero D, Rubio-Perez C, Deu-Pons J, Schroeder MP, Vivancos A, Rovira A, et al. Cancer genome interpreter annotates the biological and clinical relevance of tumor alterations. *bioRxiv* (2017) 140475. doi:10.1101/140475
- Chakravarty D, Gao J, Phillips S, Kundra R, Zhang H, Wang J, et al. OncoKB: a precision oncology knowledge base. JCO Precis Oncol (2017) 1(1):1–16. doi:10.1200/PO.17.00011
- Damodaran S, Miya J, Kautto E, Zhu E, Samorodnitsky E, Datta J, et al. Cancer driver log (CanDL): catalog of potentially actionable cancer mutations. J Mol Diagn (2015) 17(5):554–9. doi:10.1016/j.jmoldx.2015.05.002
- Micheel CM, Lovly CM, Levy MA. My cancer genome. Cancer Genet (2014) 207(6):289. doi:10.1016/j.cancergen.2014.06.016
- Hewett M, Oliver DE, Rubin DL, Easton KL, Stuart JM, Altman RB, et al. PharmGKB: the pharmacogenetics knowledge base. *Nucleic Acids Res* (2002) 30(1):163–5. doi:10.1093/nar/30.1.163
- Huang L, Fernandes H, Zia H, Tavassoli P, Rennert H, Pisapia D, et al. The cancer precision medicine knowledge base for structured clinical-grade mutations and interpretations. Journal of the American Medical Informatics Association? J Am Med Inform Assoc (2017) 24(3):513–9. doi:10.1093/jamia/ ocw148
- Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, et al. The variant call format and VCFtools. *Bioinformatics* (2011) 27(15):2156–8. doi:10.1093/bioinformatics/btr330
- Yang H, Wang K. Genomic variant annotation and prioritization with ANNOVAR and wANNOVAR. *Nat Protoc* (2015) 10(10):1556–66. doi:10.1038/ nprot.2015.105
- Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res* (2010) 38(16):e164. doi:10.1093/nar/gkq603
- Cingolani P, Platts A, Wang LL, Coon M, Nguyen T, Wang L, et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly (2012) 6(2):80–92. doi:10.4161/fly.19695
- McLaren W, Gil L, Hunt SE, Riat HS, Ritchie GRS, Thormann A, et al. The ensembl variant effect predictor. *Genome Biol* (2016) 17(1):122. doi:10.1186/ s13059-016-0974-4
- Pedersen BS, Layer RM, Quinlan AR. Vcfanno: fast, flexible annotation of genetic variants. *Genome Biol* (2016) 17(1):118. doi:10.1186/s13059-016-0973-5
- Tan A, Abecasis GR, Kang HM. Unified representation of genetic variants. *Bioinformatics* (2015) 31(13):2202-4. doi:10.1093/bioinformatics/ btv112
- Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The sequence alignment/map format and SAMtools. *Bioinformatics* (2009) 25(16):2078–9. doi:10.1093/bioinformatics/btp352
- Ramos AH, Lichtenstein L, Gupta M, Lawrence MS, Pugh TJ, Saksena G, et al. Oncotator: cancer variant annotation tool. *Hum Mutat* (2015) 36(4): E2423–9. doi:10.1002/humu.22771
- den Dunnen JT, Dalgleish R, Maglott DR, Hart RK, Greenblatt MS, McGowan-Jordan J, et al. HGVS recommendations for the description of sequence variants: 2016 update. *Hum Mutat* (2016) 37(6):564–9. doi:10.1002/ humu.22981
- Yen JL, Garcia S, Montana A, Harris J, Chervitz S, Morra M, et al. A variant by any name: quantifying annotation discordance across tools and clinical databases. *Genome Med* (2017) 9(1):7. doi:10.1186/s13073-016-0396-7
- Knepper TC, Bell GC, Hicks JK, Padron E, Teer JK, Vo TT, et al. Key lessons learned from moffitt's molecular tumor board: the clinical genomics action committee experience. *Oncologist* (2017) 22(2):144–51. doi:10.1634/theoncologist.2016-0195
- Beltran H, Eng K, Mosquera JM, Sigaras A, Romanel A, Rennert H, et al. Whole-exome sequencing of metastatic cancer and biomarkers of treatment response. *JAMA Oncol* (2015) 1(4):466–74. doi:10.1001/jamaoncol. 2015.1313
- Ghazani AA, Oliver NM, St Pierre JP, Garofalo A, Rainville IR, Hiller E, et al. Assigning clinical meaning to somatic and germ-line whole-exome sequencing data in a prospective cancer precision medicine study. *Genet Med* (2017) 19:787–95. doi:10.1038/gim.2016.191
- 95. Bryce AH, Egan JB, Borad MJ, Stewart AK, Nowakowski GS, Chanan-Khan A, et al. Experience with precision genomics and tumor board, indicates

frequent target identification, but barriers to delivery. Oncotarget (2017) 8(16):27145-54. doi:10.18632/oncotarget.16057

- Wang R-F, Wang HY. Immune targets and neoantigens for cancer immunotherapy and precision medicine. *Cell Res* (2017) 27(1):11–37. doi:10.1038/ cr.2016.155
- Yarchoan M, Johnson BA III, Lutz ER, Laheru DA, Jaffee EM. Targeting neoantigens to augment antitumour immunity. *Nat Rev Cancer* (2017) 17(4):209–22. doi:10.1038/nrc.2016.154

Conflict of Interest Statement: This work was performed while KA and HT were employed by Attain, LLC, in support of bioinformatics projects at the National Cancer Institute. The authors declare that the work was conducted in the absence of any commercial or financial relationships that constitute a potential conflict of interest.

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Therapy for Cancer: Strategy of Combining Anti-Angiogenic and Target Therapies

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The concept that blood supply is required and necessary for cancer growth and spreading is intuitive and was firstly formalized by Judah Folkman in 1971, when he demonstrated that cancer cells release molecules able to promote the proliferation of endothelial cells and the formation of new vessels. This seminal result has initiated one of the most fascinating story of the medicine, which is offering a window of opportunity for cancer treatment based on the use of molecules inhibiting tumor angiogenesis and in particular vascular-endothelial growth factor (VEGF), which is the master gene in vasculature formation and is the commonest target of anti-angiogenic regimens. However, the clinical results are far from the remarkable successes obtained in pre-clinical models. The reasons of this discrepancy have been partially understood and well addressed in many reviews (Bergers and Hanahan, 2008; Bottsford-Miller et al., 2012; El-Kenawi and El-Remessy, 2013; Wang et al., 2015; Jayson et al., 2016). At present anti-angiogenic regimens are not used as single treatments but associated with standard chemotherapies. Based on emerging knowledge of the biology of VEGF, here we sustain the hypothesis of the efficacy of a dual approach based on targeting pro-angiogenic pathways and other druggable targets such as mutated oncogenes or the immune system.

OPEN ACCESS

Edited by: Angela Re.

Angela Re, Istituto Italiano di Technologia, Italy

Reviewed by:

Ronca Roberto, University of Brescia, Italy Anca Maria Cimpean, "Victor Babes" University of Medicine and Pharmacy Timisoara, Romania

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Specialty section:

This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: 06 September 2017 Accepted: 15 November 2017 Published: 07 December 2017

Citation:

Comunanza V and Bussolino F (2017) Therapy for Cancer: Strategy of Combining Anti-Angiogenic and Target Therapies. Front. Cell Dev. Biol. 5:101. doi: 10.3389/fcell.2017.00101

Keywords: cancer, VEGF, angiogenesis, target therapy, resistance

VEGF-TARGETED ANTI-ANGIOGENIC THERAPY

During tumor progression, some clones experience the "angiogenic switch" by interrupting the balance between angiogenesis inducers and inhibitors and show pro-angiogenic phenotype. As a result, initial lesions or dormant metastases become more aggressive (Hanahan and Folkman, 1996; Wicki and Christofori, 2008). Angiogenesis inhibitors were postulated as anticancer drugs in the early 1970s (Folkman, 1971). Of all identified molecules that lead the blood vessel formation, VEGFA appears the main molecular driver of tumor angiogenesis. Indeed VEGFA is overexpressed in the majority of solid tumors and for this reason is the dominant target for antiangiogenic drugs (Carmeliet and Jain, 2000; Ferrara, 2002; Kerbel, 2008). VEGF/platelet-derived growth factor (PDGF) protein family is characterized by the presence of a structural motif with eight conserved cysteine residues forming the typical cystine-knot structure and include a wide range of angiogenic inducers: VEGFA, VEGFB, VEGFC, VEGFD, VEGFE, and placental growth factor (PLGF). The main signaling tyrosine kinase receptor (TKR) is VEGF-receptor, VEGFR2 (also known as KDR) (Ferrara and Kerbel, 2005). Two other VEGFRs are VEGFR1 and VEGFR3 (Figure 1). In embryo as well as in solid tumors VEGF expression is primarily stimulated by hypoxia and VEGFA



transcription is promoted by hypoxia-inducible factor-1 α (HIF1 α) and -2α (HIF2 α) that sense the reduced pO₂ (Semenza, 2009).

Besides favoring the tumor feed, a consequence of "angiogenic switch" is the abnormality of the vessel architecture and defects of microcirculation rheology. The aberrant amounts of angiogenic inducers accelerate the proliferation of endothelial cells with a reduced time frame to allow the whole capillary maturation. As consequence, capillaries are tortuous, irregularly fenestrated with reduced pericyte coverage and leaky. These morphological aberrations induce the increase of interstitial pressure with the decrease of convective transport of small molecules including chemotherapeutics (Nagy et al., 2006; Jain, 2014).

The rationale proposed by Dr. Folkman to exploit anti-angiogenic compounds in clinical settings was to starve cancer and induce its dormancy. Currently, preclinical data suggest that a drastic pruning of tumor vasculature results in a selection of more aggressive cancer clones, which sustain disease progression (Ebos et al., 2009; Pàez-Ribes et al., 2009; Maione et al., 2012). Of interest, some studies failed to document such effects in other preclinical models (Singh et al., 2012).

However, before reaching the whole collapse of vascular bed, VEGF pathway blockade is characterized by an early and transient phase in which vessels assume normal shape and function (Folkman, 2006; Carmeliet and Jain, 2011; Goel et al., 2011). This normalization is characterized by rescue of the balance between inhibitors and inducers of angiogenesis, reduction of leakage and interstitial pressure, improvement of tumor perfusion and oxygenation, and drug delivery. This effect is potentially sensitizing for radiotherapy and increases tumor exposure to cytotoxic chemotherapy (Jain, 2005, 2014).

The degree of vascular normalization correlated with increased survival in glioblastoma multiforme (GBM) preclinical models (Kamoun et al., 2009) and patients (Sorensen et al., 2012; Batchelor et al., 2013). Vascular normalization can also improve trafficking of immune effector cells into tumor microenvironment and prolongs the survival of tumor-bearing mice receiving active immunotherapy (Huang et al., 2013; Jain, 2014; Kwilas et al., 2015).

Anti-Angiogenic Regimens in Advanced Cancers

In general, the inhibition of tumor angiogenesis can be reached by the withdrawal of pro-angiogenic molecules or by inhibiting the signaling pathways triggered by these molecules. Most of angiogenesis inhibitors approved in human cancers targets VEGFA and its VEGFRs pro-angiogenic-mediated signals. The pioneer of angiogenesis inhibitors is the VEGFAtargeted monoclonal antibody bevacizumab (Ferrara et al., 2004; Kerbel, 2008; **Figure 1**). In contrast to most preclinical studies, monotherapy with bevacizumab failed to increase patients' overall survival (OS) (Jain, 2005), but in combination with chemotherapy it can extend progression free survival (PFS) and/or OS in several cancer types including metastatic colorectal cancer (mCRC) (Hurwitz et al., 2004; Giantonio et al., 2007; Cunningham et al., 2013) and recurrent GBM (Vredenburgh et al., 2007; Friedman et al., 2009). Based on the results of these trials, bevacizumab was approved for the treatment of patients with late stage CRC, non-small cell lung cancer (NSCLC), ovarian cancer, metastatic cervical cancer, metastatic renal cell carcinoma (RCC), and GBM, only when given in combination with chemotherapy (**Table 1**). As concern breast cancer, Food and Drug Administration (FDA) approved the combination of bevacizumab with paclitaxel for the treatment of human epidermal growth factor receptor 2 (HER2)-negative metastatic breast cancer (Miller et al., 2007). However, three further phase III trials, failed to confirm the efficacy of the association of bevacizumab with chemotherapy in metastatic breast cancer (Miles et al., 2010; Brufsky et al., 2011; Robert et al., 2011a) resulting in the withdrawal of approval by FDA.

Aflibercept, the "VEGF-trap," is a fusion protein engineered by joining the second Ig-like domain of VEGFR1 and the third Ig-like domain of VEGFR2 to a human IgG1 Fc-fragment (Holash et al., 2002). This soluble decoy receptor shows oneto-one high-affinity binding to all isoforms of VEGF and PLGF (Figure 1). Clinical randomized phase III trials using aflibercept were performed for several solid cancers (Ciombor et al., 2013) and the addition of this compound to standard therapies lengthened PFS and OS in mCRC patients who progressed on bevacizumab therapy (Van Cutsem et al., 2012a). FDA approved aflibercept in combination with leucovorin, 5-fluorouracil and irinotecan (FOLFIRI) for treating patients after progression with oxaliplatin-containing regimen (Ciombor et al., 2013; Table 1). Furthermore, promising experimental models propose aflibercept as a promising candidate to treat, hepatocarcinoma (HCC) (Torimura et al., 2016), a highly vascular tumor with the development of neoarteries in parallel with tumor growth.

Ramucirumab is a monoclonal antibody that binds the extracellular domain of VEGFR2 and interferes with VEGF binding to its receptor. FDA and EMA (European Medicines Agency) approved this compound either as single agent or in association with paclitaxel in subjects affected by metastatic gastric and gastroesophageal junction cancer after progression on fluoropyrimidine or platinum containing protocols (Fuchs et al., 2014; Wilke et al., 2014; **Figure 1**). Subsequently, ramucirumab was approved for the second-line treatment of NSCLC with active disease progression or after platinum-based therapy and for the treatment of mCRC in combination with FOLFIRI in patients whose disease was insensitive to bevacizumab, oxaliplatin and fluoropyrimidine (**Table 1**).

A number of small molecules inhibiting the TK activity of VEGFR, principally (VEGFR2) have been approved as single therapies (Figure 1). Among this class of agents, the pioneer drugs have been sorafenib and sunitinib. Sorafenib is a multikinase inhibitor that targets VEGFR1-3, PDGFRβ, FLT-3, Ret, c-kit, RAF-1, BRAF (Wilhelm et al., 2004). Due to its anti-proliferative, apoptotic, anti-angiogenic and anti-fibrotic effects, sorafenib is a compound with a potent antitumoral activity. Sorafenib is currently the only approved systemic treatment for HCC (Llovet et al., 2008) and several reports have stressed the role of VEGF in the vascularization process of this neoplasia (Miura et al., 1997). Sorafenib has also been approved for the treatment of advanced renal cell carcinoma (RCC) and thyroid cancers (Table 1). The multi-targeted kinase inhibitor sunitinib (VEGFRs, PDGFRs, FLT3, CSFF1R) has been approved for RCC and pancreatic neuroendocrine tumors (Table 1).

TABLE 1 Approved VEGF-targeted therapy for oncology.				
Drug	Brand name	Mechanism	Indications	
Bevacizumab	Avastin (Genentech)	Monoclonal anti-VEGF antibody	CRC; NSCLC; RCC; GBM; epithelial ovarian cancer; fallopian tube cancer; primary peritoneal cancer; cervical cancer	
Aflibercept	Zaltrap (Sanofi and Regeneron Pharmaceuticals)	Recombinant fusion VEGF protein	CRC	
Ramucirumab	Cyramza (Eli Lilly and Company)	Monoclonal anti-VEGFR2 antibody	CRC ; NSCLC; gastric or gastroesophageal junction adenocarcinoma	
Sorafenib	Nexavar (Bayer)	Multi-TKI (VEGFRs, PDGFRs, RAF, KIT, FLT3, RET)	RCC, HCC, thyroid cancer	
Sunitinib	Sutent (Pfizer)	Multi-TKI (VEGFRs, PDGFRs, FLT3, CSF1R, RET)	RCC, pancreatic neuroendocrine tumors, gastrointestinal stromal tumors	
Regorafenib	Stivarga (Bayer)	Multi-TKI (VEGFRs, PDGFRs, FGFRs, TIE2, KIT, RET, RAF)	GIST, CRC, HCC	
Pazopanib	Votrient (GlaxoSmithKline)	Multi-TKI (VEGFRs, PDGFRs, FGFR1, c-Kit)	RCC, soft tissue sarcoma	
Axitinib	Inlyta (Pfizer)	Multi-TKI (VEGFRs, PDGFRs, c-Kit)	RCC	
Vandetanib	Caprelsa (AstraZeneca)	Multi-TKI (VEGFRs, EGFR, RET)	medullary thyroid cancer	
Lenvatinib	Lenvima (Eisai)	Multi-TKI (VEGFRs, FGFRs, PDGFRa , RET, c-Kit)	thyroid cancer, RCC	
Cabozantinib	Cometriq (Exelixis)/Cabometyx (Exelixis)	Multi-TKI (VEGFRs, cMet, AXL)	medullary thyroid cancer, RCC	

CSFR1, colony stimulating factor 1 receptor; CRC, colorectal cancer; EGFR, epidermal growth factor receptor; FLT3, Fms-like tyrosine kinase 3; GBM, glioblastoma multiforme; GIST, gastrointestinal stromal tumor; HCC, hepatocellular carcinoma; KIT, stem cell factor receptor; MET, hepatocyte growth factor receptor; NSCLC, non-small cell lung cancer; PDGFR, platelet-derived growth factor receptor; RAF, rapidly accelerated fibrosarcoma; RCC, renal cell carcinoma; RET, rearranged during transfection; VEGFR, vascular endothelial growth factor receptor.

Anti-angiogenic therapies currently approved by the US Food and Drug Administration (FDA) for the treatment of malignancies (July 2017). For reference see http://cancer.gov.

Subsequently, other agents were developed with similar targets but are characterized by better toxicity profiles. This second-generation of multi-kinases inhibitors have improved target affinity and less off target effects thus allowing lower concentrations of active drugs to be administered with significant activity. Regorafenib belongs to this second-generation of oral multikinase inhibitors that blocks the activity of several kinases, including those involved in the regulation of tumor angiogenesis (VEGFR1-3 and TIE2), oncogenesis (KIT, RET, RAF1, BRAF and BRAF^{V600E}) and the tumor microenvironment (PDGFR β and FGFR). Moreover, it has been recently shown that regorafenib also exerts anti-metastatic activity because of its capability to inhibit epithelial-mesenchymal transition (Fan et al., 2016). This drug represents a significant improvement over the firstgeneration of TKI due to its higher specific activity leading to greater pharmacology potency (Wilhelm et al., 2011). Recently, a phase III study showed that regorafenib extended OS and PFS in mCRC patients previously progressed on standard therapies (Grothey et al., 2013). Regorafenib is now approved for the treatment of mCRC and gastrointestinal stromal tumors (Demetri et al., 2013; Table 1).

Among the second-generation multi-kinases class of inhibitors also pazopanib (Gupta and Spiess, 2013), cabozanitinib (Singh et al., 2017), lenvatinib (Fala, 2015), axitinib (Tyler, 2012), and vandetanib (Degrauwe et al., 2012) have been approved as single therapies in specific indications (**Table 1**).

Recently, based on the result of the phase III LUME-Lung 1 trial (Reck et al., 2014) EMA, but not FDA, approved the use of nintedanib, an oral multi-kinases inhibitor, targeting VEGFR1-3, FRGFR1-3, PDGFR α - β , RET, FLT3, and Src family kinases, combined with docetaxel for the second-line treatment of NSCLC (Lazzari et al., 2017). Moreover, phase II LUME-Meso trial suggested an improvement of PFS of malignant pleural mesothelioma treated with nintedanib in combination with standard treatments (Scagliotti et al., 2016).

Anti-Angiogenic Regimens in Adjuvant Settings

The use of VEGF pathway inhibitors has been started to investigate in phase II and III trials in adjuvant (post-surgical) and neoadjuvant (pre-surgical) settings. Anti-angiogenic agents are used in the adjuvant setting according to the concept that halting angiogenesis after the removal of primary tumor may prevent local relapse micrometastasis spreading tumors. However further clinical and preclinical findings raise doubts on the efficacy of VEGF pathway inhibitors in this setting (Ebos and Kerbel, 2011). Actually, many phase III adjuvant trials with VEGF-targeted therapy failed in CRC, breast cancer, RCC and HCC (de Gramont et al., 2012; Cameron et al., 2013). The reasons of these disappointing results are largely unknown. Probably the different biology of micrometastases from that of established metastatic disease may alter the response to anti-angiogenic agents (Vasudev and Reynolds, 2014).

In neoadjuvant settings, antiangiogenic treatments are used to downsized a tumor, resulting in potentially surgically treatable lesion. Furthermore, it might be used to reduce the risk of local relapse or metastasis. Interestingly the use of bevacizumab (with chemotherapy) in an neoadjuvant setting showed a pathological complete response in breast tumors (Bear et al., 2012; von Minckwitz et al., 2012; Earl et al., 2015; Sikov et al., 2015). Of interest, the efficacy of bevacizumab in promoting vascular normalization in breast tumors correlated with a high baseline microvessel density (MVD), suggesting that basal MVD is a potential biomarker of response to bevacizumab in breast cancer (Tolaney et al., 2015).

The Combination of Anti-Angiogenic Regimens with Chemotherapy

As reported above, anti-angiogenic regimens targeting the excess of angiogenic inducers (e.g., bevacizumab or aflibercept) show clinical benefits when associated with cytotoxic therapies (chemotherapy or radiation). Two different observations sustain this rationale. First, this combined strategy can destroy two separate compartments of tumors: cancer cells and endothelial cells (Teicher, 1996). Furthermore, there is a possible synergistic effect of chemotherapy on endothelial compartment by inhibiting endothelial cell cycle. Metronomic chemotherapy is based on this premise and aims at controlling tumor growth by the frequent administration of conventional chemotherapeutic agents at very low doses to target activated endothelial cells in tumors as well as cancer cells, the advantages of which include minimal adverse effects and a rare chance of developing acquired drug resistance (Kerbel, 2015). Second, the vascular normalizing effects of antiangiogenic regimen modifies the pharmacokinetics parameters of small molecules and favors the delivery of cytotoxic drugs (Zhou et al., 2008; Emblem et al., 2013).

In contrast to anti-angiogenic compounds neutralizing the excess of angiogenic inducers, TKIs do not show any clinical improvement when administered with standard therapies. For instance, attempts to combine anti-angiogenic TKIs with chemotherapy did not improve PFS in mCRC (Carrato et al., 2013) and metastatic breast cancer (Robert et al., 2011b). Indeed, VEGF receptor TKIs exhibit single-agent activity and are effective as monotherapy, while show toxicity in combination with chemotherapy (Jain et al., 2006).

MECHANISMS OF RESISTANCE TO ANTI-ANGIOGENIC REGIMENS

Despite the partial clinical success VEGF-targeted therapies in cancer, some refractory patients do not respond to the treatments (intrinsic resistance) or undergo to acquired resistance after transitory benefits (Bergers and Hanahan, 2008). The extent of refractoriness differs for VEGF blockers and for different cancer types and metastatic settings. Intrinsic and acquired modes of resistance recognize partially overlapping mechanisms, but on the clinical point of view the later represents the most difficult obstacle to achieve better clinical results with anti-angiogenic regimens.

Here we summarize the principal cellular and molecular mechanisms leading to the cancer resistance to anti-angiogenic compounds.

The Vascular Features of the Tumors

The development of anti-angiogenic strategies started before the genomic revolution signed by the first description of human genome and was largely based on a reductionist perspectives and approaches. VEGF was identified as the master tumor angiogenic inducer and "sprouting angiogenesis" (i.e., the formation of capillaries from pre-existing vessels by endothelial sprouting triggered by angiogenic inducers and followed by formation of endothelial tubes, which undergo maturation by pericyte recruitment and extracellular matrix remodeling) as the almost unique mode to sustain the tumor vascularization (Bussolino et al., 1997). The ability of a cancer clone to trigger an angiogenic response is strictly dependent on its pattern of genomic alterations (Rak et al., 1995; Arbiser, 2004), which evolve along the time of the disease and under the pressure exerted by pharmacological treatments. This situation can be exacerbated by the recent genomic findings revealing evidence of branched evolution, wherein tumors consist of multiple distinct subclones that share a common ancestor but differ in terms of subtle or deep genomic alterations that occur later in the evolution of the cancer (Swanton and Govindan, 2016). Such subclones may be intermixed within one tumor sample or regionally separated within a primary tumor, between primary and metastatic sites, or between metastatic sites (Abbosh et al., 2017; Jamal-Hanjani et al., 2017).

Moreover, communication circuits between cancer and stroma cells result in the production a plethora of angiogenic inducers that can support vascular growth and fitness in the presence blockers of VEGF action. This scenario can precede the use of anti-VEGF therapy and explain the intrinsic resistance or be triggered by VEGF inhibitors resulting in a mode of adaptive resistance (Jayson et al., 2016).

Pre-clinical studies identified numerous candidates that can substitute VEGF in sustaining tumor angiogenesis and include angiopoietins (Ang), ephrins, fibroblast growth factor-1 (FGF1) and-2 (FGF2) (Casanovas et al., 2005), prokineticin-1 (Bv8) (Shojaei et al., 2007b), hepatocyte growth factor (HGF) (Shojaei et al., 2010; Cascone et al., 2017), IL-8 (Huang et al., 2010), platelet-derived growth factor C (PDGFC) (Crawford et al., 2009), VEGFC (Li et al., 2014), and PLGF (Fischer et al., 2007). Most of these studies also show that co-targeting of VEGF and the candidate factor improves therapeutic response. In support to this, clinical evidence show that circulating levels of pro-angiogenic factors, including FGF2, HGF, PLGF, and PDGF can become elevated in patients related to the development of acquired resistance to VEGF blockade (Kopetz et al., 2010).

A more intricate connection exists between the resistance to anti-VEGF therapies and Dll4/Notch axis. In sprouting angiogenesis the expression of Dll4 and Notch are increased by VEGFA and counteract the its proangiogenic effect (Thurston et al., 2007). Interestingly, up-regulation of Dll4 induces resistance to bevacizumab in GBM preclinical models, and is in turn overcame by Notch inhibition by γ -secretase inhibitors (Li et al., 2011). Moreover, it has been also reported that high Dll4 expression is predictive of favorable clinical response to anti-VEGF regimen in ovarian cancer (Hu et al., 2011). VEGF blockers and more in general all compounds devised to interfere with an angiogenic inducer halt sprouting angiogenesis. However, established evidences indicate that the tumor mass can be vascularized by vessel co-option, a process wherewith tumor cells simply incorporate pre-existing capillaries from surrounding tissue (Holash et al., 1999). Recently, it has reported in metastases blood supply occurs by the non-angiogenic mechanism of vessel co-option (Donnem et al., 2013; Frentzas et al., 2016). The prevalence of vessel co-option in breast cancer and in liver metastasis of CRC (Frentzas et al., 2016) could explain why anti-angiogenic therapies were poorly effective in approaching metastatic breast cancer and showed a moderate efficacy in metastatic CRC.

Pre-clinical evidences support the role of vessel co-option in the onset of resistance to anti-angiogenic regimens in GBM (Rubenstein et al., 2000), HCC (Kuczynski et al., 2016), and in metastasis to lungs (Bridgeman et al., 2017), lymph nodes (Jeong et al., 2015), and liver (Frentzas et al., 2016). Adjuvant trials in thousands of patients with breast cancer and CRC (de Gramont et al., 2012; Cameron et al., 2013) have been negative probably because micrometastases co-opt existing vessels. Therefore, vessel co-option—mediated blood delivery to the growing tumors and metastases can contribute to both adaptive (e.g., in metastatic disease) and intrinsic resistance.

Besides vessel co-option other tumor vascularization mechanisms have been described and are not sustained by sprouting angiogenesis. They include vascular mimicry, in which tumor cells replace endothelial cells to form the capillary wall; tumor vasculogenesis, which is characterized by the recruitment of endothelial precursors from bone marrow and intussusceptive angiogenesis characterized the duplication of a pre-existing vessel by a splitting mechanism. However the clinical relevance of these mechanisms in mediating resistance to anti-angiogenic compounds remain unclear (Lyden et al., 2001; Semela et al., 2007; Kirschmann et al., 2012).

Finally some tumors but in particular pancreatic ductal adenocarcinomas can exhibit primary refractoriness, manifest as a tumor type that is poorly vascularized with a prominent fibrotic reaction and able to survive in adverse and most probably hypoxic conditions (Ryan et al., 2014). A similar circumstance can explain the lack of the effect of anti-angiogenic regimens in the treatment of prostate cancer (Taverna et al., 2013; Jayson et al., 2016).

Tumor Microenvironment

The features of the stroma cells (leucocytes, pericytes, and fibroblasts) in tumors can deeply influence the initial response to angiogenic-regimens as well as the establishment of acquired resistance.

A wide range of myeloid and lymphoid cells can dynamically visit solid tumors. The presence of M2 polarized macrophages or immature Tie2⁺ monocytes can configure a precise circumstance rendering poorly effective the effects of VEGF blockers and specific myeloid-mediated circuits are activated by anti-VEGF therapies and trigger the onset of acquired resistance (Mantovani and Allavena, 2015). Of notice, refractoriness to antiangiogenic therapies, in GBM patients, is associated with higher numbers of CD68⁺ TAMs and CD11b⁺ myeloid cells and the increase of these populations is associated with poor survival (Lu-Emerson et al., 2013).

In particular, a specific circuit eliciting the acquired resistance to anti-VEGF antibodies has been described and involves CD11b⁺/Gr1⁺myeloid cells and T_H17 lymphocytes, which represent a subset CD4⁺ T cells producing IL-17. It is plausible to envisage that in response to VEGF removal more T_H17 are recruited and/or start to produce IL-17, which in turn activates the release of G-CSF from stroma cells (Chung et al., 2013). G-CSF is an angiogenic inducer (Bussolino et al., 1989) and is crucial for the mobilization and recruitment of CD11b^{+/}Gr1⁺ population to the cancer microenvironment that are capable of promoting VEGF-independent tumorigenesis (Shojaei et al., 2007a). A second circuit entails Ly6Clo monocytes. Anti-VEGF therapy up-regulates CX3CL1 expression, which facilitates CX3CR1-dependent infiltration of Ly6C^{lo} monocytes. These cells attract neutrophils via CXCL5, resulting in the formation of an immunosuppressive microenvironment with a reduction of cytotoxic T lymphocytes (Jung et al., 2017).

Pericytes are mesenchymal cells with contractile properties that patch the capillary outer surface and play a part in vascular physiology. Pericytes are recruited on vessels by PDGFB/PDGFR β signaling both in physiologic and pathological conditions (Abramsson et al., 2003). In most tumor, vessels are surrounded by few pericytes, but in others a dense pericyte coat with thick basement membrane is present; such vessels are usually less sensitive to VEGF blockers (Bergers et al., 2003). Of interest, several anti-angiogenic TKIs clinical-approved are efficient blockers of both VEGF and PDGF receptors (e.g., sunitinib, sorafenib, pazopanib) and therefore may interfere in pericyte coverage.

Finally, cancer associated fibroblasts (CAFs) or fibrocytic cells recruited from bone marrow are cells that take part to the acquisition of resistance to VEGF-blockers by producing alternative pro-angiogenic substances (Crawford et al., 2009; Mitsuhashi et al., 2015).

Besides the cellular components of the stroma, the features of extracellular matrix can influence the refractoriness to antiangiogenic therapies. It has been recently noted in human and mouse models of CRC liver metastatization that anti-VEGF therapy results in abnormal deposition of proteoglycans, in particular hyaluronic acid and sulfated glycosaminoglycans (Rahbari et al., 2016). Interestingly, the depletion of hyaluronic acid results in improved tumor perfusion and treatment efficacy in the mouse model of liver mCRC (Rahbari et al., 2016). These findings parallel the observation that anti-angiogenic therapy increases collagen expression, as a consequence of increased hypoxia, in murine models of pancreatic ductal adenocarcinoma (Aguilera et al., 2014) and HCC (Chen et al., 2014).

Adaption of Tumor Cells to Stressed Conditions

As discussed above, a negative consequence of a prolonged treatment with anti-angiogenic regimens is the reduced blood perfusion and metabolites' exchanges, which evolve in hypoxia and acidosis (Jain, 2014). Besides induction of epithelial-tomesenchymal program that favors an invasive and metastatic tumor cell phenotype hypoxia is thought to select for tumor cells with cancer stem cell properties that might further mediate resistance to cytotoxic agents (Semenza, 2014). In mouse models, hypoxic stress promoted by short-term treatment with anti-VEGF molecules amplified tumor invasiveness and metastatic progression (Ebos et al., 2009; Loges et al., 2009; Pàez-Ribes et al., 2009). The rationale of this paradox is based on the effect on tumor metabolism exerted by the massive vessel pruning and the reduced blood perfusion. The generated hypoxic and acidotic stresses kill a huge amount of cancer cells, but few of them change their features to survive in these hostile conditions by adapting their metabolism, changing the expression of proton pumps, or through autophagy by activating AMP-kinase (Hu et al., 2012; Xu et al., 2013; Fais et al., 2014).

In particular, HIF1-mediated response favors the selection of more aggressive cancer clones (Semenza, 2009) and their metastatic phenotype (Maione et al., 2012) thus explaining the clinical observation that in some solid tumors anti-angiogenic molecules are effective in increasing PFS but they show a negligible effect on OS.

Furthermore, hypoxia favors an immunosuppressive microenvironment by reducing the activity of cytotoxic T cells and antigen-presenting cells and by skewing the polarization of TAMs toward protumorigenic and immunosuppressive M2 phenotype (Mantovani and Allavena, 2015). In HCC, it was demonstrated that increased hypoxia after sorafenib treatment induced $Gr1^+$ myeloid-derived suppressor cell recruitment (Chen et al., 2014).

Several pre-clinical studies report that VEGF-targeted therapy can promote increased tumor invasion and metastasis in a hypoxia-independent manner. It was demonstrated that VEGF suppresses HGF-dependent MET phosphorylation and tumor cell migration through the formation of a VEGFR2/MET heterocomplex. This mechanism could explain why VEGF blockade leads to a proinvasive phenotype in preclinical mouse models of GBM and in a subset of GBM patients treated with bevacizumab (Lu et al., 2012).

Biomarkers and Anti-Angiogenic Therapies

The clinical efficacy of an anti-angiogenic regimen is based on strategies mainly set-up to monitor the tumor cyto-reduction along chemotherapic and radiotherapic treatments. However, the effect of this kind of treatment does not necessarily induce a rapid reduction of tumor mass detectable by imaging approaches or by analyzing the decrease of plasmatic levels of molecules released by the tumor (specific proteins, microRNA, mutated DNAs). Similarly, the present knowledge does not allow predicting which cancer patient can really benefit of an anti-angiogenic treatment.

Huge efforts have been made to evaluate the potential value of circulating angiogenic inducers to address clinical strategies. However high plasmatic levels of VEGF do not predict a response to anti-VEGF/VEGFR2 compounds, and its fluctuation along the treatment is independent from the clinical efficacy (Kopetz et al., 2010). Recent studies have assessed the potential for other biomarkers detectable in plasma. In particular, the pretreatment levels of soluble VEGFR1 inversely correlated with the outcome of either bevacizumab and TKIs because it acts as an endogenous VEGF trap (Meyerhardt et al., 2012; Zhu et al., 2013). Another postulated biomarker is the increased amount of CXCL12, which increased in subjects who escape to anti-angiogenic regimens (Zhu et al., 2009; Batchelor et al., 2010) while low amount of IL-8 at the baseline seems to predict a poor response to bevacizumab treatment in HCC (Boige et al., 2012). Conversely, low pretreatment levels of Ang-2 were associated with a prolonged PFS in CRC treated with bevacizumab (Goede et al., 2010). Many other works showed an increase of angiogenic molecules along anti-angiogenic regimens and in particular bevacizumab, suggesting that the VEGF removal can trigger the activation of alternative pathways sustaining vascularization, reviewed in Lambrechts et al. (2013).

An emerging diagnostic area still not investigated in antiangiogenic regimens is represented by circulating exosomes and their cargos (Wang et al., 2016), including microRNA that are promising markers in oncology (Lin and Gregory, 2015).

A second investigative area is the presence in primary tumors of molecules or vascular features, which can predict the response to angiogenesis inhibitors. Generally speaking, many data have been provided such as microvessel density and the expression of pro-angiogenic molecules (VEGFs, VEGFRs, HGF, PDGFs, chemokines, and Ang) but the results are largely contradictory and poorly robust in term of clinical analysis (Lambrechts et al., 2013). In this context one of the more promising result is the correlation between low level of neuropilin-1 expressed in a large cohort of gastric cancers and the prolonged OS after bevacizumab treatment (Van Cutsem et al., 2012b).

Tumors release a plethora of soluble molecules that have a major impact on the biology of bone marrow. Besides modifying the differentiation and the mobilization in particular of myeloid cells, these molecules can promote the mobilization of endothelial precursors. In particular it has been reported that VEGFA or PLGF released by tumor, through a mechanism dependent on metalloproteinase-9 and soluble Kit ligand, increase the number of these cells in bloodstream, while CXCL12 and CXCR4 receptor favor their retention in perivascular site of injured issues (Kopp et al., 2006). The preclinical observation that the number of circulation endothelial precursors was increased by vascular disrupting molecules (Shaked, 2006), many studies focused on the possibility that these cells could be used to monitor or predict the efficacy of anti-angiogenic drugs. Besides the lack of a solid consensus on their phenotype (Ingram et al., 2005) the clinical data on this approach in clinical oncology are conflicting (Bertolini et al., 2006). For instance anti-angiogenic treatment reduces circulating endothelial cells (Dellapasqua et al., 2008), while metronomic therapy shows an opposite effect (Mancuso et al., 2006).

A further promising area is the role exerted by specific single nucleotide polymorphisms (SNPs) of candidate genes to stratify responder and non-responder patients to anti-angiogenic regimen associated with standard therapies. VEGFR1 rs9582036 associated with an improvement of PFS and OS in patients with metastatic pancreatic adenocarcinoma treated with bevacizumab associated with chemotherapy. On the contrary in renal-clear carcinoma VEGFR1 rs7993418 correlated with PFS but not OS in the bevacizumab group (Lambrechts et al., 2012). Another example was reported in metastatic CRC where VEGFA rs833061 and VEGFR1 rs9513070 respectively associated with the objective response rate and the OS in subjects treated with cytotoxic chemotherapy plus bevacizumab (Sohn et al., 2014). Analysis of genetic variants of other angiogenic-related genes in breast cancer using neoadjuvant bevacizumab in combination with chemotherapy compared to chemotherapy alone showed a correlation between specific SNPs in term of pathologic complete response but not in OS (Makhoul et al., 2017).

In recent years, dynamic contrast-enhanced (DCE)-MRI, which enable non-invasive quantification of microvascular structure and function in tumors, has been extensively evaluated in clinical trials as a biomarker for predicting tumor vascular response to anti-angiogenic treatments (Morotti et al., 2017). VEGF blockade is believed to reduce tumor vascular permeability and perfusion. Significant reductions in capillary permeability have been observed in different studies of bevacizumab and TKI in monotherapy or combination with cytotoxic agents (O'Connor et al., 2012). More recently a further exploitation of DCE-MRI termed vessel architectural imaging allowed the vessel caliber estimation and can be considered a powerful biomarker of the vascular normalization induced by anti-angiogenic therapies (Emblem et al., 2013).

COMBINATION STRATEGIES

The partial effect of anti-angiogenic regimens in human cancers and the wide range of mechanisms sustaining intrinsic and acquired resistance represent a driving force for innovative strategies. For example, the anti-angiogenic regimens could improve their efficacy when associated with compounds targeting other major biological processes (e.g., tumor proliferation or apoptosis). In this context, the combination anti-angiogenic molecules with other approaches such as kinase inhibitors, chemotherapy, DNA repair inhibitors, radiotherapy, and immunotherapy have been reported in many experimental and human settings (Jayson et al., 2016).

Furthermore, nanotechnologies approaches could improve the current pharmacokinetic profiles of anti-angiogenic drugs and favor their selective accumulation in tumors and/or induce a shift the microenvironmental equilibria toward tumorunfavorable conditions (El-Kenawi and El-Remessy, 2013).

Targeting Simultaneously VEGF and Other Angiogenic Mechanisms

Multiple inhibition of concomitant proangiogenic pathways may hamper cancer resistance or extend PFS. A first example deals the simultaneous or sequential blocking of the VEGF and Ang pathways in order to improve efficacy without increasing toxicity (Monk et al., 2014). The tyrosine kinase (TIE2) receptor is activated by its ligand Ang-1, which stabilizes vessels. Ang-2, which antagonizes Ang-1 effects, is highly expressed in cancer, destabilizing vessels and enabling sprouts under a chemotactic gradient of VEGFA. However, the scenario is more intricate

because Ang-2 has a partial agonist activity and has a proangiogenic effect independent of its cognate receptor TIE2. Increased amount of Ang-2 may be instrumental in eluding the anti-VEGF therapy. Preclinical and clinical studies in GMB reported that Ang-2 levels declined temporarily following inhibition of the VEGF pathway but later rebounded as tumors became resistant to the therapy (Batchelor et al., 2010; Chae et al., 2010). More recently it has been hypothesized that dual inhibition of VEGF and Ang-2 signaling respectively with TKI cediranib and MEDI3617 (an anti-Ang-2-neutralizing antibody) could prolong the temporal window of vascular normalization and thereby enhances the survival benefit of anti-VEGF therapy in two orthotopic murine model of GBM (Peterson et al., 2016). This combinatorial effect is related to an increased amount of recruited M1 polarized TAMs, which have anti-tumor effects. This observation is further supported by the data of another study showing that concurrent blockade of VEGF and Ang-2, using a bispecific Ang-2/VEGF antibody, similarly increased the M1/M2 ratio compared with VEGF-inhibition alone (Kloepper et al., 2016). These results match previous preclinical studies reporting a greater efficacy of combined VEGF and Ang-2 signaling inhibition as compared to single treatment (Brown et al., 2010; Hashizume et al., 2010; Koh et al., 2010; Daly et al., 2013; Kienast et al., 2013).

A second example is the association between VEGF blockade with therapies targeting FGF. Pan inhibitors of the FGF receptor (FGFR1-3), such as AZD4547 and BGJ398, elicited potent anti-tumor activities in preclinical investigations and are currently being evaluated in clinical trials (Chae et al., 2017). In this context, the dual inhibition of VEGFRs and FGFRs using brivanib produced enduring tumors stasis and angiogenic blockade following the failure of VEGF-targeted therapies (Allen et al., 2011).

A third approach exploits the possibility to target VEGF signals and Notch pathway, which is fundamental in regulation tip-stalk endothelial cell dynamics in sprouting angiogenesis (Jakobsson et al., 2009). Down-modulation of the Notch ligand Dll4 in combination with anti-VEGF therapy results in a greater tumor growth inhibition than with each agent alone in ovarian cancer models (Huang et al., 2016).

Fourth, HGF/c-MET pathway is driver and biomarker of VEGFR-inhibitor resistance in NSCLC. Dual VEGFR/c-MET pathway inhibition provide superior therapeutic benefit by delaying the onset of the resistant phenotype (Cascone et al., 2017). The efficacy of combining MET and VEGF inhibitors showed beneficial effect in murine GBM overexpressing MET (Okuda et al., 2017) and in pancreatic neuroendocrine tumors (Sennino et al., 2012).

Simultaneous inhibition of angiogenesis and vessel co-option may represent a further improvement of current therapeutic approaches. It has been recently reported that inhibition of angiogenesis and vessel co-option, by the knockdown of Arp2/3mediated cancer cell motility, is more effective than targeting angiogenesis alone in a preclinical orthotopic model of advanced CRC liver metastasis (Frentzas et al., 2016).

Finally, tumor angiogenesis may be also affected and regulated by TGF β family members, that exert a contradictory role in

endothelial cells by inhibiting cell migration and proliferation but also acting as a proangiogenic factor and cooperating with VEGF, PDGF, and FGF in autocrine/paracrine signaling (Guerrero and McCarty, 2017). Preclinical studies have shown the antiangiogenic effect elicited by the TGF β inhibition in HCC, CRC, and GBM xenografts (Mazzocca et al., 2009; Zhang et al., 2011; Akbari et al., 2014) offering the rationale for the combination of TGF β inhibitors with VEGF targeting agents (Neuzillet et al., 2015). In particular, are under clinical investigation the efficacy of the combination of galunisertib, a small molecule inhibitor of TGF β RI, with sorafenib and ramucirumab in HCC and PF-03446962, a monoclonal antibody against TGF β , in combination with regorafenib in CRC.

Targeting Simultaneously VEGF and Oncogenic Drivers

Different oncogenic hits can perturb the balance between proan anti-angiogenic molecules thereby promoting pathological angiogenesis (Arbiser, 2004). For example, MAPK and PI3K-AKT pathways, which are often altered in cancers, are strictly connected with an increased transcription or translation of angiogenic factors. Consequently, specific inhibitors of signaling nodes of these pathways can induce vascular normalization and improve blood perfusion and tumor oxygenation (Qayum et al., 2009).

In particular, RAS activation increases VEGF and IL8 levels and the inhibition of RAS activity by gene silencing suppresses VEGF expression (Mizukami et al., 2005; Matsuo et al., 2009). Moreover, when VEGF expression is inhibited in CRC cells harboring KRAS mutations it has been reported a reduction of in vivo tumorigenic potential, highlighting the relevance of VEGF in exploiting the oncogenic potential of mutated KRAS (Okada et al., 1998). The role of KRAS in supporting angiogenesis is confirmed in NSCLC, where VEGF expression correlates with KRAS activating mutations (Konishi et al., 2000). We also described how mutated BRAF affected tumor angiogenesis and proved that targeting BRAF^{V600E} stabilized the tumor vascular bed and abrogated hypoxia in mouse xenografts (Bottos et al., 2012). It has been suggested that EGFRdriven intracellular signaling may control angiogenesis and pharmacological inhibition of EGFR reduces VEGF expression in cancer cells (Ciardiello et al., 2001). It has been reported that a mechanism of acquired resistance to EGFR inhibitors is mediated by the increased secretion of VEGF, suggesting a key role for tumor-induced angiogenesis in the development of anti-EGFR resistance (Ciardiello et al., 2004). In NSCLC preclinical models it was found possible overcome acquired resistance to EGFR inhibitors by adding a VEGF blocker (Naumov et al., 2009). Human epidermal growth factor receptor 2 (HER2) is an oncogene overexpressed in more malignant breast cancer. Trastuzumab, which targets HER2positive tumors strongly affect vascular shape and function and caused vessel normalization, down-regulating the secretion of VEGF and Ang-1 and in parallel up-regulating the expression of the anti-angiogenic factor thrombospondin 1 (Izumi et al., 2002).

These data suggest that pharmacological inhibition of oncogenes in tumor cells can restore a functional vasculature and potentially blocks the specific angiogenic program activated by individual tumors. Alternative strategy to target tumor angiogenesis could rescue the equilibrium of angiogenic signals by targeting the mutated oncogenes, which play a central role in this process. In order to potentially reduce acquired resistance combined strategy of anti-angiogenic and target therapies are explored in the recent years in pre-clinical and clinical trials.

Cetuximab and panitumumab are monoclonal antibodies that block the activation of EGFR and downstream RAS-RAF-MAPK and the PTEN-PIK3CA-AKT pathways (Ciardiello and Tortora, 2008; Figure 2). These two drugs are currently approved for the treatment of mCRC patients with all-RAS wild-type tumors. It has been recently reported that combined treatment with cetuximab and regorafenib induced synergistic anti-proliferative and pro-apoptotic effects by blocking MAPK and AKT pathways in orthotopic CRC xenograft models with primary or acquired resistance to anti-EGFR (Napolitano et al., 2015). This beneficial effect can be dependent on the inhibitor activities of regorafenib on different tyrosine kinase receptors involved in angiogenesis and potentially in the mechanism of resistance to cetuximab. The results provide the rationale for the clinical development of this combination. A phase I study was designed to evaluate the antitumor property of this combination among patients with advanced cancer refractory to several lines of therapy (Table 2). This study demonstrated that the combination of regorafenib and cetuximab showed a clinical benefit in all patients. It a plausible that inhibition of one of the molecular targets of regorafenib contributes to overcome resistance to previous anti-VEGF or anti-EGFR therapy (Subbiah et al., 2017). These results sustain the results of a previous work showing the cooperative antitumor activity of cetuximab or erlotinib and sorafenib in a xenograft model of NSCLC (Martinelli et al., 2010). More recently, it has been also shown the prolonged antitumor activity exerted by the combination of erlotinib with bevacizumab in a xenograft model of EGFR-mutated NSCLC (Masuda et al., 2017).

In the clinical setting, several studies are exploring the possibility of combining anti-EGFR drugs such as cetuximab, panitumumab or erlotinib, with different antiangiogenic drugs, including bevacizumab or sorafenib (Table 2). The results in unselected NSCLC or CRC cancer patients have been contradictory. Two large randomized phase III studies have evaluated the efficacy of the addition of cetuximab (CAIRO-2) or panitumumab (PACCE) to an oxaliplatin-containing chemotherapy doublet plus bevacizumab (Hecht et al., 2009; Tol et al., 2009). Both studies showed that the addition of the anti-EGFR antibodies did not improve the therapeutic efficacy. However, the results of randomized phase II study in NSCLC cancer patients selected for the presence of activating EGFR gene mutations demonstrated a clinically relevant increase of PFS by the combined treatment with erlotinib associated with bevacizumab compared erlotinib alone (Seto



TABLE 2 | Selected Clinical Trials of VEGF-targeted therapy in combination with oncogene-targeted therapy (July 2017).

Anti-angiogenic	Target Therapy	Phase	Indications	ClinicalTrials.gov Identifier
Bevacizumab	Trastuzumab	2	Stage IV metastatic breast cancer	NCT00428922
Bevacizumab	Trastuzumab	3	Metastatic HER2+ breast cancer	NCT00391092
Bevacizumab	Trastuzumab	2	Breast cancer	NCT01321775
Bevacizumab	Trastuzumab	2	Metastatic HER2+ breast cancer	NCT00364611
Bevacizumab	Trastuzumab	2	Metastatic HER2+ breast cancer	NCT00670982
Bevacizumab	Trastuzumab	2	Metastatic HER2+ breast cancer	NCT00392392
Bevacizumab	Trastuzumab	2	Metastatic breast cancer	NCT00405938
Sorafenib	Trametinib	1	HCC	NCT02292173
Sorafenib	Refametinib	2	HCC	NCT01204177
Sorafenib	Refametinib	2	HCC RAS-mutated	NCT01915602
Regorafenib	Refametinib	1	Neoplasm	NCT02168777
Bevacizumab	Erlotinib	3	CRC	NCT00265824
Bevacizumab	Erlotinib	2	NSCLC EGFR-mutated	NCT01562028
Bevacizumab	Erlotinib	2	NSCLC EGFR-mutated	NCT01532089
Regorafenib	Cetuximab	1	Advanced cancers	NCT02095054
Sorafenib	Cetuximab	2	Squamos cell carcinoma of the Head and Neck	NCT00815295
Sorafenib	Cetuximab	2	CRC	NCT00326495
Bevacizumab	Trastuzumab	3	HER2-positive breast cancer	NCT00625898
Pazopanib	Lapatinib	2	HER2-positive breast cancer	NCT00558103.

CRC, colorectal cancer; EGFR, epiderma growth factor receptor; HCC, hepatocellular carcinoma; HER2, human epidermal growth factor receptor 2; NSCLC, non-small cell lung cancer. For reference see https://clinicaltrials.gov.

et al., 2014). This study provided the first evidence that the addition of bevacizumab to erlotinib confers a significant clinical improvement when used as first-line treatment for patients with NSCLC carrying activating EGFR mutations. More recently, the BELIEF trial (NCT01562028) provided further evidences of benefit for the combined use of erlotinib and bevacizumab in patients with EGFR-mutant NSCLC. Of notice, this study was stratified by the presence of the pretreatment of the T790M mutation with EGFR TKI (Rosell et al., 2017). Further, the efficacy and safety of sorafenib and cetuximab association are under evaluation also in patients with head and neck cancer and CRC (**Table 2**).

It has been demonstrated a positive correlation between elevated HER2 and VEGF levels and the poor outcome of breast cancer (Konecny et al., 2004). Trastuzumab, a monoclonal antibody against HER2, induces normalization and regression of the vasculature in HER2-overexpressing human breast cancer xenografts by lowering the amount of expressed proangiogenic molecules while up-regulating thrombospondin-1, which has anti-angiogenic activity (Izumi et al., 2002; Figure 2). Moreover, in a breast cancer xenograft model, VEGF was found to be elevated in the trastuzumab-resistant group, and sensitivity to trastuzumab was restored upon treatment with bevacizumab (Rugo, 2004). The small molecule inhibitor lapatinib, which inhibits EGFR and HER2, associated with regorafenib showed a greater anti-tumor activity than the compound alone in xenograft models of CRC associated with a relevant reduction of angiogenesis (Zhang et al., 2017). The result of this study has provided the rationale for using HER2 and VEGF inhibitors in clinical practice. Two large phase III trials evaluated the efficacy of bevacizumab and trastuzumab with chemotherapy in HER2⁺ metastatic breast cancer (BETH and AVAREL) (**Table 2**). A modest improvement was seen in PFS, but the most intriguing finding was a more specific benefit from bevacizumab in the subgroup of patients with high levels of plasmatic VEGFA (Gianni et al., 2013). Moreover, the inhibition of VEGFRs and PDGFRs by pazopanib has been assessed in a phase II trial in combination with lapatinib in HER2-positive breast cancer. In this study the combination of lapatinib and pazopanib was associated with a numerically higher response rate without increase in PFS (Cristofanilli et al., 2013; **Table 2**).

We have recently demonstrated that targeting the vascular compartment with bevacizumab modulated the response to BRAF^{V600E} inhibition in melanoma and CRC xenograft models. The final result is a synergistic antitumor effect and a delay of the appearance of the acquired resistance to BRAF inhibition. Of interest, we highlighted that this effect is the result of two biological processes: 1) the recruitment of TAMs polarized toward an M1-like phenotype and 2) the stroma remodeling characterized by the reduction of collagen deposition and the number of activated and tumor associated fibroblasts (Comunanza et al., 2017).

In the recent years, there has been great interest in developing clinically effective small-molecule inhibitors of the Ras-Raf-MEK-ERK1/2 pathway (Samatar and Poulikakos, 2014). Recently, (Bridgeman et al., 2016) provide preclinical evidence that combining TKI, such as sunitinib or pazopanib, with a MEK inhibitor (MEKI) is a rationale and efficacious treatment regimen for RCC, showing a more effective suppression of
tumor growth and tumor angiogenesis in vivo. Furthermore, it has also been reported the enhanced antitumor activity of a new combination regimen containing MEK inhibitor, binimetinib (MEK162), paclitaxel and bevacizumab in platinumrelapsing ovarian patient-derived xenografts (PDX) (Ricci et al., 2017). These results support the ongoing clinical development of MEK inhibitors and VEGF targeted combination therapy (Table 2). In particular, a phase II clinical trial (NCT01204177) investigating refametinib, a potent MEK1/2 inhibitor, in combination with sorafenib as a first-line treatment for subjects with advanced HCC showed that the combination of the two drugs appeared clinically active. Of notice, the majority of patients who responded to this regimen had mutant KRAS tumors (Lim et al., 2014). Further clinical trials are currently being conducted to explore this observation (Table 2).

Targeting VEGF in Combination with Immunotherapy

Immune checkpoint inhibition is exploiting in various tumors with promising results. The programmed death protein 1 (PD-1), its ligand the programmed death ligand 1 (PD-L1) and the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) are negative regulators of T-cell immune function (Figure 2). Direct stimulation of the immune system with immune checkpoint inhibitors, such as antibody against PD1-1/PD-L1 and CTLA-4 has been reported in multiple cancers, resulting in several promising clinical trials (Mahoney et al., 2015; Callahan et al., 2016). Despite these exciting results, clinical responses are of limited duration (Sharma and Allison, 2017). A challenging aspect for the development of immune-therapies will be their inclusion in the current therapeutic strategies. Notably, tumor vasculature is an important co-regulator of the immune system and different anti-angiogenesis pathways interact with antitumor immunity through multiple mechanisms (Motz et al., 2014). Of great interest, VEGF was recognized as one of the critical molecule of immune suppression. VEGF reduces adhesion molecules expression on endothelial cells, such as intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) impairing leukocyte-endothelial interactions and leukocyte entry in injured tissues. This process results in a dysfunctional tumor vasculature and hinders the immune T effector cell infiltration into the tumors (Ohm, 2003; Motz et al., 2014) and correlates with increased PD-1 expression on CD8 T cells (Voron et al., 2015). In addition to direct effects on T cells, VEGF suppresses dendritic cell differentiation and activity (Gabrilovich et al., 1998) and expands T regulatory cell (Terme et al., 2013) and myeloid-derived suppressor cells (Huang et al., 2007). In patients with CRC bevacizumab improved the antigen-presenting capacity of circulating dendritic cells (Osada et al., 2008), revealing an additional mechanism for bevacizumab on immune functions in the context of checkpoint blockade. Interestingly, it has been recently shown that high serum levels of VEGF were associated with decreased OS in advanced melanoma patients treated with ipilimumab, an anti-CTLA4 antibody (Yuan et al., 2014). In line with this, VEGF was decreased in patients with metastatic melanoma responding to sequential anti-CTLA4 and anti-PD-1 therapy but increased in non-responders (Chen et al., 2016) indicating a mechanism of therapeutic resistance and a potential target to therapy (Ott et al., 2015; Voron et al., 2015).

Besides to the effects on tumor vasculature, VEGF blockade may have a positive impact on the immune mechanisms leading to an anti-tumor response and preclinical studies support the possibility to exploit anti-angiogenesis inhibitors in association with molecule regulating innate and adaptive immunity. It has been reported in preclinical models of melanoma that blockade of the VEGF/VEGFR2 pathway increased the antitumor activity of adoptively transferred T-cells (Shrimali et al., 2010) and the combination of blocking VEGFR2 by the specific monoclonal antibody DC101 with a cancer vaccination showed a great anti-tumor effect by favoring CD8⁺ T cell recruitment and reducing the number of regulatory T cells, which have tumor immune-suppressive function (Huang et al., 2012).

The positive effect on immune response obtained by halting VEGF pathway can be further increased by combining the block of Ang-2. A bispecific antibody, which bind both VEGFA and Ang-2 showed a better effect as compared to the single block, in many pre-clinical models and synergized with PD-1 blockade. Mechanistically, the antagonistic effect on these two angiogenic molecules favors the vascular normalization with a more efficient recruitment of CD8⁺ T, which is concomitantly characterized by the up-regulation of PD-L1 on perivascular T cells (Schmittnaegel et al., 2017).

Further, the addition of anti-PD1 antibody to the CXCR4 inhibitor AMD3100 and sorafenib augments the antitumor immune responses mediated by CD8⁺ T cells in an orthotopic murine models of HCC. The triple association showed a significant activity both on primary tumors and on the lung metastatic spreading (Chen et al., 2015).

More recently, a preclinical study provided evidences that anti-PD-1 or anti PD-L1 therapy sensitized and prolonged the efficacy of antiangiogenic therapy, and conversely, antiangiogenic therapy improved anti-PD-L1 treatment by supporting vascular changes, such as vessel normalization and high endothelial venules formation, that facilitate enhanced cytotoxic T cell infiltration and subsequent tumor cell destruction (Allen et al., 2017).

Based on these preclinical and translational data supporting synergy between angiogenesis inhibitors and checkpoint blockers, multiple trials of combinatorial therapies are under way and some have produced encouraging results. For example a phase I trial data of combination of bevacizumab and ipilimumab in patients with advanced melanoma showed disease control and increased CD8 T-cell tumor infiltration, resulting in durable patient response of more than 6 months (Hodi et al., 2014; Ott et al., 2015).

Other clinical trials are evaluating the combination between anti-angiogenic regimens and antibody targeting PD1 (nivolumab, pembrolizumab) and PDL1 (MPDL-3280A) (**Table 3**). TABLE 3 Selected Clinical Trials of VEGF-targeted therapy in combination with immune checkpoint inhibitors (July 2017).

Anti-angiogenic	Immunotherapy	Phase	Indications	ClinicalTrials.gov Identifier
Bevacizumab	Ipilimumab	2	Melanoma	NCT01950390
Bevacizumab	Ipilimumab	1	Melanoma	NCT00790010
Bevacizumab	Atezolizumab	2	CRC	NCT02982694
Bevacizumab	Atezolizumab	2	Melanoma brain metastases	NCT03175432
Bevacizumab	Atezolizumab	2	RCC	NCT02724878
Bevacizumab	Atezolizumab	3	RCC	NCT02420821
Bevacizumab	Nivolumab	2	Ovarian, Fallopian Tube Or Peritoneal Cancer	NCT02873962
Bevacizumab	Nivolumab	3	Glioblastoma	NCT02017717
Bevacizumab	Nivolumab	1	NSCLC	NCT01454102
Bevacizumab	Nivolumab	1	RCC	NCT02210117
Bevacizumab	Pembrolizumab	2	RCC	NCT02348008
Bevacizumab	Pembrolizumab	1/2	NSCLC	NCT02039674
Bevacizumab	Pembrolizumab	2	Glioblastoma	NCT02337491
Bevacizumab	Pembrolizumab	2	Melanoma/NSCLC brain metastases	NCT02681549
Aflibercept	Pembrolizumab	1	Solid tumors	NCT02298959
Sunitinib	Nivolumab	1	RCC	NCT01472081
Axitinib	Pembrolizumab	3	RCC	NCT02853331
Axitinib	Avelumab	3	RCC	NCT02684006
Cabozantinib	Nivolumab	3	RCC	NCT03141177

CRC, colorectal cancer; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma. For reference see https://clinicaltrials.gov.

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CONCLUSION

Preclinical findings show that single-drug antiangiogenic therapy delayed tumor growth but it was unable to determine tumor regression (Jayson et al., 2016) and in general, clinical efficacy of anti-angiogenic agents is lower than that observed in preclinical cancer models (Ebos and Kerbel, 2011) with significant adverse effects. The mechanisms that restrain the therapeutic efficacy of anti-angiogenic drugs in cancer are still poor comprehended. Moreover, an essential issue in the smart development of these compound is the identification of predictive biomarkers to find responder and non-responder patients. However biomarkers that are predictive of response to anti-angiogenic therapy in patients remain elusive (Jain et al., 2009; Vasudev and Reynolds, 2014; Jayson et al., 2016) and the patients' stratification on the basis of the drivers mutations and on feature of transcriptomic landscape including both gene coding and noncoding RNAs could really ameliorate the selection of responder patients.

Furthermore, biomarkers analysis and identification could represent the rationale for novel and combinatorial

REFERENCES

- Abbosh, C., Birkbak, N. J., Wilson, G. A., Jamal-Hanjani, M., Constantin, T., Salari, R., et al. (2017). Phylogenetic ctDNA analysis depicts early-stage lung cancer evolution. *Nature* 545, 446–451. doi: 10.1038/nature22364
- Abramsson, A., Lindblom, P., and Betsholtz, C. (2003). Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence

approaches, which could improve the clinical outcome exerted by angiogenesis inhibition. In particular oncogenes and immune response play a central role in the regulation of tumor angiogenesis and for this reason represent two attractive targets to develop combinatorial strategies. Many preclinical studies encourage the clinical exploitation of this approach.

AUTHOR CONTRIBUTIONS

VC contributed to the research, figure design, and writing of manuscript. FB contributed to the research, editing, and overall design of manuscript.

ACKNOWLEDGMENTS

This work was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC) investigator grants IG (18652) and AIRC 5 X 1000 (12182); Fondo investimenti per la Ricerca di Base (codes: RBAP11BYNP), Fondazione Cassa di Risparmio di Torino and University of Torino- Compagnia di San Paolo.

vascular pattern formation in tumors. J. Clin. Invest. 112, 1142–1151. doi: 10.1172/JCI200318549

Aguilera, K. Y., Rivera, L. B., Hur, H., Carbon, J. G., Toombs, J. E., Goldstein, C. D., et al. (2014). Collagen signaling enhances tumor progression after anti-VEGF therapy in a murine model of pancreatic ductal adenocarcinoma. *Cancer Res.* 74, 1032–1044. doi: 10.1158/0008-5472.CAN-13-2800

- Akbari, A., Amanpour, S., Muhammadnejad, S., Ghahremani, M. H., Ghaffari, S. H., Dehpour, A. R., et al. (2014). Evaluation of antitumor activity of a TGFbeta receptor I inhibitor (SD-208) on human colon adenocarcinoma. DARU J. Pharm. Sci. 22:47. doi: 10.1186/2008-2231-22-47
- Allen, E., Jabouille, A., Rivera, L. B., Lodewijckx, I., Missiaen, R., Steri, V., et al. (2017). Combined antiangiogenic and anti–PD-L1 therapy stimulates tumor immunity through HEV formation. *Sci. Transl. Med.* 9:eaak9679. doi: 10.1126/scitranslmed.aak9679
- Allen, E., Walters, I. B., and Hanahan, D. (2011). Brivanib, a dual FGF/VEGF inhibitor, is active both first and second line against mouse pancreatic neuroendocrine tumors developing adaptive/evasive resistance to VEGF inhibition. *Clin. Cancer Res.* 17, 5299–5310. doi: 10.1158/1078-0432.CCR-10-2847
- Arbiser, J. L. (2004). Molecular regulation of angiogenesis and tumorigenesis by signal transduction pathways: evidence of predictable and reproducible patterns of synergy in diverse neoplasms. *Semin. Cancer Biol.* 14, 81–91. doi: 10.1016/j.semcancer.2003.09.013
- Batchelor, T. T., Duda, D. G., di Tomaso, E., Ancukiewicz, M., Plotkin, S. R., Gerstner, E., et al. (2010). Phase II study of cediranib, an oral pan-vascular endothelial growth factor receptor tyrosine kinase inhibitor, in patients with recurrent glioblastoma. J. Clin. Oncol. 28, 2817–2823. doi: 10.1200/JCO.2009.26.3988
- Batchelor, T. T., Gerstner, E. R., Emblem, K. E., Duda, D. G., Kalpathy-Cramer, J., Snuderl, M., et al. (2013). Improved tumor oxygenation and survival in glioblastoma patients who show increased blood perfusion after cediranib and chemoradiation. *Proc. Natl. Acad. Sci. U.S.A.* 110, 19059–19064. doi: 10.1073/pnas.1318022110
- Bear, H. D., Tang, G., Rastogi, P., Geyer, C. E., Robidoux, A., Atkins, J. N., et al. (2012). Bevacizumab added to neoadjuvant chemotherapy for breast cancer. N. Engl. J. Med. 366, 310–320. doi: 10.1056/NEJMoa1111097
- Bergers, G., and Hanahan, D. (2008). Modes of resistance to anti-angiogenic therapy. Nat. Rev. Cancer 8, 592–603. doi: 10.1038/nrc2442
- Bergers, G., Song, S., Meyer-Morse, N., Bergsland, E., and Hanahan, D. (2003). Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *J. Clin. Invest.* 111, 1287–1295. doi: 10.1172/JCI200317929
- Bertolini, F., Shaked, Y., Mancuso, P., and Kerbel, R. S. (2006). The multifaceted circulating endothelial cell in cancer: towards marker and target identification. *Nat. Rev. Cancer* 6, 835–845. doi: 10.1038/nrc1971
- Boige, V., Malka, D., Bourredjem, A., Dromain, C., Baey, C., Jacques, N., et al. (2012). Efficacy, Safety, and Biomarkers of Single-Agent Bevacizumab Therapy in Patients with Advanced Hepatocellular Carcinoma. *Oncology* 17, 1063–1072. doi: 10.1634/theoncologist.2011-0465
- Bottos, A., Martini, M., Di Nicolantonio, F., Comunanza, V., Maione, F., Minassi, A., et al. (2012). Targeting oncogenic serine/threonine-protein kinase BRAF in cancer cells inhibits angiogenesis and abrogates hypoxia. *Proc. Natl. Acad. Sci.* U.S.A. 109, E353–E359. doi: 10.1073/pnas.1105026109
- Bottsford-Miller, J. N., Coleman, R. L., and Sood, A. K. (2012). Resistance and escape from antiangiogenesis therapy: clinical implications and future strategies. J. Clin. Oncol. 30, 4026–4034. doi: 10.1200/JCO.2012. 41.9242
- Bridgeman, V. L., Vermeulen, P. B., Foo, S., Bilecz, A., Daley, F., Kostaras, E., et al. (2017). Vessel co-option is common in human lung metastases and mediates resistance to anti-angiogenic therapy in preclinical lung metastasis models. *J. Pathol.* 241, 362–374. doi: 10.1002/path.4845
- Bridgeman, V. L., Wan, E., Foo, S., Nathan, M. R., Welti, J. C., Frentzas, S., et al. (2016). Preclinical evidence that trametinib enhances the response to antiangiogenic tyrosine kinase inhibitors in renal cell carcinoma. *Mol. Cancer Ther.* 15, 172–183. doi: 10.1158/1535-7163.MCT-15-0170
- Brown, J. L., Cao, Z. A., Pinzon-Ortiz, M., Kendrew, J., Reimer, C., Wen, S., et al. (2010). A Human Monoclonal Anti-ANG2 antibody leads to broad antitumor activity in combination with VEGF inhibitors and chemotherapy agents in preclinical models. *Mol. Cancer Ther.* 9, 145–156. doi: 10.1158/1535-7163.MCT-09-0554
- Brufsky, A. M., Hurvitz, S., Perez, E., Swamy, R., Valero, V., O'Neill, V., et al. (2011). RIBBON-2: a randomized, double-blind, placebo-controlled, phase III trial evaluating the efficacy and safety of bevacizumab in combination with

chemotherapy for second-line treatment of human epidermal growth factor receptor. J. Clin. Oncol. 29, 4286–4293. doi: 10.1200/JCO.2010.34.1255

- Bussolino, F., Mantovani, A., and Persico, G. (1997). Molecular mechanisms of blood vessel formation. *Trends Biochem. Sci.* 22, 251–256. doi: 10.1016/S0968-0004(97)01074-8
- Bussolino, F., Wang, J. M., Defilippi, P., Turrini, F., Sanavio, F., Edgell, C. J., et al. (1989). Granulocyte- and granulocyte- macrophage-colony stimulating factors induce human endothelial cells to migrate and proliferate. *Nature* 337, 471–473. doi: 10.1038/337471a0
- Callahan, M. K., Postow, M. A., and Wolchok, J. D. (2016). Targeting T cell co-receptors for cancer therapy. *Immunity* 44, 1069–1078. doi: 10.1016/j.immuni.2016.04.023
- Cameron, D., Brown, J., Dent, R., Jackisch, C., Mackey, J., Pivot, X., et al. (2013). Adjuvant bevacizumab-containing therapy in triple-negative breast cancer (BEATRICE): primary results of a randomised, phase 3 trial. *Lancet Oncol.* 14, 933–942. doi: 10.1016/S1470-2045(13)70335-8
- Carmeliet, P., and Jain, R. K. (2000). Angiogenesis in cancer and other diseases. *Nature* 407, 249–257. doi: 10.1038/35025220
- Carmeliet, P., and Jain, R. K. (2011). Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. *Nat. Rev. Drug Discov.* 10, 417–427. doi: 10.1038/nrd3455
- Carrato, A., Swieboda-Sadlej, A., Staszewska-Skurczynska, M., Lim, R., Roman, L., Shparyk, Y., et al. (2013). Fluorouracil, leucovorin, and irinotecan plus either sunitinib or placebo in metastatic colorectal cancer: a randomized, phase III trial. J. Clin. Oncol. 31, 1341–1347. doi: 10.1200/JCO.2012.45.1930
- Casanovas, O., Hicklin, D. J., Bergers, G., and Hanahan, D. (2005). Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in latestage pancreatic islet tumors. *Cancer Cell* 8, 299–309. doi: 10.1016/j.ccr.2005. 09.005
- Cascone, T., Xu, L., Lin, H. Y., Liu, W., Tran, H. T., Liu, Y., et al. (2017). The HGF/c-MET pathway is a driver and biomarker of VEGFR-inhibitor resistance and vascular remodeling in non-small cell lung cancer. *Clin. Cancer Res. Clincanres.* 23, 5489–5501. doi: 10.1158/1078-0432.CCR-16-3216
- Chae, S. S., Kamoun, W. S., Farrar, C. T., Kirkpatrick, N. D., Niemeyer, E., de Graaf, A. M., et al. (2010). Angiopoietin-2 interferes with anti-VEGFR2-induced vessel normalization and survival benefit in mice bearing gliomas. *Clin. Cancer Res.* 16, 3618–3627. doi: 10.1158/1078-0432.CCR-09-3073
- Chae, Y. K., Ranganath, K., Hammerman, P. S., Vaklavas, C., Mohindra, N., Kalya, A., et al. (2017). Inhibition of the fibroblast growth factor receptor (FGFR) pathway: the current landscape and barriers to clinical application. *Oncotarget* 8, 16052–16074. doi: 10.18632/oncotarget.14109
- Chen, P. L., Roh, W., Reuben, A., Cooper, Z. A., Spencer, C. N., Prieto, P. A., et al. (2016). Analysis of immune signatures in longitudinal tumor samples yields insight into biomarkers of response and mechanisms of resistance to immune checkpoint blockade. *Cancer Discov.* 6, 827–837. doi: 10.1158/2159-8290.CD-15-1545
- Chen, Y., Huang, Y., Reiberger, T., Duyverman, A. M., Huang, P., Samuel, R., et al. (2014). Differential effects of sorafenib on liver versus tumor fibrosis mediated by stromal-derived factor 1 alpha/C-X-C receptor type 4 axis and myeloid differentiation antigen-positive myeloid cell infiltration in mice. *Hepatology* 59, 1435–1447. doi: 10.1002/hep.26790
- Chen, Y., Ramjiawan, R. R., Reiberger, T., Ng, M. R., Hato, T., Huang, Y., et al. (2015). CXCR4 inhibition in tumor microenvironment facilitates anti-programmed death receptor-1 immunotherapy in sorafenibtreated hepatocellular carcinoma in mice. *Hepatology* 61, 1591–1602. doi: 10.1002/hep.27665
- Chung, A. S., Wu, X., Zhuang, G., Ngu, H., Kasman, I., Zhang, J., et al. (2013). An interleukin-17-mediated paracrine network promotes tumor resistance to anti-angiogenic therapy. *Nat. Med.* 19, 1114–1123. doi: 10.1038/ nm.3291
- Ciardiello, F., Bianco, R., Caputo, R., Caputo, R., Damiano, V., Troiani, T., et al. (2004). Antitumor activity of ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in human cancer cells with acquired resistance to antiepidermal growth factor receptor therapy. *Clin. Cancer Res.* 10, 784–793. doi: 10.1158/1078-0432.CCR-1100-03
- Ciardiello, F., Caputo, R., Bianco, R., Damiano, V., Fontanini, G., Cuccato, S., et al. (2001). Inhibition of growth factor production and angiogenesis in human

cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. *Clin. Cancer Res.* 7, 1459–1465.

- Ciardiello, F., and Tortora, G. (2008). EGFR antagonists in cancer treatment. N. Engl. J. Med. 358, 1160–1174. doi: 10.1056/NEJMra0707704
- Ciombor, K. K., Berlin, J., and Chan, E. (2013). Aflibercept. Clin. Cancer Res. 19, 1920–1925. doi: 10.1158/1078-0432.CCR-12-2911
- Comunanza, V., Corà, D., Orso, F., Consonni, F. M., Middonti, E., Di Nicolantonio, F., et al. (2017). VEGF blockade enhances the antitumor effect of BRAF^{V 600E} inhibition. *EMBO Mol. Med.* 9, 219–237. doi: 10.15252/emmm.201505774
- Crawford, Y., Kasman, I., Yu, L., Zhong, C., Wu, X., Modrusan, Z., et al. (2009). PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment. *Cancer Cell* 15, 21–34. doi: 10.1016/j.ccr.2008.12.004
- Cristofanilli, M., Johnston, S. R., Manikhas, A., Gomez, H. L., Gladkov, O., Shao, Z., et al. (2013). A randomized phase II study of lapatinib + pazopanib versus lapatinib in patients with HER2+ inflammatory breast cancer. *Breast Cancer Res. Treat*. 137, 471–482. doi: 10.1007/s10549-012-2369-x
- Cunningham, D., Lang, I., Marcuello, E., Lorusso, V., Ocvirk, J., Shin, D. B., et al. (2013). Bevacizumab plus capecitabine versus capecitabine alone in elderly patients with previously untreated metastatic colorectal cancer (AVEX): An open-label, randomised phase 3 trial. *Lancet Oncol.* 14, 1077–1085. doi: 10.1016/S1470-2045(13)70154-2
- Daly, C., Eichten, A., Castanaro, C., Pasnikowski, E., Adler, A., Lalani, A. S., et al. (2013). Angiopoietin-2 functions as a Tie2 agonist in tumor models, where it limits the effects of VEGF inhibition. *Cancer Res.* 73, 108–118. doi: 10.1158/0008-5472.CAN-12-2064
- de Gramont, A., Van Cutsem, E., Schmoll, H. J., Tabernero, J., Clarke, S., Moore, M. J., et al. (2012). Bevacizumab plus oxaliplatin-based chemotherapy as adjuvant treatment for colon cancer (AVANT): a phase 3 randomised controlled trial. *Lancet Oncol.* 13, 1225–1233. doi: 10.1016/S1470-2045(12)70 509-0
- Degrauwe, N., Sosa, J. A., Roman, S., and Deshpande, H. A. (2012). Vandetanib for the treatment of metastatic medullary Thyroid Cancer. *Clin. Med. Insights. Oncol.* 6, 243–252. doi: 10.4137/CMO.S7999
- Dellapasqua, S., Bertolini, F., Bagnardi, V., Campagnoli, E., Scarano, E., Torrisi, R., et al. (2008). Metronomic Cyclophosphamide and Capecitabine Combined with Bevacizumab in Advanced Breast Cancer. J. Clin. Oncol. 26, 4899–4905. doi: 10.1200/JCO.2008.17.4789
- Demetri, G. D., Reichardt, P., Kang, Y. K., Blay, J. Y., Rutkowski, P., Gelderblom, H., et al. (2013). Efficacy and safety of regorafenib for advanced gastrointestinal stromal tumours after failure of imatinib and sunitinib (GRID): an international, multicentre, randomised, placebo-controlled, phase 3 trial. *Lancet* 381, 295–302. doi: 10.1016/S0140-6736(12)61857-1
- Donnem, T., Hu, J., Ferguson, M., Adighibe, O., Snell, C., Harris, A. L., et al. (2013). Vessel co-option in primary human tumors and metastases: an obstacle to effective anti-angiogenic treatment? *Cancer Med.* 2, 427–436. doi: 10.1002/cam4.105
- Earl, H. M., Hiller, L., Dunn, J. A., Blenkinsop, C., Grybowicz, L., Vallier, A. L., et al. (2015). Efficacy of neoadjuvant bevacizumab added to docetaxel followed by fluorouracil, epirubicin, and cyclophosphamide, for women with HER2-negative early breast cancer (ARTemis): an open-label, randomised, phase 3 trial. *Lancet Oncol.* 16, 656–666. doi: 10.1016/S1470-2045(15)70 137-3
- Ebos, J. M., and Kerbel, R. S. (2011). Antiangiogenic therapy: impact on invasion, disease progression, and metastasis. *Nat. Rev. Clin. Oncol.* 8, 210–221. doi: 10.1038/nrclinonc.2011.21
- Ebos, J. M. L., Lee, C. R., Cruz-Munoz, W., Bjarnason, G. A., Christensen, J. G., and Kerbel, R. S. (2009). Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell* 15, 232–239. doi: 10.1016/j.ccr.2009.01.021
- El-Kenawi, A. E., and El-Remessy, A. B. (2013). Angiogenesis inhibitors in cancer therapy: mechanistic perspective on classification and treatment rationales. *Br. J. Pharmacol.* 170, 712–729. doi: 10.1111/bph.12344
- Emblem, K. E., Mouridsen, K., Bjornerud, A., Farrar, C. T., Jennings, D., Borra, R. J., et al. (2013). Vessel architectural imaging identifies cancer patient responders to anti-angiogenic therapy. *Nat. Med.* 19, 1178–1183. doi: 10.1038/nm.3289

- Fais, S., Venturi, G., and Gatenby, B. (2014). Microenvironmental acidosis in carcinogenesis and metastases: new strategies in prevention and therapy. *Cancer Metastasis Rev.* 33, 1095–1108. doi: 10.1007/s10555-014-9531-3
- Fala, L. (2015). Lenvima (Lenvatinib), a multireceptor tyrosine kinase inhibitor, approved by the FDA for the treatment of patients with differentiated Thyroid Cancer. *Am. Heal. Drug Benefits* 8, 176–179.
- Fan, L. C., Teng, H. W., Shiau, C. W., Tai, W. T., Hung, M. H., Yang, S. H., et al. (2016). Regorafenib (Stivarga) pharmacologically targets epithelialmesenchymal transition in colorectal cancer. *Oncotarget* 7, 64136–64147. doi: 10.18632/oncotarget.11636
- Ferrara, N. (2002). VEGF and the quest for tumour angiogenesis factors. Nat. Rev. Cancer 2, 795–803. doi: 10.1038/nrc909
- Ferrara, N., and Kerbel, R. S. (2005). Angiogenesis as a therapeutic target. *Nature* 438, 967–974. doi: 10.1038/nature04483
- Ferrara, N., Hillan, K. J., Gerber, H.-P., and Novotny, W. (2004). Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat. Rev. Drug Discov.* 3, 391–400. doi: 10.1038/nrd1381
- Fischer, C., Jonckx, B., Mazzone, M., Zacchigna, S., Loges, S., Pattarini, L., et al. (2007). Anti-PIGF inhibits growth of VEGF(R)-inhibitorresistant tumors without affecting healthy vessels. *Cell* 131, 463–475. doi: 10.1016/j.cell.2007.08.038
- Folkman, J. (1971). Tumor angiogenesis: therapeutic implications. N. Engl. J. Med. 285, 1182–1186. doi: 10.1056/NEJM197111182852108
- Folkman, J. (2006). Angiogenesis. Annu. Rev. Med. 57, 1–18. doi: 10.1146/annurev.med.57.121304.131306
- Frentzas, S., Simoneau, E., Bridgeman, V. L., Vermeulen, P. B., Foo, S., Kostaras, E., et al. (2016). Vessel co-option mediates resistance to anti-angiogenic therapy in liver metastases. *Nat. Med.* 22, 1294–1302. doi: 10.1038/nm.4197
- Friedman, H. S., Prados, M. D., Wen, P. Y., Mikkelsen, T., Schiff, D., Abrey, L. E., et al. (2009). Bevacizumab alone and in combination with irinotecan in recurrent glioblastoma. J. Clin. Oncol. 27, 4733–4740. doi: 10.1200/JCO.2008.19.8721
- Fuchs, C. S., Tomasek, J., Yong, C. J., Dumitru, F., Passalacqua, R., Goswami, C., et al. (2014). Ramucirumab monotherapy for previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (REGARD): an international, randomised, multicentre, placebo-controlled, phase 3 trial. *Lancet* 383, 31–39. doi: 10.1016/S0140-6736(13)61719-5
- Gabrilovich, D., Ishida, T., Oyama, T., Ran, S., Kravtsov, V., Nadaf, S., et al. (1998). Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages *in vivo. Blood* 92, 4150–4166.
- Gianni, L., Romieu, G. H., Lichinitser, M., Serrano, S. V., Mansutti, M., Pivot, X., et al. (2013). AVEREL: a randomized phase III Trial evaluating bevacizumab in combination with docetaxel and trastuzumab as first-line therapy for HER2-positive locally recurrent/metastatic breast cancer. J. Clin. Oncol. 31, 1719–1725. doi: 10.1200/JCO.2012.44.7912
- Giantonio, B. J., Catalano, P. J., Meropol, N. J., O'Dwyer, P. J., Mitchell, E. P., Alberts, S. R., et al. (2007). Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200. J. Clin. Oncol. 25, 1539–1544. doi: 10.1200/JCO.2006.09.6305
- Goede, V., Coutelle, O., Neuneier, J., Reinacher-Schick, A., Schnell, R., Koslowsky, T. C., et al. (2010). Identification of serum angiopoietin-2 as a biomarker for clinical outcome of colorectal cancer patients treated with bevacizumabcontaining therapy. Br. J. Cancer 103, 1407–1414. doi: 10.1038/sj.bjc.6605925
- Goel, S., Duda, D. G., Xu, L., Munn, L. L., Boucher, Y., Fukumura, D., et al. (2011). Normalization of the vasculature for treatment of cancer and other diseases. *Physiol. Rev.* 91, 1071–1121. doi: 10.1152/physrev.00038.2010
- Grothey, A., Van Cutsem, E., Sobrero, A., Siena, S., Falcone, A., Ychou, M., et al. (2013). Regorafenib monotherapy for previously treated metastatic colorectal cancer (CORRECT): an international, multicentre, randomised, placebo-controlled, phase 3 trial. *Lancet* 381, 303–312. doi: 10.1016/S0140-6736(12)61900-X
- Guerrero, P. A., and McCarty, J. H. (2017). "TGF-β activation and signaling in angiogenesis," in *Physiologic and Pathologic Angiogenesis - Signaling Mechanisms and Targeted Therapy*, eds D. Simionescu and A. Simionescu (Rijeka: InTech). doi: 10.5772/66405

- Gupta, S., and Spiess, P. E. (2013). The prospects of pazopanib in advanced renal cell carcinoma. *Ther. Adv. Urol.* 5, 223–232. doi: 10.1177/17562872134 95099
- Hanahan, D., and Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86, 353–364. doi: 10.1016/S0092-8674(00)80108-7
- Hashizume, H., Falcón, B. L., Kuroda, T., Baluk, P., Coxon, A., Yu, D., et al. (2010). Complementary actions of inhibitors of angiopoietin-2 and VEGF on tumor angiogenesis and growth. *Cancer Res.* 70, 2213–2223. doi: 10.1158/0008-5472.CAN-09-1977
- Hecht, J. R., Mitchell, E., Chidiac, T., Scroggin, C., Hagenstad, C., Spigel, D., et al. (2009). A randomized phase IIIB trial of chemotherapy, bevacizumab, and panitumumab compared with chemotherapy and bevacizumab alone for metastatic colorectal cancer. J. Clin. Oncol. 27, 672–680. doi: 10.1200/JCO.2008.19.8135
- Hodi, F. S., Lawrence, D., Lezcano, C., Wu, X., Zhou, J., Sasada, T., et al. (2014). Bevacizumab plus ipilimumab in patients with metastatic melanoma. *Cancer Immunol. Res.* 2, 632–642. doi: 10.1158/2326-6066.CIR-14-0053
- Holash, J., Davis, S., Papadopoulos, N., Croll, S. D., Ho, L., Russell, M., et al. (2002). VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11393–11398. doi: 10.1073/pnas.172398299
- Holash, J., Maisonpierre, P. C., Compton, D., Boland, P., Alexander, C. R., Zagzag, D., et al. (1999). Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. *Science* 284, 1994–1998. doi: 10.1126/science.284.5422.1994
- Hu, W., Lu, C., Dong, H. H., Huang, J., Shen, D. Y., Stone, R. L., et al. (2011). Biological roles of the delta family notch ligand Dll4 in tumor and endothelial cells in ovarian cancer. *Cancer Res.* 71, 6030–6039. doi: 10.1158/0008-5472.CAN-10-2719
- Hu, Y. L., DeLay, M., Jahangiri, A., Molinaro, A. M., Rose, S. D., Carbonell, W. S., et al. (2012). Hypoxia-induced autophagy promotes tumor cell survival and adaptation to antiangiogenic treatment in glioblastoma. *Cancer Res.* 72, 1773–1783. doi: 10.1158/0008-5472.CAN-11-3831
- Huang, D., Ding, Y., Zhou, M., Rini, B. I., Petillo, D., Qian, C. N., et al. (2010). Interleukin-8 mediates resistance to antiangiogenic agent sunitinib in renal cell carcinoma. *Cancer Res.* 70, 1063–1071. doi: 10.1158/0008-5472.CAN-09-3965
- Huang, J., Hu, W., Hu, L., Previs, R. A., Dalton, H. J., Yang, X. Y., et al. (2016). Dll4 Inhibition plus Aflibercept Markedly Reduces Ovarian Tumor Growth. *Mol. Cancer Ther.* 15, 1344–1352. doi: 10.1158/1535-7163.MCT-15-0144
- Huang, Y., Chen, X., Dikov, M. M., Novitskiy, S. V., Mosse, C. A., Yang, L., et al. (2007). Distinct roles of VEGFR-1 and VEGFR-2 in the aberrant hematopoiesis associated with elevated levels of VEGF. *Blood* 110, 624–631. doi: 10.1182/blood-2007-01-065714
- Huang, Y., Goel, S., Duda, D. G., Fukumura, D., and Jain, R. K. (2013). Vascular normalization as an emerging strategy to enhance cancer immunotherapy. *Cancer Res.* 73, 2943–2948. doi: 10.1158/0008-5472.CAN-12-4354
- Huang, Y., Yuan, J., Righi, E., Kamoun, W. S., Ancukiewicz, M., Nezivar, J., et al. (2012). Vascular normalizing doses of antiangiogenic treatment reprogram the immunosuppressive tumor microenvironment and enhance immunotherapy. *Proc. Natl. Acad. Sci. U.S.A.* 109, 17561–17566. doi: 10.1073/pnas.12153 97109
- Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., et al. (2004). Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N. Engl. J. Med.* 350, 2335–2342. doi: 10.1056/NEJMoa032691
- Ingram, D. A., Caplice, N. M., and Yoder, M. C. (2005). Unresolved questions, changing definitions, and novel paradigms for defining endothelial progenitor cells. *Blood* 106:1525 LP-1531. doi: 10.1182/blood-2005-04-1509
- Izumi, Y., Xu, L., di Tomaso, E., Fukumura, D., and Jain, R. K. (2002). Tumour biology: herceptin acts as an anti-angiogenic cocktail. *Nature* 416, 279–280. doi: 10.1038/416279b
- Jain, R. K. (2005). Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 307, 58–62. doi: 10.1126/science.1104819
- Jain, R. K. (2014). Antiangiogenesis strategies revisited: from starving tumors to alleviating hypoxia. *Cancer Cell* 26, 605–622. doi: 10.1016/j.ccell.2014.10.006
- Jain, R. K., Duda, D. G., Clark, J. W., and Loeffler, J. S. (2006). Lessons from phase III clinical trials on anti-VEGF therapy for cancer. *Nat. Clin. Pract. Oncol.* 3, 24–40. doi: 10.1038/ncponc0403

- Jain, R. K., Duda, D. G., Willett, C. G., Sahani, D. V., Zhu, A. X., Loeffler, J. S., et al. (2009). Biomarkers of response and resistance to antiangiogenic therapy. *Nat. Rev. Clin. Oncol.* 6, 327–338. doi: 10.1038/nrclinonc.2009.63
- Jakobsson, L., Bentley, K., and Gerhardt, H. (2009). VEGFRs and Notch: a dynamic collaboration in vascular patterning. *Biochem. Soc. Trans.* 37, 1233–1236. doi: 10.1042/BST0371233
- Jamal-Hanjani, M., Wilson, G. A., McGranahan, N., Birkbak, N. J., Watkins, T. B. K., Veeriah, S., et al. (2017). Tracking the evolution of non-small-cell lung cancer. N. Engl. J. Med. 376, 2109–2121. doi: 10.1056/NEJMoa1616288
- Jayson, G. C., Kerbel, R., Ellis, L. M., and Harris, A. L. (2016). Antiangiogenic therapy in oncology: current status and future directions. *Lancet* 388, 518–529. doi: 10.1016/S0140-6736(15)01088-0
- Jeong, H. S., Jones, D., Liao, S., Wattson, D. A., Cui, C. H., Duda, D. G., et al. (2015). Investigation of the lack of angiogenesis in the formation of lymph node metastases. J. Natl. Cancer Inst. 107:djv155. doi: 10.1093/jnci/djv155
- Jung, K., Heishi, T., Khan, O. F., Kowalski, P. S., Incio, J., Rahbari, N. N., et al. (2017). Ly6Clo monocytes drive immunosuppression and confer resistance to anti-VEGFR2 cancer therapy. J. Clin. Invest. 127, 3039–3051. doi: 10.1172/JCI93182
- Kamoun, W. S., Ley, C. D., Farrar, C. T., Duyverman, A. M., Lahdenranta, J., Lacorre, D. A., et al. (2009). Edema control by cediranib, a vascular endothelial growth factor receptor-targeted kinase inhibitor, prolongs survival despite persistent brain tumor growth in mice. J. Clin. Oncol. 27, 2542–2552. doi: 10.1200/JCO.2008.19.9356
- Kerbel, R. (2008). Tumor angiogenesis. N. Engl. J. Med. 358, 2039–2049. doi: 10.1056/NEJMra0706596
- Kerbel, R. S. (2015). A decade of experience in developing preclinical models of advanced- or early-stage spontaneous metastasis to study antiangiogenic drugs, metronomic chemotherapy, and the tumor microenvironment. *Cancer J.* 21, 274–283. doi: 10.1097/PPO.00000000000134
- Kienast, Y., Klein, C., Scheuer, W., Raemsch, R., Lorenzon, E., Bernicke, D., et al. (2013). Ang-2-VEGF-A crossmab, a novel bispecific human IgG1 antibody Blocking VEGF-A and Ang-2 functions simultaneously, mediates potent antitumor, antiangiogenic, and antimetastatic efficacy. *Clin. Cancer Res.* 19, 6730–6740. doi: 10.1158/1078-0432.CCR-13-0081
- Kirschmann, D. A., Seftor, E. A., Hardy, K. M., Seftor, R. E., and Hendrix, M. J. (2012). Molecular pathways: vasculogenic mimicry in tumor cells: diagnostic and therapeutic implications. *Clin. Cancer Res.* 18, 2726–2732. doi: 10.1158/1078-0432.CCR-11-3237
- Kloepper, J., Riedemann, L., Amoozgar, Z., Seano, G., Susek, K., Yu, V., et al. (2016). Ang-2/VEGF bispecific antibody reprograms macrophages and resident microglia to anti-tumor phenotype and prolongs glioblastoma survival. *Proc. Natl. Acad. Sci. U.S.A.* 113, 4476–4481. doi: 10.1073/pnas.1525360113
- Koh, Y. J., Kim, H. Z., Hwang, S. I., Lee, J. E., Oh, N., Jung, K., et al. (2010). Double antiangiogenic protein, DAAP, targeting VEGF-A and angiopoietins in tumor angiogenesis, metastasis, and vascular leakage. *Cancer Cell* 18, 171–184. doi: 10.1016/j.ccr.2010.07.001
- Konecny, G. E., Meng, Y. G., Untch, M., Wang, H.-J., Bauerfeind, I., Epstein, M., et al. (2004). Association between HER-2/neu and vascular endothelial growth factor expression predicts clinical outcome in primary breast cancer patients. *Clin. Cancer Res.* 10, 1706–1716. doi: 10.1158/1078-0432.CCR-0951-3
- Konishi, T., Huang, C. L., Adachi, M., Taki, T., Inufusa, H., Kodama, K., et al. (2000). The K-ras gene regulates vascular endothelial growth factor gene expression in non-small cell lung cancers. *Int. J.Oncol.* 16, 501–511. doi: 10.3892/ijo.16.3.501
- Kopetz, S., Hoff, P. M., Morris, J. S., Wolff, R. A., Eng, C., Glover, K. Y., et al. (2010). Phase II trial of infusional fluorouracil, irinotecan, and bevacizumab for metastatic colorectal cancer: efficacy and circulating angiogenic biomarkers associated with therapeutic resistance. J. Clin. Oncol. 28, 453–459. doi: 10.1200/JCO.2009.24.8252
- Kopp, H. G., Ramos, C. A., and Rafii, S. (2006). Contribution of endothelial progenitors and proangiogenic hematopoietic cells to vascularization of tumor and ischemic tissue. *Curr. Opin. Hematol.* 13, 175–181. doi: 10.1097/01.moh.0000219664.26528.da
- Kuczynski, E. A., Yin, M., Bar-Zion, A., Lee, C. R., Butz, H., Man, S., et al. (2016). Co-option of liver vessels and not sprouting angiogenesis drives acquired sorafenib resistance in hepatocellular carcinoma. *J. Natl. Cancer Inst.* 108:djw030. doi: 10.1093/jnci/djw030

- Kwilas, A. R., Donahue, R. N., Tsang, K. Y., and Hodge, J. W. (2015). Immune consequences of tyrosine kinase inhibitors that synergize with cancer immunotherapy. *Cancer Cell Microenviron*. 2, 1–11. doi: 10.14800/ccm.677
- Lambrechts, D., Claes, B., Delmar, P., Reumers, J., Mazzone, M., Yesilyurt, B. T., et al. (2012). VEGF pathway genetic variants as biomarkers of treatment outcome with bevacizumab: an analysis of data from the AVITA and AVOREN randomised trials. *Lancet Oncol.* 13, 724–733. doi: 10.1016/S1470-2045(12)70231-0
- Lambrechts, D., Lenz, H.-J., de Haas, S., Carmeliet, P., and Scherer, S. J. (2013). Markers of response for the antiangiogenic agent bevacizumab. J. Clin. Oncol. 31, 1219–1230. doi: 10.1200/JCO.2012.46.2762
- Lazzari, C., Karachaliou, N., Gregorc, V., Bulotta, A., Gonzalez-Cao, M., Verlicchi, A., et al. (2017). Second-line therapy of squamous non-small cell lung cancer: an evolving landscape. *Expert Rev. Respir. Med.* 11, 469–479. doi: 10.1080/17476348.2017.1326822
- Li, D., Xie, K., Ding, G., Li, J., Chen, K., Li, H., et al. (2014). Tumor resistance to anti-VEGF therapy through up-regulation of VEGF-C expression. *Cancer Lett.* 346, 45–52. doi: 10.1016/j.canlet.2013.12.004
- Li, J. L., Sainson, R. C. A., Oon, C. E., Turley, H., Leek, R., Sheldon, H., et al. (2011). DLL4-Notch signaling mediates tumor resistance to anti-VEGF therapy *in vivo*. *Cancer Res.* 71, 6073–6083. doi: 10.1158/0008-5472.CAN-11-1704
- Lim, H. Y., Heo, J., Choi, H. J., Lin, C.-Y., Yoon, J.-H., Hsu, C., et al. (2014). A phase II study of the efficacy and safety of the combination therapy of the MEK inhibitor refametinib (BAY 86-9766) Plus Sorafenib for Asian Patients with Unresectable Hepatocellular Carcinoma. *Clin. Cancer Res.* 20, 5976–5985. doi: 10.1158/1078-0432.CCR-13-3445
- Lin, S., and Gregory, R. I. (2015). MicroRNA biogenesis pathways in cancer. *Nat Rev Cancer* 15, 321–333. doi: 10.1038/nrc3932
- Llovet, J. M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J. F., et al. (2008). Sorafenib for advanced hepatocellular carcinoma. *N. Engl. J. Med.* 359, 378–390. doi: 10.1056/NEJMoa0708857
- Loges, S., Mazzone, M., Hohensinner, P., and Carmeliet, P. (2009). Silencing or fueling metastasis with VEGF inhibitors: antiangiogenesis revisited. *Cancer Cell* 15, 167–170. doi: 10.1016/j.ccr.2009.02.007
- Lu, K. V., Chang, J. P., Parachoniak, C. A., Pandika, M. M., Aghi, M. K., Meyronet, D., et al. (2012). VEGF inhibits tumor cell invasion and mesenchymal transition through a MET/VEGFR2 complex. *Cancer Cell* 22, 21–35. doi: 10.1016/j.ccr.2012.05.037
- Lu-Emerson, C., Snuderl, M., Kirkpatrick, N. D., Goveia, J., Davidson, C., Huang, Y., et al. (2013). Increase in tumor-associated macrophages after antiangiogenic therapy is associated with poor survival among patients with recurrent glioblastoma. *Neuro. Oncol.* 15, 1079–1087. doi: 10.1093/neuonc/ not082
- Lyden, D., Hattori, K., Dias, S., Costa, C., Blaikie, P., Butros, L., et al. (2001). Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat. Med.* 7, 1194–1201. doi: 10.1038/nm1101-1194
- Mahoney, K. M., Rennert, P. D., and Freeman, G. J. (2015). Combination cancer immunotherapy and new immunomodulatory targets. *Nat. Rev. Drug Discov.* 14, 561–584. doi: 10.1038/nrd4591
- Maione, F., Capano, S., Regano, D., Zentilin, L., Giacca, M., Casanovas, O., et al. (2012). Semaphorin 3A overcomes cancer hypoxia and metastatic dissemination induced by antiangiogenic treatment in mice. *J. Clin. Invest.* 122, 1832–1848. doi: 10.1172/JCI58976
- Makhoul, I., Todorova, V. K., Siegel, E. R., Erickson, S. W., Dhakal, I., Raj, V. R., et al. (2017). Germline Genetic Variants in TEK, ANGPT1, ANGPT2, MMP9, FGF2 and VEGFA are associated with pathologic complete response to Bevacizumab in Breast Cancer Patients. *PLoS ONE* 12:e0168550. doi: 10.1371/journal.pone.0168550
- Mancuso, P., Colleoni, M., Calleri, A., Orlando, L., Maisonneuve, P., Pruneri, G., et al. (2006). Circulating endothelial-cell kinetics and viability predict survival in breast cancer patients receiving metronomic chemotherapy. *Blood* 108, 452–459. doi: 10.1182/blood-2005-11-4570
- Mantovani, A., and Allavena, P. (2015). The interaction of anticancer therapies with tumor-associated macrophages. J. Exp. Med. 212, 435–445. doi: 10.1084/jem.20150295
- Martinelli, E., Troiani, T., Morgillo, F., Rodolico, G., Vitagliano, D., Morelli, M. P., et al. (2010). Synergistic antitumor activity of sorafenib in combination with

epidermal growth factor receptor inhibitors in colorectal and lung cancer cells. *Clin. Cancer Res.* 16, 4990–5001. doi: 10.1158/1078-0432.CCR-10-0923

- Masuda, C., Yanagisawa, M., Yorozu, K., Kurasawa, M., Furugaki, K., Ishikura, N., et al. (2017). Bevacizumab counteracts VEGF-dependent resistance to erlotinib in an EGFR-mutated NSCLC xenograft model. *Int. J. Oncol.* 51, 425–434. doi: 10.3892/ijo.2017.4036
- Matsuo, Y., Campbell, P. M., Brekken, R. A., Sung, B., Ouellette, M. M., Fleming, J. B., et al. (2009). K-Ras promotes angiogenesis mediated by immortalized human pancreatic epithelial cells through mitogenactivated protein kinase signaling pathways. *Mol. Cancer Res.* 7, 799–808. doi: 10.1158/1541-7786.MCR-08-0577
- Mazzocca, A., Fransvea, E., Lavezzari, G., Antonaci, S., and Giannelli, G. (2009). Inhibition of transforming growth factor β receptor I kinase blocks hepatocellular carcinoma growth through neo-angiogenesis regulation. *Hepatology* 50, 1140–1151. doi: 10.1002/hep.23118
- Meyerhardt, J. A., Ancukiewicz, M., Abrams, T. A., Schrag, D., Enzinger, P. C., Chan, J. A., et al. (2012). Phase I Study of Cetuximab, Irinotecan, and Vandetanib (ZD6474) as therapy for patients with previously treated metastastic colorectal cancer. *PLoS ONE* 7:e38231. doi: 10.1371/journal.pone.0038231
- Miles, D. W., Chan, A., Dirix, L. Y., Cortés, J., Pivot, X., Tomczak, P., et al. (2010). Phase III study of bevacizumab plus docetaxel compared with placebo plus docetaxel for the first-line treatment of human epidermal growth factor receptor 2-negative metastatic breast cancer. J. Clin. Oncol. 28, 3239–3247. doi: 10.1200/JCO.2008.21.6457
- Miller, K., Wang, M., Gralow, J., Dickler, M., Cobleigh, M., Perez, E. A., et al. (2007). Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. N. Engl. J. Med. 357, 2666–2676. doi: 10.1056/NEJMoa072113
- Mitsuhashi, A., Goto, H., Saijo, A., Trung, V. T., Aono, Y., Ogino, H., et al. (2015).
 Fibrocyte-like cells mediate acquired resistance to anti-angiogenic therapy with bevacizumab. *Nat. Commun.* 6:8792. doi: 10.1038/ncomms9792
- Miura, H., Miyazaki, T., Kuroda, M., Oka, T., Machinami, R., Kodama, T., et al. (1997). Increased expression of vascular endothelial growth factor in human hepatocellular carcinoma. J. Hepatol. 27, 854–861. doi: 10.1016/S0168-8278(97)80323-6
- Mizukami, Y., Jo, W.-S., Duerr, E.-M., Gala, M., Li, J., Zhang, X., et al. (2005). Induction of interleukin-8 preserves the angiogenic response in HIF-1αdeficient colon cancer cells. *Nat. Med.* 11, 992–997. doi: 10.1038/nm1294
- Monk, B. J., Poveda, A., Vergote, I., Raspagliesi, F., Fujiwara, K., Bae, D. S., et al. (2014). Anti-angiopoietin therapy with trebananib for recurrent ovarian cancer (TRINOVA-1): a randomised, multicentre, double-blind, placebo-controlled phase 3 trial. *Lancet Oncol.* 15, 799–808. doi: 10.1016/S1470-2045(14)70244-X
- Morotti, M., Dass, P. H., Harris, A. L., and Lord, S. (2017). Pharmacodynamic and pharmacokinetic markers for anti-angiogenic cancer therapy: implications for dosing and selection of patients. *Eur. J. Drug Metab. Pharmacokinet.* doi: 10.1007/s13318-017-0442-x. [Epub ahead of print].
- Motz, G. T., Santoro, S. P., Wang, L.-P., Garrabrant, T., Lastra, R. R., Hagemann, I. S., et al. (2014). Tumor endothelium FasL establishes a selective immune barrier promoting tolerance in tumors. *Nat. Med.* 20, 607–615. doi: 10.1038/nm.3541
- Nagy, J. A., Feng, D., Vasile, E., Wong, W. H., Shih, S.-C., Dvorak, A. M., et al. (2006). Permeability properties of tumor surrogate blood vessels induced by VEGF-A. *Lab. Investig.* 86, 767–780. doi: 10.1038/labinvest.3700436
- Napolitano, S., Martini, G., Rinaldi, B., Martinelli, E., Donniacuo, M., Berrino, L., et al. (2015). Primary and acquired resistance of colorectal cancer to anti-EGFR monoclonal antibody can be overcome by combined treatment of regorafenib with cetuximab. *Clin. Cancer Res.* 21, 2975–2983. doi: 10.1158/1078-0432.CCR-15-0020
- Naumov, G. N., Nilsson, M. B., Cascone, T., Briggs, A., Straume, O., Akslen, L. A., et al. (2009). Combined vascular endothelial growth factor receptor and Epidermal Growth Factor Receptor (EGFR) blockade inhibits tumor growth in xenograft models of EGFR inhibitor resistance. *Clin. Cancer Res.* 15, 3484–3494. doi: 10.1158/1078-0432.CCR-08-2904
- Neuzillet, C., Tijeras-Raballand, A., Cohen, R., Cros, J., Faivre, S., Raymond, E., et al. (2015). Targeting the TGFβ pathway for cancer therapy. *Pharmacol. Ther.* 147, 22–31. doi: 10.1016/j.pharmthera.2014.11.001
- O'Connor, J. P., Jackson, A., Parker, G. J., Roberts, C., and Jayson, G. C. (2012). Dynamic contrast-enhanced MRI in clinical trials of antivascular therapies. *Nat. Rev. Clin. Oncol.* 9, 167–177. doi: 10.1038/nrclinonc.2012.2

- Ohm, J. E. (2003). VEGF inhibits T-cell development and may contribute to tumor-induced immune suppression. *Blood* 101, 4878–4886. doi: 10.1182/blood-2002-07-1956
- Okada, F., Rak, J. W., Croix, B. S., Lieubeau, B., Kaya, M., Roncari, L., et al. (1998). Impact of oncogenes in tumor angiogenesis: mutant K-ras up-regulation of vascular endothelial growth factor/vascular permeability factor is necessary, but not sufficient for tumorigenicity of human colorectal carcinoma cells. *Proc. Natl. Acad. Sci. U.S.A.* 95, 3609–3614. doi: 10.1073/pnas.95.7.3609
- Okuda, T., Tasaki, T., Nakata, S., Yamashita, K., Yoshioka, H., Izumoto, S., et al. (2017). Efficacy of Combination Therapy with MET and VEGF Inhibitors for MET-overexpressing Glioblastoma. *Anticancer Res.* 37, 3871–3876.
- Osada, T., Chong, G., Tansik, R., Hong, T., Spector, N., Kumar, R., et al. (2008). The effect of anti-VEGF therapy on immature myeloid cell and dendritic cells in cancer patients. *Cancer Immunol. Immunother.* 57, 1115–1124. doi: 10.1007/s00262-007-0441-x
- Ott, P. A., Hodi, F. S., and Buchbinder, E. I. (2015). Inhibition of immune checkpoints and vascular endothelial growth factor as combination therapy for metastatic melanoma: an overview of rationale, preclinical evidence, and initial clinical data. *Front. Oncol.* 5:202. doi: 10.3389/fonc.2015.00202
- Pàez-Ribes, M., Allen, E., Hudock, J., Takeda, T., Okuyama, H., Viñals, F., et al. (2009). Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 15, 220–231. doi: 10.1016/j.ccr.2009.01.027
- Peterson, T. E., Kirkpatrick, N. D., Huang, Y., Farrar, C. T., Marijt, K. A., Kloepper, J., et al. (2016). Dual inhibition of Ang-2 and VEGF receptors normalizes tumor vasculature and prolongs survival in glioblastoma by altering macrophages. *Proc. Natl. Acad. Sci. U.S.A.* 113, 4470–4475. doi: 10.1073/pnas.1525349113
- Qayum, N., Muschel, R. J., Im, J. H., Balathasan, L., Koch, C. J., Patel, S., et al. (2009). Tumor vascular changes mediated by inhibition of oncogenic signaling. *Cancer Res.* 69, 6347–6354. doi: 10.1158/0008-5472.CAN-09-0657
- Rahbari, N. N., Kedrin, D., Incio, J., Liu, H., Ho, W. W., Nia, H. T., et al. (2016). Anti-VEGF therapy induces ECM remodeling and mechanical barriers to therapy in colorectal cancer liver metastases. *Sci. Transl. Med.* 8:360ra135. doi: 10.1126/scitranslmed.aaf5219
- Rak, J., Filmus, J., Finkenzeller, G., Grugel, S., Marmé, D., and Kerbel, R. S. (1995). Oncogenes as inducers of tumor angiogenesis. *Cancer Metastasis. Rev.* 14, 263–277. doi: 10.1007/BF00690598
- Reck, M., Kaiser, R., Mellemgaard, A., Douillard, J.-Y., Orlov, S., Krzakowski, M., et al. (2014). Docetaxel plus nintedanib versus docetaxel plus placebo in patients with previously treated non-small-cell lung cancer (LUME-Lung 1): a phase 3, double-blind, randomised controlled trial. *Lancet Oncol.* 15, 143–155. doi: 10.1016/S1470-2045(13)70586-2
- Ricci, F., Guffanti, F., Damia, G., and Broggini, M. (2017). Combination of paclitaxel, bevacizumab and MEK162 in second line treatment in platinumrelapsing patient derived ovarian cancer xenografts. *Mol. Cancer* 16:97. doi: 10.1186/s12943-017-0662-3
- Robert, N. J., Diéras, V., Glaspy, J., Brufsky, A. M., Bondarenko, I., Lipatov, O. N., et al. (2011a). RIBBON-1: randomized, double-blind, placebo-controlled, phase III trial of chemotherapy with or without bevacizumab for first-line treatment of human epidermal growth factor receptor 2-negative, locally recurrent or metastatic breast cancer. J. Clin. Oncol. 29, 1252–1260. doi: 10.1200/JCO.2010.28.0982
- Robert, N. J., Saleh, M. N., Paul, D., Generali, D., Gressot, L., Copur, M. S., et al. (2011b). Sunitinib plus paclitaxel versus bevacizumab plus paclitaxel for firstline treatment of patients with advanced breast cancer: a phase III, randomized, open-label trial. *Clin. Breast Cancer* 11, 82–92. doi: 10.1016/j.clbc.2011.03.005
- Rosell, R., Dafni, U., Felip, E., Curioni-Fontecedro, A., Gautschi, O., Peters, S., et al. (2017). Erlotinib and bevacizumab in patients with advanced non-small-cell lung cancer and activating EGFR mutations (BELIEF): an international, multicentre, single-arm, phase 2 trial. *Lancet Respir. Med.* 5, 435–444. doi: 10.1016/S2213-2600(17)30129-7
- Rubenstein, J. L., Kim, J., Ozawa, T., Zhang, M., Westphal, M., Deen, D. F., et al. (2000). Anti-VEGF antibody treatment of glioblastoma prolongs survival but results in increased vascular cooption. *Neoplasia (New York, NY)* 2, 306–314. doi: 10.1038/sj.neo.7900102
- Rugo, H. S. (2004). Bevacizumab in the Treatment of Breast Cancer: rationale and current data. *Oncologist* 9, 43–49. doi: 10.1634/theoncologist.9-suppl_1-43

- Ryan, D. P., Hong, T. S., and Bardeesy, N. (2014). Pancreatic adenocarcinoma. N. Engl. J. Med. 35, 353–354. doi: 10.1056/NEJMra1404198
- Samatar, A. A., and Poulikakos, P. I. (2014). Targeting RAS-ERK signalling in cancer: promises and challenges. *Nat. Rev. Drug Discov.* 13, 928–942. doi: 10.1038/nrd4281
- Scagliotti, G. V., Gaafar, R., Nowak, A., Vogelzang, N. J., Von Wangenheim, U., Morsli, N., et al. (2016). P2.01: LUME-MeSO: Phase II/III Study of Nintedanib + Pemetrexed/Cisplatin in Patients With Malignant Pleural Mesothelioma. J. Thorac. Oncol. 11:S216. doi: 10.1016/j.jtho.2016.08.075
- Schmittnaegel, M., Rigamonti, N., Kadioglu, E., Cassará, A., Wyser Rmili, C., Kiialainen, A., et al. (2017). Dual angiopoietin-2 and VEGFA inhibition elicits antitumor immunity that is enhanced by PD-1 checkpoint blockade. *Sci. Transl. Med.* 9:eaak9670. doi: 10.1126/scitranslmed.aak9670
- Semela, D., Piguet, A. C., Kolev, M., Schmitter, K., Hlushchuk, R., Djonov, V., et al. (2007). Vascular remodeling and antitumoral effects of mTOR inhibition in a rat model of hepatocellular carcinoma. *J. Hepatol.* 46, 840–848. doi: 10.1016/j.jhep.2006.11.021
- Semenza, G. L. (2009). Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene 29, 625–634. doi: 10.1038/onc.2009.441
- Semenza, G. L. (2014). Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. Annu. Rev. Pathol. 9, 47–71. doi: 10.1146/annurev-pathol-012513-104720
- Sennino, B., Ishiguro-Oonuma, T., Wei, Y., Naylor, R. M., Williamson, C. W., Bhagwandin, V., et al. (2012). Suppression of tumor invasion and metastasis by concurrent inhibition of c-Met and VEGF signaling in pancreatic neuroendocrine tumors. *Cancer Discov.* 2, 270–287. doi:10.1158/2159-8290.CD-11-0240
- Seto, T., Kato, T., Nishio, M., Goto, K., Atagi, S., Hosomi, Y., et al. (2014). Erlotinib alone or with bevacizumab as first-line therapy in patients with advanced nonsquamous non-small-cell lung cancer harbouring EGFR mutations (JO25567): an open-label, randomised, multicentre, phase 2 study. *Lancet Oncol.* 15, 1236–1244. doi: 10.1016/S1470-2045(14)70381-X
- Shaked, Y. (2006). Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors. *Science* 313, 1785–1787. doi: 10.1126/science.1127592
- Sharma, P., and Allison, J. P. (2017). Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential. *Cell* 161, 205–214. doi: 10.1016/j.cell.2015.03.030
- Shojaei, F., Lee, J. H., Simmons, B. H., Wong, A., Esparza, C. O., Plumlee, P. A., et al. (2010). HGF/c-Met acts as an alternative angiogenic pathway in sunitinib-resistant tumors. *Cancer Res.* 70, 10090–10100. doi: 10.1158/0008-5472.CAN-10-0489
- Shojaei, F., Wu, X., Malik, A. K., Zhong, C., Baldwin, M. E., Schanz, S., et al. (2007a). Tumor refractoriness to anti-VEGF treatment is mediated by CD11b⁺Gr1⁺ myeloid cells. *Nat. Biotechnol.* 25, 911–920. doi: 10.1038/nbt1323
- Shojaei, F., Wu, X., Zhong, C., Yu, L., Liang, X.-H., Yao, J., et al. (2007b). Bv8 regulates myeloid-cell-dependent tumour angiogenesis. *Nature* 450, 825–831. doi: 10.1038/nature06348
- Shrimali, R. K., Yu, Z., Theoret, M. R., Chinnasamy, D., Restifo, N. P., and Rosenberg, S. A. (2010). Antiangiogenic agents can increase lymphocyte infiltration into tumor and enhance the effectiveness of adoptive immunotherapy of cancer. *Cancer Res.* 70, 6171–6180. doi: 10.1158/0008-5472.CAN-10-0153
- Sikov, W. M., Berry, D. A., Perou, C. M., Singh, B., Cirrincione, C. T., Tolaney, S. M., et al. (2015). Impact of the addition of carboplatin and/or bevacizumab to neoadjuvant once-per-week paclitaxel followed by dose-dense doxorubicin and cyclophosphamide on pathologic complete response rates in stage ii to iii triple-negative breast cancer: CALGB 40603 (Alliance). J. Clin. Oncol. 33, 13–21. doi: 10.1200/JCO.2014.57.0572
- Singh, H., Brave, M., Beaver, J. A., Cheng, J., Tang, S., Zahalka, E., et al. (2017). U.S. food and drug administration approval: cabozantinib for the treatment of advanced renal cell carcinoma. *Clin. Cancer Res.* 23, hbox330–335. doi: 10.1158/1078-0432.CCR-16-1073
- Singh, M., Couto, S. S., Forrest, W. F., Lima, A., Cheng, J. H., Molina, R., et al. (2012). Anti-VEGF antibody therapy does not promote metastasis in genetically engineered mouse tumour models. *J. Pathol.* 227, 417–430. doi: 10.1002/path.4053

- Sohn, B. S., Park, S. J., Kim, J. E., Kim, K. P., Hong, Y. S., Suh, C., et al. (2014). Single-nucleotide polymorphisms in the vascular endothelial growth factor pathway and outcomes of patients treated with first-line cytotoxic chemotherapy combined with bevacizumab for advanced colorectal cancer. *Oncology* 87, 280–292. doi: 10.1159/000365593
- Sorensen, A. G., Emblem, K. E., Polaskova, P., Jennings, D., Kim, H., Ancukiewicz, M., et al. (2012). Increased survival of glioblastoma patients who respond to antiangiogenic therapy with elevated blood perfusion. *Cancer Res.* 72, 402–407. doi: 10.1158/0008-5472.CAN-11-2464
- Subbiah, V., Khawaja, M. R., Hong, D. S., Amini, B., Yungfang, J., Liu, H., et al. (2017). First-in-human trial of multikinase VEGF inhibitor regorafenib and anti-EGFR antibody cetuximab in advanced cancer patients. *JCI Insight* 2:90380. doi: 10.1172/jci.insight.90380
- Swanton, C., and Govindan, R. (2016). Clinical implications of genomic discoveries in Lung Cancer. N. Engl. J. Med. 374, 1864–1873. doi: 10.1056/NEJMra1504688
- Taverna, G., Grizzi, F., Colombo, P., and Graziotti, P. (2013). Is angiogenesis a hallmark of prostate cancer? Front. Oncol. 3:15. doi: 10.3389/fonc.2013.00015
- Teicher, B. A. (1996). A systems approach to cancer therapy. (Antioncogenics + standard cytotoxics→mechanism(s) of interaction). *Cancer Metast. Rev.* 15, 247–272. doi: 10.1007/BF00437479
- Terme, M., Pernot, S., Marcheteau, E., Sandoval, F., Benhamouda, N., Colussi, O., et al. (2013). VEGFA-VEGFR pathway blockade inhibits tumor-induced regulatory T-cell proliferation in colorectal cancer. *Cancer Res.* 73, 539–549. doi: 10.1158/0008-5472.CAN-12-2325
- Thurston, G., Noguera-Troise, I., and Yancopoulos, G. D. (2007). The Delta paradox: DLL4 blockade leads to more tumour vessels but less tumour growth. *Nat. Rev. Cancer* 7, 327–331. doi: 10.1038/nrc2130
- Tol, J., Koopman, M., Cats, A., Rodenburg, C. J., Creemers, G. J. M., Schrama, J. G., et al. (2009). Chemotherapy, Bevacizumab, and Cetuximab in Metastatic Colorectal Cancer. N. Engl. J. Med. 360, 563–572. doi: 10.1056/NEJMoa0808268
- Tolaney, S. M., Boucher, Y., Duda, D. G., Martin, J. D., Seano, G., Ancukiewicz, M., et al. (2015). Role of Vascular Density and Normalization in Response to Neoadjuvant Bevacizumab and Chemotherapy in Breast Cancer Patients. *Proc. Natl. Acad. Sci. U.S.A.* 112, 14325–14330. doi: 10.1073/pnas.1518808112
- Torimura, T., Iwamoto, H., Nakamura, T., Abe, M., Ikezono, Y., Wada, F., et al. (2016). Antiangiogenic and antitumor activities of aflibercept, a soluble VEGF receptor-1 and-2, in a mouse model of hepatocellular carcinoma. *Neoplasia* 18, 413–424. doi: 10.1016/j.neo.2016.05.001
- Tyler, T. (2012). Axitinib: newly approved for renal cell Carcinoma. J. Adv. Pract. Oncol. 3, 333–335.
- Van Cutsem, E., de Haas, S., Kang, Y.-K., Ohtsu, A., Tebbutt, N. C., Ming Xu, J., et al. (2012b). Bevacizumab in combination with chemotherapy as first-line therapy in advanced Gastric Cancer: a biomarker evaluation from the AVAGAST randomized phase III trial. J. Clin. Oncol. 30, 2119–2127. doi: 10.1200/JCO.2011.39.9824
- Van Cutsem, E., Tabernero, J., Lakomy, R., Prenen, H., Prausová, J., Macarulla, T., et al. (2012a). Addition of aflibercept to fluorouracil, leucovorin, and irinotecan improves survival in a phase III randomized trial in patients with metastatic colorectal cancer previously treated with an oxaliplatin-based regimen. J. Clin. Oncol. 30, 3499–3506. doi: 10.1200/JCO.2012.42.8201
- Vasudev, N. S., and Reynolds, A. R. (2014). Anti-angiogenic therapy for cancer: current progress, unresolved questions and future directions. *Angiogenesis* 17, 471–494. doi: 10.1007/s10456-014-9420-y
- von Minckwitz, G., Eidtmann, H., Rezai, M., Fasching, P. A., Tesch, H., Eggemann, H., et al. (2012). Neoadjuvant Chemotherapy and Bevacizumab for HER2-Negative Breast Cancer. N. Engl. J. Med. 366, 299–309. doi: 10.1056/NEJMoa1111065
- Voron, T., Colussi, O., Marcheteau, E., Pernot, S., Nizard, M., Pointet, A.-L., et al. (2015). VEGF-A modulates expression of inhibitory checkpoints on CD8+ T cells in tumors. *J. Exp. Med.* 212, 139–148. doi: 10.1084/jem.20140559
- Vredenburgh, J. J., Desjardins, A., Herndon, J. E., Marcello, J., Reardon, D. A., Quinn, J. A., et al. (2007). Bevacizumab plus irinotecan in recurrent glioblastoma multiforme. *J. Clin. Oncol.* 25, 4722–4729. doi: 10.1200/JCO.2007.12.2440

- Wang, Z., Chen, J.-Q., Liu, J., and Tian, L. (2016). Exosomes in tumor microenvironment: novel transporters and biomarkers. J. Transl. Med. 14:297. doi: 10.1186/s12967-016-1056-9
- Wang, Z., Dabrosin, C., Yin, X., Fuster, M. M., Arreola, A., Rathmell, W. K., et al. (2015). Broad targeting of angiogenesis for cancer prevention and therapy. *Semin. Cancer Biol.* 35, S224–S243. doi: 10.1016/j.semcancer.2015.01.001
- Wicki, A., and Christofori, G. (2008). "The angiogenic switch in tumorigenesis," in *Tumor Angiogenesis*, eds D. Marmé and N. Fusenig (Springer; Berlin; Heidelberg), 67–88. doi: 10.1007/978-3-540-33177-3_4
- Wilhelm, S. M., Carter, C., Tang, L. Y., Wilkie, D., McNabola, A., Rong, H., et al. (2004). BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res.* 64, 7099–7109. doi: 10.1158/0008-5472.CAN-04-1443
- Wilhelm, S. M., Dumas, J., Adnane, L., Lynch, M., Carter, C. A., Schütz, G., et al. (2011). Regorafenib (BAY 73-4506): a new oral multikinase inhibitor of angiogenic, stromal and oncogenic receptor tyrosine kinases with potent preclinical antitumor activity. *Int. J. Cancer* 129, 245–255. doi: 10.1002/ijc.25864
- Wilke, H., Muro, K., Van Cutsem, E., Oh, S.-C., Bodoky, G., Shimada, Y., et al. (2014). Ramucirumab plus paclitaxel versus placebo plus paclitaxel in patients with previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (RAINBOW): a double-blind, randomised phase 3 trial. *Lancet Oncol.* 15, 1224–1235. doi: 10.1016/S1470-2045(14)70420-6
- Xu, J., Wang, J., Xu, B., Ge, H., Zhou, X., and Fang, J.-Y. (2013). Colorectal cancer cells refractory to Anti-VEGF Treatment are vulnerable to glycolytic blockade due to persistent impairment of Mitochondria. *Mol. Cancer Ther.* 12, 717–724. doi: 10.1158/1535-7163.MCT-12-1016-T
- Yuan, J., Zhou, J., Dong, Z., Tandon, S., Kuk, D., Panageas, K. S., et al. (2014). Pretreatment Serum VEGF Is Associated with Clinical Response and Overall Survival in Advanced Melanoma Patients Treated with Ipilimumab. *Cancer Immunol. Res.* 2:127LP-132. doi: 10.1158/2326-6066.CIR-13-0163
- Zhang, M., Kleber, S., Röhrich, M., Timke, C., Han, N., Tuettenberg, J., et al. (2011). Blockade of TGF-β signaling by the TGFβR-I kinase inhibitor LY2109761 enhances radiation response and prolongs survival in Glioblastoma. *Cancer Res.* 71:7155LP-7167. doi: 10.1158/0008-5472.CAN-11-1212
- Zhang, W.-J., Li, Y., Wei, M.-N., Chen, Y., Qiu, J.-G., Jiang, Q.-W., et al. (2017). Synergistic antitumor activity of regorafenib and lapatinib in preclinical models of human colorectal cancer. *Cancer Lett.* 386, 100–109. doi: 10.1016/j.canlet.2016.11.011
- Zhou, Q., Guo, P., and Gallo, J. M. (2008). Impact of angiogenesis inhibition by sunitinib on tumor distribution of temozolomide. *Clin. Cancer Res.* 19, 1557–1566. doi: 10.1158/1078-0432.CCR-07-4544
- Zhu, A. X., Ancukiewicz, M., Supko, J. G., Sahani, D. V., Blaszkowsky, L. S., Meyerhardt, J. A., et al. (2013). Efficacy, safety, pharmacokinetics, and biomarkers of cediranib monotherapy in advanced hepatocellular carcinoma: a phase II study. *Clin. Cancer Res.* 19, 1557–1566. doi: 10.1158/1078-0432.CCR-12-3041
- Zhu, A. X., Sahani, D. V., Duda, D. G., di Tomaso, E., Ancukiewicz, M., Catalano, O. A., et al. (2009). Efficacy, safety, and potential biomarkers of sunitinib monotherapy in advanced hepatocellular carcinoma: a phase II study. J. Clin. Oncol. 27, 3027–3035. doi: 10.1200/JCO.2008.20.9908

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a past co-authorship with one of the author FB.

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ALK1Fc Suppresses the Human Prostate Cancer Growth in *in Vitro* and *in Vivo* Preclinical Models

OPEN ACCESS

Edited by:

Caterina Nardella, University of Trento, Italy

Reviewed by:

Nan-Shan Chang, National Cheng Kung University, Taiwan Anca Maria Cimpean, "Victor Babes" University of Medicine and Pharmacy Timisoara, Romania

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Specialty section:

This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: 31 May 2017 Accepted: 22 November 2017 Published: 05 December 2017

Citation:

Astrologo L, Zoni E, Karkampouna S, Gray PC, Klima I, Grosjean J, Goumans MJ, Hawinkels LJAC, van der Pluijm G, Spahn M, Thalmann GN, ten Dijke P and Kruithof-de Julio M (2017) ALK1Fc Suppresses the Human Prostate Cancer Growth in in Vitro and in Vivo Preclinical Models. Front. Cell Dev. Biol. 5:104. doi: 10.3389/fcell.2017.00104 Letizia Astrologo^{1‡}, Eugenio Zoni^{1,2‡}, Sofia Karkampouna^{1,3‡}, Peter C. Gray^{4†}, Irena Klima¹, Joël Grosjean¹, Marie J. Goumans³, Lukas J. A. C. Hawinkels^{3,5}, Gabri van der Pluijm², Martin Spahn¹, George N. Thalmann¹, Peter ten Dijke³ and Marianna Kruithof-de Julio^{1,2,3*}

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Prostate cancer is the second most common cancer in men and lethality is normally associated with the consequences of metastasis rather than the primary tumor. Therefore, targeting the molecular pathways that underlie dissemination of primary tumor cells and the formation of metastases has a great clinical value. Bone morphogenetic proteins (BMPs) play a critical role in tumor progression and this study focuses on the role of BMP9- Activin receptor-Like Kinase 1 and 2 (ALK1 and ALK2) axis in prostate cancer. In order to study the effect of BMP9 in vitro and in vivo on cancer cells and tumor growth, we used a soluble chimeric protein consisting of the ALK1 extracellular domain (ECD) fused to human Fc (ALK1Fc) that prevents binding of BMP9 to its cell surface receptors and thereby blocks its ability to activate downstream signaling. ALK1Fc sequesters BMP9 and the closely related BMP10 while preserving the activation of ALK1 and ALK2 through other ligands. We show that ALK1Fc acts in vitro to decrease BMP9-mediated signaling and proliferation of prostate cancer cells with tumor initiating and metastatic potential. In line with these observations, we demonstrate that ALK1Fc also reduces tumor cell proliferation and tumor growth in vivo in an orthotopic transplantation model, as well as in the human patient derived xenograft BM18. Furthermore, we also provide evidence for crosstalk between BMP9 and NOTCH and find that ALK1Fc inhibits NOTCH signaling in human prostate cancer cells and blocks the induction of the NOTCH target Aldehyde dehydrogenase member ALDH1A1, which is a clinically relevant marker associated with poor survival and advanced-stage prostate cancer. Our study provides the first demonstration that ALK1Fc inhibits prostate cancer progression, identifying BMP9 as a putative therapeutic target and ALK1Fc as a potential therapy. Altogether, these findings support the validity of ongoing clinical development of drugs blocking ALK1 and ALK2 receptor activity.

Keywords: BMP9, ALK1, ALK2, ALK1Fc, NOTCH, prostate cancer

INTRODUCTION

Prostate cancer is the second most common cancer in men worldwide (Jemal et al., 2010). Currently prostate cancer, when still in its first phase of androgen dependency, can be successfully treated surgically. However, if the tumor becomes androgen independent, therapy is no longer possible and lethality is almost invariably due to the consequences of metastasis. Therefore, understanding the molecular pathways that underlie the emergence and spread of metastases from primary tumors is of great biological and clinical value.

Expression of several BMPs has been examined in prostatic tissue with benign prostatic hyperplasia (BPH), non-metastatic and metastatic prostatic adenocarcinoma and has been associated with cancer aggressiveness (Ye et al., 2007; Ye and Jiang, 2016). Among the BMPs, BMP9 is one of the most recently identified (Song et al., 1995). Little is known about the roles of BMP9 and its cell surface signaling receptors, ALK1 and ALK2, in prostate cancer and particularly in androgen independent and metastatic prostate cancer. Current research has not only attributed a tumor-promoting role to BMP9 (Herrera et al., 2009, 2013; Li et al., 2014) but also tumor suppressing properties (Ye et al., 2008; Wang et al., 2011; Olsen et al., 2014) in different types of cancer, including prostate cancer.

Previous studies have highlighted the role of ALK1, which is predominantly expressed by endothelial cells (van Meeteren et al., 2012), as key regulator of angiogenesis in normal tissue and in tumors (Hawinkels et al., 2013; Bendell et al., 2014). BMP9 and BMP10 are high affinity ligands for ALK1, while BMP9 signals through the BMP type I receptor ALK2 (David et al., 2007; Herrera et al., 2009; Bragdon et al., 2011). Binding of BMP9/BMP10 to ALK1/ALK2 results in phosphorylation and activation of downstream effectors SMAD1 and/or SMAD5 (David et al., 2007; Scharpfenecker et al., 2007; Herrera et al., 2009). BMP9 promotes human epithelial ovarian cancer and human immortalized ovarian surface epithelial cell proliferation through ALK2/SMAD1/SMAD4 pathway (Herrera et al., 2009). Similarly, BMP9 stimulates proliferation of liver cancer cells (Herrera et al., 2013) and osteosarcoma growth (Li et al., 2014).

Several studies have highlighted the role of BMP9/ALK1 in blood vessel formation, outlining its critical involvement in pathological and tumor angiogenesis (Urness et al., 2000; Cunha and Pietras, 2011). Interestingly, alterations of signal transduction pathways that are important for blood vessel formation, such as the NOTCH pathway, have also been associated with arterio-venous malformations (Gale et al., 2004; Krebs et al., 2004). Recently, BMP9 and BMP10 signaling were linked to NOTCH signaling, one of the major pathways involved in prostate cancer development, progression and bone metastasis (Carvalho et al., 2014; Kron et al., 2017; Zhang et al., 2017). Expression profiling studies have shown that members of the NOTCH pathway are characteristic of high-grade (Gleason 4 + 4 = 8) micro-dissected prostate cancer cells compared to low-grade (Gleason 3 + 3 = 6) (Ross et al., 2011). Moreover, inhibition of NOTCH1 reduces prostate cancer cell growth, migration and invasion (Wang et al., 2010). Interestingly, the NOTCH signaling indirectly activates the enzymatic activity of ALDH1A1, a well-known marker of prostate cancer stem cells (Ginestier et al., 2007; Li et al., 2010; Le Magnen et al., 2013; Zhao et al., 2014), which are thought to be responsible for tumor recurrence, metastasis and cancer related death (Moltzahn and Thalmann, 2013).

In order to understand the role of BMP9 in prostate cancer progression, we employed the soluble chimeric protein ALK1Fc (ACE-041) (Seehra et al., 2009) which binds BMP9 and BMP10 with high affinity and blocks their signaling via ALK1 and ALK2 receptors by acting as a ligand trap (Cunha et al., 2010; Mitchell et al., 2010). Phase I clinical trials have been completed using ALK1Fc as anti-angiogenesis therapy in myeloma (clinicaltrials. gov identifier NCT00996957). Here we show that ALK1Fc reduces BMP9 signaling and decreases proliferation of highly metastatic human prostate cancer cells in vitro. We further demonstrate that ALK1Fc impairs tumor angiogenesis, affects tumor cell proliferation and reduces tumor growth in vivo. Taken together these data suggest BMP9 as a possible therapeutic target in prostate cancer and provide a new rationale for ongoing clinical development of drugs blocking BMP9 signaling via ALK1 and ALK2.

MATERIALS AND METHODS

Cell Line and Culture Conditions

The human osteotropic prostate cancer cell line PC-3M-Pro4-Luc2 (Kroon et al., 2014; Zoni et al., 2015, 2017) was maintained in DMEM supplemented with 10% FCII, 0.8 mg/ml Neomycin (Santacruz, Dallas, USA) and 1% Penicillin-Streptomycin (Life Technologies, Carlsbad, USA).

Recombinant Proteins and Chemical Inhibitors

ALK1Fc (de Vinuesa et al., 2016) is a fusion protein comprised of the extracellular domain (ECD) of human ALK1 fused to the Fc region of IgG and was obtained from Acceleron Pharma, Cambridge, USA. As a control we used either the Fc domain of IgG₁ (MOPC-21; Bio Express, West Lebanon NH) or normal goat IgG from R&D System.

Recombinant human BMP9 was obtained from R&D System, whereas the chemical inhibitor LDN193189 was purchased from Axon Medchem. The final concentration for the *in vitro* experiments was 1 nM for BMP9 and 120 nM for LDN193189.

Lentiviral-Mediated RNA Interference of NOTCH1

shRNAi for NOTCH1 (TRC000000350253, TRC000000350330, TRC0000003361, TRC0000003360) were obtained from Sigma MISSION library and used for lentiviral vector production and transduction as described previously (Zoni et al., 2017). Scramble shRNA (NT; SHC002, Sigma) was used as control. The transduced cells underwent puromycin selection and used for further experiments as described below. The experiments were carried out in accordance with standard biosecurity procedures.

Luciferase Reporter Assays and Constructs

PC-3MPro4 cells were seeded at density of 50,000 cells in 500 μ L medium in a 24-well plate. Transient transfection of reporter constructs was performed with Lipofectamine2000 (Life Technologies) according to the manufacturer's protocol. For each well, 100 ng of NICD-ff-luciferase, 10 ng CAGGS-Renilla luciferase, 100 ng BRE renilla (Korchynskyi and ten Dijke, 2002) and 100 ng BREluc/well were transfected. After 24 h, medium was replaced and cells were treated with BMP9 for 24 h. The *Firefly* luciferase and *Renilla* luciferase levels in the lysates were measured using Dual Luciferase Assay (Promega, Madison, USA).

RNA Isolation and Real-Time Q-PCR

Total RNA was isolated from PC-3M-Pro4-Luc2 cells with Trizol Reagent (Invitrogen, Waltham, USA) and cDNA was synthesized by reverse transcription (Promega, Madison, USA) according to the manufacturer's protocol. qRT-PCR was performed with Biorad CFX96 system (Biorad, Veenendaal, The Netherlands). Gene expression was normalized to *GAPDH* or β -actin. Total RNA from frozen section (5 μ m) was isolated with Qiagen Mini Isolation kit (Venlo, The Netherlands) according to the manufacturer's protocol. Primer sequences are listed in Supplementary Table I.

MTS Assay

Cells were seeded at density of 2,000 cells/well in low serum condition (0.3% FCII), treated with ALK1Fc or Control-Fc (CFc) (10 μ g/ml, Acceleron, USA) and allowed to grow for 24, 48, 72, and 96 h. After incubation, 20 μ l of 3-(4,5 dimethylthiazol- 2-yl)- 5 -(3 -carboxymethoxyphenyl)- 2 -(4 -sulfophenyl)- 2 H-tetrazolium (MTS) was added and mitochondrial activity was measured after 2 h incubation at 37°C. MTS absorbance values are positively proportional to total number of metabolically active cells providing an indirect correlation with cell proliferation rate (CellTiter96 Aqueous Non-radioactive Cell proliferation assay, Promega) (Berridge et al., 2005).

Animals

Male 6–8 week-old athymic nude (Balb/c *nu/nu*) or CB17 SCID mice, purchased from Charles River (L'Arbresle, France), were used in all *in vivo* experiments. Mice were housed in individual ventilated cages under sterile condition, and sterile food and water were provided *ad libitum*. Animal experiments were approved by the local committee for animal health ethics and research of Leiden University (DEC #11246) and Canton of Bern, Switzerland (Permit Number: BE55/16), and carried out in accordance with European Communities Council Directive 86/609/EEC and Swiss Guidelines for the Care and Use of Laboratory Animals.

Orthotopic Prostate Transplantation and ALK1Fc Treatment

25,000 PC-3M-Pro4-Luc2 cells (10 μ l final volume) were injected in the dorsal prostate lobe of anesthetized male nude mice. In brief: After anesthetizing the mice with isoflurane, each mouse was placed on its back and a small incision was made along the lower midline of the peritoneum for about 1 cm. The prostate dorsal lobes were exteriorized and stabilized gently. A 30-gauge needle attached to a 1-cc syringe was inserted into the right dorsal lobe of the prostate. 10 µl of the cell suspension was slowly injected. A well-localized bleb indicates a successful injection. After retracting the needle, a Q-tip was placed over the injection site for about 1 min to prevent bleeding and spillage of material. The prostate was then returned to the peritoneum and the abdominal wall and skin layer was sutured. After establishment of the primary tumor, at 10 days after the orthotopic transplantation, mice were intraperitoneally injected with Control-Fc (CFc) or ALK1Fc compounds (10 mg/kg) twice per week. Administration of compounds was performed for 4 weeks. After the experimental periods, mice were injected with hypoxia probe (6 mg/kg, Burlington, Massachusetts, USA) and lectin-Tomato (1 mg/kg, Vector Laboratories, USA) intravenously prior to perfusion and sacrificed according to our mouse protocol. Tumors were dissected and processed for further histomorphological analysis as described below.

Subcutaneously BM18 Transplantation and ALK1Fc Treatment

BM18 xenografts were transplanted subcutaneously in CB17 SCID mice anesthetized with a cocktail of medetomidin (1 mg/kg body weight), midazolam (10 mg/kg) and fentanyl (0.1 mg/kg) (Schwaninger et al., 2007). After 1 week, the animals were intraperitoneally injected with ALK1Fc or IgG at the dose of 10 mg/kg once a week, for 5 weeks. Every week the tumors were measured with the caliper and finally dissected and fixed in 4% paraformaldehyde (PFA) for paraffin embedding and hematoxylin and eosin staining.

Whole Body Bioluminescent Imaging (BLI)

Tumor growth from orthotopic injection was monitored weekly by whole body bioluminescent imaging (BLI) using an intensified-charge-coupled device (I-CCD) video camera of the *in vivo* Imaging System (IVIS100, Xenogen/Perkin Elmer, Alameda, CA, USA) as described previously (Buijs et al., 2007; van den Hoogen et al., 2010). In the orthotopic transplantation experiment the newer IVIS Lumina II (Xenogen/Perkin Elmer, Alameda, CA, USA) was used for BLI measurements. Mice were anesthetized using isoflurane and injected intraperitoneally with 2 mg D-luciferin (Per bio Science Nederland B.V., Etten-Leur, the Netherlands). Analyses for each metastatic site were performed after definition of the region of interest and quantified with Living Image 4.2 (Caliper Life Sciences, Teralfene, Belgium). Values are expressed as relative light units (RLU) in photons/s.

Immunofluorescence

Immunofluorescence staining was performed on 5- μ m paraffin embedded sections. For antigen retrieval, sections were boiled in antigen unmasking solution (Vector Labs, Peterborough, UK) and stained with anti- pH3 (Millipore), cleaved CASP3 (Cell Signaling), CD31 (Sigma) or ALDH1A1 (Abcam) antibodies. Sections were blocked with 1% bovine serum albumin (BSA)-PBS-0.1% v/v Tween-20 and incubated with primary antibodies diluted in the blocking solution, overnight at 4°C. Sections were then incubated with secondary antibodies labeled with Alexa Fluor 488, 555, or 647 (Invitrogen/Molecular Probes, Waltham, USA) at 1:250 in PBS-0.1% Tween-20. Nuclei were visualized by TO-PRO3 (Invitrogen/Molecular Probes, 1:1000 diluted in PBS-0.1% Tween-20) (Karkampouna et al., 2014).

Western Immunoblotting

Cell lysates were prepared using RIPA buffer (Thermo Scientific) and protein concentrations were quantified according to manufacturer's protocol (Thermo Scientific). Proteins (20 μ g per sample) were separated by 15% SDS-PAGE followed by transfer to a blotting membrane. The membrane was blocked with 5% Milk, dissolved in PBS-Tween, for 1 h at room temperature. The membrane was incubated with 1:1,000 primary antibody (anti-NOTCH1, Cell Signaling, catalog number 3608) at 4°C overnight. Subsequently, the membrane was incubated with 1:10,000 secondary horseradish peroxidase (HRP) antibody. All antibodies were dissolved in PBS-Tween. Chemiluminescence was used to visualize the bands.

Clonogenic Assay

Clonogenic assay was performed in 6 well plate. 100 cells were seeded in 2 mL of medium and incubated at 37° C in presence of 5% CO₂ for 2 weeks. Plates were washed with PBS and cells fixed for 5 min with a solution of 4% PFA. Colonies were stained with 0.1% crystal violet (Sigma-Aldrich, The Netherlands) and plates were imaged before processing the data with ImageJ software (Franken et al., 2006; Rafehi et al., 2011; Guzman et al., 2014).

Prostate Cancer Dataset Analysis

The Taylor MSKCC prostate dataset was queried for BMP9 and ALK1 expression in prostate cancer patients through the online biomarker validation tool SurvExpress (http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp). The data are censored as "recurrence month," and the risk groups are defined estimating a prognostic index by the Cox model algorithm (Aguirre-Gamboa et al., 2013).

R2: Genomics Analysis and Visualization Platform (http://r2. amc.nl) was used to investigate the ALK2, JAG1 and NOTCH1 expression in benign (n = 48) vs. tumor (n = 47) tissues using the GEO accession number GSE29079 dataset (Borno et al., 2012).

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad software) using *t*-test or ANOVA for comparison between more groups. Data is presented as mean \pm SEM. *P*-values < 0.05 were considered to be statistically significant (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

RESULTS

High BMP9 and ALK1 Correlate with Recurrence in Prostate Cancer Patients

The role of BMP9 in cancer development and progression is still controversial. We analyzed a Taylor MSKCC Prostate dataset (GSE21032) through the online tool SurvExpress to assess how the expression of BMP9 and ALK1 is related with recurrence in prostate cancer. We found that the group of patients with high expression of BMP9 and ALK1 has higher probability to encounter biochemical recurrence than the group with lower levels of BMP9 and ALK1 (**Figures 1A,B** top). The SurvExpress tool also defines "high-" and "low-" risk group of patients based on the risk prognosis calculated as described in the Material and Methods. According to that definition, we also found that the high-risk group of patients had higher expression of BMP9 and ALK1 than lower risk patients (**Figures 1A,B** bottom). Therefore, we explored whether inhibition of BMP9 signaling in mouse models of prostate cancer interferes with tumor growth.

ALK1Fc Reduces Primary Prostate Tumor Burden *in Vivo*

To investigate the role of BMP9 in prostate cancer progression, the BMP9 ligand trap ALK1Fc was administered in an orthotopic mouse model of prostate cancer. Primary prostate tumor growth was induced by intra-prostatic inoculation of human prostate cancer PC-3M-Pro4-Luc2 cells in Balb/c nude mice and tumor progression was followed by bioluminescence imaging (BLI) (Kroon et al., 2014) (Figure 2A). Based on the BLI signal the mice were randomized in two treatment groups: ALK1Fc or control (C) Fc (n = 15 per group). The recombinant proteins were injected twice weekly and tumor imaging and body weights were monitored weekly for 5 weeks (Supplementary Figure 1). Tumor burden was quantitatively assessed for each animal during the course of treatment. The group of animals that received ALK1Fc exhibited smaller tumor size compared to the animals that received CFc based on bioluminescence quantification (**Figure 2B**, *p* < 0.01).

ALK1Fc Reduces Cell Proliferation in the Primary Prostate Tumor

The degree of tumor angiogenesis is critical for progressive tumor growth beyond a few mm³ in size. Intravital lectin perfusion was used to map the perfused elements of the tumor vasculature in mice. Fluorescent-conjugated lectin (lectin-Tomato) was visualized in tumor tissue sections and quantified. We observed a trend in decreased vascular density, indicated by the overall lectin presence, in the tumors treated with ALK1Fc compared to the CFc group (Supplementary Figures 2A,B). We evaluated the presence of endothelial cells in tumor sections by CD31 immunofluorescence. A trend of decreased CD31 expression was also observed after treatment with ALK1Fc suggesting fewer endothelial cells and vessels (Supplementary Figures 2C,D). Hypoxia is an important component of angiogenesis and critical for tumor formation. A hypoxia-induced probe was injected in tumor bearing mice just prior to sacrifice and the hypoxic areas within the tumors were visualized after tumor resection (Figure 3A; left panels). Although hypoxic areas were found in both treatment groups, the overall amount of hypoxia seemed to be higher in ALK1Fc-treated mice relative to the CFc-treated mice (Figure 3A; right graph, p = 0.050). We assessed the presence of cell proliferation and cell death in these tumors by immunofluorescence for the mitosis marker phosphorylated histone 3 (PH3) and the apoptosis marker



cleaved caspase 3 (CASP3), respectively. Dividing PH3 positive cells are predominantly located in normoxic areas (**Figure 3A**; left panel). Quantification of immunofluorescence signal shows that the number of dividing cells is lower in the ALK1Fc-treated animals (**Figure 3A**; right graph p < 0.05). Detection of apoptotic cells (Caspase-3 positive) is higher in the ALK1Fc-treated tumors (**Figure 3A**; right graph p < 0.05) and occurs mostly, but not exclusively, in hypoxic areas (**Figure 3A**; left panel), suggesting a correlation between the hypoxia and tumor cell death.

ALK1Fc Decreases Proliferation of Human Prostate Cancer Cells *in Vitro*

To investigate how ALK1Fc can decrease tumor growth, we studied the effect of ALK1Fc on prostate cancer cells. We measured the mRNA levels of the BMP9 type I receptors ALK1 and ALK2 in the PC-3M-Pro4-Luc2 (Kroon et al., 2014) human prostate cancer cell line and tested their response to BMP9. Consistent with a previous report in highly metastatic PC-3 and PC-3M prostate cancer cells (Craft et al., 2007), qRT-PCR analysis in osteotropic PC-3M-Pro4-Luc2 cells revealed undetectable levels of ALK1 but measurable levels of ALK2 (Supplementary Figure 3A). Treatment with BMP9 showed a dose-dependent induction of BRE-*Renilla* luciferase (luc) activity in PC-3M-Pro4 cells (p-value = 0.005 and 0.05 with 0.5 nM and 1 nM BMP9, respectively) indicative of conserved and active

canonical Smad signaling machinery (Supplementary Figure 3B). We subsequently tested the combined effect of treating cells with 1 nM BMP9 and either ALK1Fc or CFc on BRE reporter assay and found that treatment with ALK1Fc ($10 \mu g/mL$) completely abolished BMP9 signaling (Supplementary Figure 3C; BMP9+ALK1Fc) as evidenced by BRE-luc activity levels similar to that of cells without BMP9 treatment (Untreated). Treatment with BMP9+CFc ($10 \mu g/mL$) led to induction of BRE-luc activity that was similar to the level of BMP9 treatment alone (Supplementary Figure 3C; *p*-value < 0.05). Taken together, these results indicate that ALK1Fc blocks BMP9 signaling via ALK2 in PC-3MPro4 cells.

Moreover, ALK1Fc treatment, but not CFc, strongly reduced BMP9-induced cell proliferation in PC-3M-Pro4-Luc2 (p < 0.001 at day 4 comparing vehicle vs. BMP9 or ALK1Fc treatment, respectively; **Figure 3B**). This effect appeared to be specific (Supplementary Figure 3D) since treatment of PC-3M-Pro4-Luc2 cells with BMP9 in combination with an ALK2 small molecule kinase inhibitor (LDN193189, LDN) (Cuny et al., 2008; Shi et al., 2011) similarly resulted in the complete loss of BMP9-induced cell proliferation (**Figure 3C**). LDN treatment also blocked BMP9 stimulation of the BRE-luc reporter in PC-3M-Pro4-Luc2 cells (Supplementary Figure 3E). Together, these data indicate that ALK1Fc strongly reduces BMP9- induced proliferation in human prostate cancer cells.



ALK1Fc Inhibits ALDH1A1 Expression in Vivo and Interferes with NOTCH Signaling

Given its ability to reduce primary tumor burden and block BMP9-induced tumor cell proliferation *in vitro* and *in vivo*, we assessed the effects of ALK1Fc on the relative expression of *ALDH1A1*, a marker previously associated with cancer stem cell-like properties and poor patient prognosis (Li et al., 2010; Le Magnen et al., 2013). Treatment of prostate tumor bearing mice with ALK1Fc affected the number of ALDH1A1 positive cells in the prostate tumor tissues both at the protein (**Figure 4A**) and mRNA levels (**Figure 4B**). *In vitro* stimulation with BMP9 of the same cell line used to induce tumors in the xenograft mouse model confirmed that treatment with BMP9 or BMP9+CFc upregulates ALDH1A1 expression while BMP9+ALK1Fc treatment does not have any effect (**Figure 4C**).

We tested the effects of BMP9 treatment on the colony forming capacity of PC-3M-Pro4-Luc2 cells (Figure 4D) and found that it alters cell proliferation and strongly increasing

the size of the colonies (**Figure 4E**, p < 0.05). However, BMP9 showed no effect on colony formation ability of PC-3M-Pro4-Luc2 since the total number of colonies formed with or without BMP9 treatment is similar (**Figure 4F**).

ALDH1A1 is known to be regulated by NOTCH signaling (Ginestier et al., 2007; Le Magnen et al., 2013; Zhao et al., 2014) and NOTCH1 plays a prominent role in prostate cancer cell proliferation and migration (Shou et al., 2001; Zhang et al., 2006; Leong and Gao, 2008; Bin Hafeez et al., 2009; Wang et al., 2010). Larrivee et al. have shown that ALK1 and NOTCH converge on common downstream pathways and that BMP9 treatment is sufficient to upregulate expression of the NOTCH pathway ligand JAG1 in HUVEC non-transformed cells (Larrivee et al., 2012).

To assess the clinical relevance of crosstalk between BMP9/ALK2 signaling and NOTCH pathway activation in human prostate cancer, we performed bioinformatics analysis in 48 benign prostate tumors and 47 malignant prostate tumors (Borno et al., 2012) using R2 data mining platform (source: GEO



FIGURE 3 [ALK1Fc increases hypoxia and apoptosis and decreases cell proliferation *in vivo*. (A) Left panels: Representative images of hypoxia immunofluorescence staining (red) in primary prostate tumor samples after 5 weeks of treatment with either ALK1Fc or CFc. Hypoxia probe was injected prior to sacrifice and was detected by a specific fluorescent antibody. Immunofluorescence images for colocalization of apoptotic or proliferating cells in hypoxic/normoxic area within the prostate tumor area in ALK1Fc and CFc treated animals. pH3: PhosphoHistone 3 proliferation marker (green); cleaved caspase 3 apoptosis marker (green); Hypoxic probe-antibody: hypoxic area (red); TOPRO: nuclear dye (blue). Right graphs: Quantification of hypoxia, pH3 and cl.CASP3 positive area in all tumor samples of each group (n = 6 for CFc, n = 7 for ALK1Fc). (B) MTS assay (24, 48, 72, 96 h) was performed in PC-3M-Pro4-Luc2 cells stimulated with recombinant BMP9 (1 nM), BMP9 (1 nM) + ALK1Fc (10 µg/ml), or BMP9 (1 nM) + CFc (10 µg/ml). Accumulation of MTS was measured based on absorbance at 490 nm. Values are normalized to the basal measurements at 24 h after cell seeding and treatments. Graph represents values for three independent experiments (n = 3). Error bars indicate \pm SEM. ***P*-value < 0.01 BMP9 vs. Untreated and ****P*-value < 0.01 BMP9 (1 nM), LDN (BMP type I receptor inhibitor LDN193189, 120 nM) or LDN+BMP9. (n = 2). Values are normalized to the basal measurements at the time of cell seeding and treatments. Error bars indicate SEM. ***P*-value < 0.01 BMP9 vs. LDN+-BMP9; **P*-value < 0.01 BMP9 vs. LDN; ###BMP9 vs. LDN+BMP9; **P*-value < 0.01 BMP9 vs. LDN and BMP9 vs. LDN+-BMP9; **P*-value < 0.001 BMP9 vs. Untreated; \$\$

ID: GSE29079). Transcript levels of *ALK2*, the NOTCH ligand *JAG1*, and *NOTCH1* were significantly higher in the malignant tumor group compared to the benign group (**Figures 5A–C**).

We targeted the expression of NOTCH1 in PC-3M-Pro4-Luc2 using a specific shRNA (shNOTCH1) and assessed resulting NOTCH1 levels by western blot and reporter assay (Supplementary Figures 4A,B). NOTCH1 knockdown led to a decreased proliferation rate compared to cells transduced with non-targeting shRNA lentivirus (p < 0.05 at 48 h and at 72 h) (**Figure 5D**). Notably, we observed that that shNOTCH1-cells display decreased levels of JAG1 mRNA (Supplementary Figure 4C) relative to control cells and that stimulation of shNOTCH1cells with BMP9 rescued their proliferation rate (**Figure 5D**). To verify the effect of BMP9 on NOTCH signaling activation in our cancer model, we used qRT-PCR to quantify the expression of *JAG1* after BMP9 stimulation in presence of ALK1Fc or CFc. Our transcriptional analysis showed that BMP9 and BMP9+CFc induce mRNA expression of *JAG1* and that ALK1Fc treatment reduces this induction (**Figure 5E**). These data reinforce the hypothesis that the BMP9/ALK2 pathway can drive activation of NOTCH signaling linking two pathways that are associated with tumor progression in prostate cancer.

ALK1Fc Reduces Tumor Burden in the BM18 Patient Derived Xenograft Model

While cell lines and mouse models are of great help in addressing biological questions, there is an increased need for personalized treatments and precision medicine based on screening of human material is becoming increasingly important. Therefore, we tested the antitumoral effect of ALK1Fc on the human patient derived xenograft (PDX) BM18. This PDX was derived



FIGURE 4 [ALK1Fc treatment reduces ALDH1A1 expression. (A) Representative images of ALDH1A1 immunofluorescence in prostate tumor samples from ALK1Fc and CFc treated animals. ALDH1A1: red; TOPRO: blue. (B) Quantification of ALDH1A1 mRNA by Q-PCR in tumor samples of each group (n = 5 for CFc, n = 5 for ALK1Fc). (C) Expression of *ALDH1A1* in PC-3M-Pro4-Luc2 cells. Relative mRNA expression was measured by Q-PCR from cDNA obtained from PC-3M-Pro4-Luc2 cells treated with BMP9, BMP9+ALK1Fc, BMP9+CFc, for 48, 72, and 96 h. Values are normalized to β -actin expression. Error bars are \pm SEM (n = 3). ^{\$}*P*-value < 0.05; ^{\$\$}*P*-value < 0.01. (D) Clonogenic assay of PC-3M-Pro4-Luc2 cells. Low-density cultures (100 cells per well of 6 well plate) were stimulated with BMP9, CFc, ALK1Fc, BMP9+CFc, BMP9+ALK1Fc. Colony formation was assessed after 10 days by crystal violet staining. Representative images are shown. (**E**, **F**) Quantification of surface covered by crystal violet positive colonies (colony area) and colony number. Graph shows percentage of positive surface normalized per condition (average of three independent experiments). **P*-value < 0.05. Error bars indicate SEM.



values for three independent experiments (n = 3). Error bars indicate SEM. **P*-value < 0.05. (**E**) Expression of *JAG1* in PC-3M-Pro4-Luc2 cells. Relative mRNA expression was measured by Q-PCR from cDNA obtained from PC-3M-Pro4-Luc2 cells treated with BMP9, ALK1Fc, CFc, BMP9+ALK1Fc or BMP9+CFc for 96 h. Values are normalized to β -actin expression. Error bars are \pm SEM (n = 3). **P*-value < 0.01.

from prostate cancer tissue harvested from femoral metastasis (McCulloch et al., 2005) and it is vitally maintained through serial passage in immunocompromised mice (Germann et al., 2012). We transplanted BM18 cells subcutaneously in severe combined immunodeficiency SCID mice and after 1 week the animals were treated with 10 mg/kg ALK1Fc or an IgG control once a week for an additional 5 weeks. Body weights of the mice were monitored weekly (Supplementary Figure 5) and the tumor growth was assessed by caliper measurement. We observed significant reduction in tumor burden upon ALK1Fc but not IgG administration (**Figures 6A,B**) and assessed the prostate epithelial phenotype of the tumors by the hematoxylin eosin (H&E) staining (**Figure 6C**).

DISCUSSION

In this study, we found that BMP9 has a tumor-promoting effect on human prostate cancer cells both *in vitro* and *in vivo*. We demonstrate that blocking BMP9 signaling with ALK1Fc efficiently diminishes prostate cancer cell proliferation and substantially attenuates tumor growth in both an orthotopic model of human prostate cancer and a prostate cancer derived PDX.

BMP9 was first identified in the liver (Song et al., 1995) and active forms are present in serum (Herrera et al., 2009). BMP9 is a ligand for ALK1 in endothelial cells (van Meeteren et al., 2012) and exerts stimulatory or inhibitory effects on endothelial cell growth and migration depending on the cellular context (David et al., 2007; Suzuki et al., 2010; Liao et al., 2017). Aberrant regulation of transforming growth factor- β (TGF-β) and BMP signaling often results in cancer progression (Siegel and Massague, 2003; Massagué, 2008). In particular, BMP ligands, such as BMP9 as well as BMP type I receptors (e.g., ALK1 and ALK2) have been associated with tumor angiogenesis and cancer progression. BMP9 signals through ALK2 in nonendothelial cells including those in ovarian epithelium, where it has been shown to promote proliferation of ovarian cancer cells (Herrera et al., 2009). Similarly, in hepatocellular carcinoma BMP9 has been reported to act as a factor that promotes cell proliferation and survival (Herrera et al., 2013). More recently, the BMP9/ALK2 axis has also been involved in erythroblast cancer cells proliferation (Kim et al., 2017). By contrast, studies have also highlighted the role of BMP9 in reducing breast cancer cell growth and metastasis (Wang et al., 2011, 2017; Ren et al., 2014a,b). Overall, the role of BMP9 and ALKs in promoting or suppressing different cancer types remains controversial and the



effect of BMP9 on tumor promotion vs. tumor suppression is likely to be context and cancer-type specific. This provided the rationale for us to elucidate the role of BMP9 in prostate cancer, for which no information is available to our knowledge.

In our search of publicly available databases of human prostate cancer specimens we found that BMP9 was expressed at significantly higher levels in high risk prostate cancer patients compared to the low risk group and that ALK2 was significantly upregulated in malignant vs. benign tissue samples. These data are consistent with our model in which the tumor-promoting effects of BMP9 are mediated by ALK2. Additionally, microarray analysis of data from mouse prostate intraepithelial neoplasia (PIN) vs. invasive cancer in a multistage model of prostate carcinogenesis showed up regulation of ALK2 and BMP9 at the invasive stage in the stromal compartment (Bacac et al., 2006). These data, together with the anti-tumorigenic effect of ALK1Fc documented here, suggest a tumor-promoting role of BMP9 during prostate cancer progression.

Our *in vitro* findings strengthen the afore-mentioned expression data and suggest that BMP9 increases proliferation of human prostate cancer cells. Moreover, our studies with the ALK2 inhibitor LDN193189 support the notion that ALK2 is critically involved in mediating BMP9-induced proliferation in PC-3M-Pro4-Luc2 cells. As depicted in the Results and Supplementary Data sections, treatment with ALK1Fc or LDN193189 alone did not affect proliferation of human prostate cancer cells suggesting a paracrine effect of stroma-derived BMP9 on tumor cells.

We also used an orthotopic model of prostate cancer to demonstrate that ALK1Fc reduces prostate tumor burden and vascular density compared to the controls. Lectin distribution appeared to be less diffuse in ALK1Fc treated animals, suggesting an effect on vessel maintenance rather than angiogenesis. Strikingly, ALK1Fc treatment of tumor-bearing animals resulted in highly hypoxic tumors with a trend in decreased number of CD31+ tumor capillaries suggesting that ALK1Fc may block BMP9-induced neovascularization. As expected, areas of tumor proliferation and apoptosis were found to be mutually exclusive in their distribution. Apoptotic regions overlapped with hypoxic areas, suggesting that blockade of BMP9 by ALK1Fc might have an effect on proliferation and apoptosis of human prostate cancer cells in addition to targeting vessel maintenance (Mitchell et al., 2010).

SMAD1 and SMAD5 are downstream intracellular effectors of BMP9 signaling and can directly interact with the JAG1 promoter and induce transcription of the NOTCH ligand JAG1 (Larrivee et al., 2012) following BMP9 treatment (Morikawa et al., 2011). Transcriptional analysis revealed that ALK1Fc systemically blocks the induction of JAG1 mRNA in the presence of BMP9 (Morikawa et al., 2011) supporting our hypothesis that the crosstalk between BMP9 and NOTCH signaling may have clinical implications in prostate cancer. Indeed, in silico analysis of a previously published dataset of human prostate cancer specimens confirms that both NOTCH1 and JAG1 are upregulated at the tumor stage (Borno et al., 2012). In particular, NOTCH signaling seems to promote epithelial-mesenchymal transition in prostate cancer cells (Zhang et al., 2017). Moreover, recent publication shows how NOTCH pathway inhibition antagonizes the growth and invasion of TMPERSS2-ERG positive prostate cancer cells (ERG overexpressing prostate tumor) (Kron et al., 2017) suggesting an important role of the cascade in tumor growth.

Interestingly, NOTCH activates ALDH1A1, an established marker for highly tumorigenic prostate cancer stem cell-like cells (Ginestier et al., 2007; Le Magnen et al., 2013; Zhao et al., 2014; Harris and Kerr, 2017). The ALDH1A1 subpopulation contributes to both tumor initiation and progression and when highly expressed in advanced-stage cancers correlates with poor survival in hormone-naïve patients (Le Magnen et al., 2013). Notably, we show here that ALK1Fc-treated tumors showed significant reduction of ALDH1A1. Taken together, these data suggest that ALK1Fc might potentially interfere with NOTCH signaling in the regulation of ALDH1A1.

Our conclusion that BMP9 promotes aggressive prostate cancer growth was further supported by our demonstration that administration of ALK1Fc inhibited the growth of BM18 PDX, an androgen-dependent bone metastatic prostate tumor. Importantly, these data confirm the ability of ALK1Fc to treat a tumor derived from human patient and open new perspectives in the clinical application of this ligand trap for the cure of prostate cancer.

Our findings provide novel information on the role of BMP9 in human prostate cancer and suggest the promising use of BMP9 targeting molecules for the treatment of tumor and supportive microenvironment in prostate cancer patients.

AUTHOR CONTRIBUTIONS

LA, EZ, and SK: designed the work, acquired and interpreted the data and drafted the manuscript; PG interpreted the data and drafted and revised the manuscript; IK and JG acquired the data and revised the manuscript; MG, LH, GvdP, MS, GT interpreted the data and revised the manuscript; PtD conceived the work, interpreted the data and revised the manuscript; MK conceived the work, acquired and interpreted the data, drafted and revised the manuscript. All authors approved the version to be published and are accountable for all aspects of the work

REFERENCES

- Aguirre-Gamboa, R., Gomez-Rueda, H., Martinez-Ledesma, E., Martinez-Torteya, A., Chacolla-Huaringa, R., Rodriguez-Barrientos, A., et al. (2013). SurvExpress: an online biomarker validation tool and database for cancer gene expression data using survival analysis. *PLoS ONE* 8:e74250. doi: 10.1371/journal.pone.0074250
- Bacac, M., Provero, P., Mayran, N., Stehle, J. C., Fusco, C., and Stamenkovic, I. (2006). A mouse stromal response to tumor invasion predicts prostate and breast cancer patient survival. *PLoS ONE* 1:e32. doi: 10.1371/journal.pone.0000032
- Bendell, J. C., Gordon, M. S., Hurwitz, H. I., Jones, S. F., Mendelson, D. S., Blobe, G. C., et al. (2014). Safety, pharmacokinetics, pharmacodynamics, and antitumor activity of dalantercept, an activin receptor-like kinase-1 ligand trap, in patients with advanced cancer. *Clin. Cancer Res.* 20, 480–489. doi: 10.1158/1078-0432.CCR-13-1840
- Berridge, M. V., Herst, P. M., and Tan, A. S. (2005). Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol. Ann. Rev.* 11, 127–152. doi: 10.1016/S1387-2656(05)11004-7
- Bin Hafeez, B., Adhami, V. M., Asim, M., Siddiqui, I. A., Bhat, K. M., Zhong, W., et al. (2009). Targeted knockdown of Notch1 inhibits invasion of human prostate cancer cells concomitant with inhibition of matrix metalloproteinase-9 and urokinase plasminogen activator. *Clin. Cancer Res.* 15, 452–459. doi: 10.1158/1078-0432.CCR-08-1631
- Borno, S. T., Fischer, A., Kerick, M., Falth, M., Laible, M., Brase, J. C., et al. (2012). Genome-wide DNA methylation events in TMPRSS2-ERG fusion-negative prostate cancers implicate an EZH2-dependent mechanism with miR-26a hypermethylation. *Cancer Discov.* 2, 1024–1035. doi: 10.1158/2159-8290.CD-12-0041
- Bovelstad, H. M., and Borgan, O. (2011). Assessment of evaluation criteria for survival prediction from genomic data. *Biom. J.* 53, 202–216. doi: 10.1002/bimj.201000048
- Bragdon, B., Moseychuk, O., Saldanha, S., King, D., Julian, J., and Nohe, A. (2011). Bone morphogenetic proteins: a critical review. *Cell. Signal.* 23, 609–620. doi: 10.1016/j.cellsig.2010.10.003

in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

FUNDING

The research leading to these results has received funding from the FP7 Marie Curie ITN under grant agreement No. 264817–BONE-NET (EZ) and from the Netherlands Initiative of Regenerative Medicine (NIRM, grant No. FES0908). This work was also supported in part by the Clayton Medical Research Foundation, Inc. (PG).

ACKNOWLEDGMENTS

We thank Acceleron Pharma for providing the ALK1Fc (RAP-041), Laurens van Meeteren, Marjan van de Merbel and Federico La Manna.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2017. 00104/full#supplementary-material

- Buijs, J. T., Rentsch, C. A., van der Horst, G., van Overveld, P. G., Wetterwald, A., Schwaninger, R., et al. (2007). BMP7, a putative regulator of epithelial homeostasis in the human prostate, is a potent inhibitor of prostate cancer bone metastasis *in vivo. Am. J. Pathol.* 171, 1047–1057. doi: 10.2353/ajpath.2007.070168
- Carvalho, F. L., Simons, B. W., Eberhart, C. G., and Berman, D. M. (2014). Notch signaling in prostate cancer: a moving target. *Prostate* 74, 933–945. doi: 10.1002/pros.22811
- Craft, C. S., Romero, D., Vary, C. P., and Bergan, R. C. (2007). Endoglin inhibits prostate cancer motility via activation of the ALK2-Smad1 pathway. *Oncogene* 26, 7240–7250. doi: 10.1038/sj.onc.1210533
- Cunha, S. I., Pardali, E., Thorikay, M., Anderberg, C., Hawinkels, L., Goumans, M.-J., et al. (2010). Genetic and pharmacological targeting of activin receptorlike kinase 1 impairs tumor growth and angiogenesis. *J. Exp. Med.* 207, 85–100. doi: 10.1084/jem.20091309
- Cunha, S. I., and Pietras, K. (2011). ALK1 as an emerging target for antiangiogenic therapy of cancer. Blood 117, 6999–7006. doi: 10.1182/blood-2011-01-330142
- Cuny, G. D., Yu, P. B., Laha, J. K., Xing, X., Liu, J. F., Lai, C. S., et al. (2008). Structure-activity relationship study of bone morphogenetic protein (BMP) signaling inhibitors. *Bioorg. Med. Chem. Lett.* 18, 4388–4392. doi: 10.1016/j.bmcl.2008.06.052
- David, L., Mallet, C., Mazerbourg, S., Feige, J. J., and Bailly, S. (2007). Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. *Blood* 109, 1953–1961. doi: 10.1182/blood-2006-07-034124
- de Vinuesa, A. G., Bocci, M., Pietras, K., and Ten Dijke, P. (2016). Targeting tumour vasculature by inhibiting activin receptor-like kinase (ALK)1 function. *Biochem. Soc. Trans.* 44, 1142–1149. doi: 10.1042/BST20160093
- Franken, N. A., Rodermond, H. M., Stap, J., Haveman, J., and van Bree, C. (2006). Clonogenic assay of cells *in vitro*. *Nat. Protoc.* 1, 2315–2319. doi: 10.1038/nprot.2006.339
- Gale, N. W., Dominguez, M. G., Noguera, I., Pan, L., Hughes, V., Valenzuela, D. M., et al. (2004). Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proc. Natl. Acad. Sci. U.S.A.* 101, 15949–15954. doi: 10.1073/pnas.0407290101

- Germann, M., Wetterwald, A., Guzman-Ramirez, N., van der Pluijm, G., Culig, Z., Cecchini, M. G., et al. (2012). Stem-like cells with luminal progenitor phenotype survive castration in human prostate cancer. *Stem Cells* 30, 1076–1086. doi: 10.1002/stem.1087
- Ginestier, C., Hur, M. H., Charafe-Jauffret, E., Monville, F., Dutcher, J., Brown, M., et al. (2007). ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* 1, 555–567. doi: 10.1016/j.stem.2007.08.014
- Guzman, C., Bagga, M., Kaur, A., Westermarck, J., and Abankwa, D. (2014). ColonyArea: an ImageJ plugin to automatically quantify colony formation in clonogenic assays. *PLoS ONE* 9:e92444. doi: 10.1371/journal.pone.0092444
- Harris, K. S., and Kerr, B. A. (2017). Prostate cancer stem cell markers drive progression, therapeutic resistance, and bone metastasis. *Stem Cells Int.* 2017:8629234. doi: 10.1155/2017/8629234
- Hawinkels, L. J., Garcia de Vinuesa, A., and Ten Dijke, P. (2013). Activin receptorlike kinase 1 as a target for anti-angiogenesis therapy. *Expert Opin. Investig. Drugs* 22, 1371–1383. doi: 10.1517/13543784.2013.837884
- Herrera, B., Garcia-Alvaro, M., Cruz, S., Walsh, P., Fernandez, M., Roncero, C., et al. (2013). BMP9 is a proliferative and survival factor for human hepatocellular carcinoma cells. *PLoS ONE* 8:e69535. doi: 10.1371/journal.pone.0069535
- Herrera, B., van Dinther, M., Ten Dijke, P., and Inman, G. J. (2009). Autocrine bone morphogenetic protein-9 signals through activin receptor-like kinase-2/Smad1/Smad4 to promote ovarian cancer cell proliferation. *Cancer Res.* 69, 9254–9262. doi: 10.1158/0008-5472.CAN-09-2912
- Jemal, A., Center, M. M., DeSantis, C., and Ward, E. M. (2010). Global patterns of cancer incidence and mortality rates and trends. *Cancer Epidemiol. Biomark. Prev.* 19, 1893–1907. doi: 10.1158/1055-9965.EPI-10-0437
- Karkampouna, S., Kruithof, B. P., Kloen, P., Obdeijn, M. C., van der Laan, A. M., Tanke, H. J., et al. (2014). Novel *ex vivo* culture method for the study of Dupuytren's disease: effects of TGFβ type 1 receptor modulation by antisense oligonucleotides. *Mol. Ther. Nucleic Acids* 3:e142. doi: 10.1038/mtna.2013.69
- Kim, M., Choi, O., Pyo, S., Choi, S. U., and Park, C. H. (2017). Identification of novel ALK2 inhibitors and their effect on cancer cells. *Biochem. Biophys. Res. Commun.* 492, 121–127. doi: 10.1016/j.bbrc.2017.08.016
- Korchynskyi, O., and ten Dijke, P. (2002). Identification and functional characterization of distinct critically important bone morphogenetic proteinspecific response elements in the Id1 promoter. J. Biol. Chem. 277, 4883–4891. doi: 10.1074/jbc.M111023200
- Krebs, L. T., Shutter, J. R., Tanigaki, K., Honjo, T., Stark, K. L., and Gridley, T. (2004). Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. *Genes Dev.* 18, 2469–2473. doi: 10.1101/gad.1239204
- Kron, K. J., Murison, A., Zhou, S., Huang, V., Yamaguchi, T. N., Shiah, Y. J., et al. (2017). TMPRSS2-ERG fusion co-opts master transcription factors and activates NOTCH signaling in primary prostate cancer. *Nat. Genet.* 49, 1336–1345. doi: 10.1038/ng.3930
- Kroon, J., In 't Veld, L. S., Buijs, J. T., Cheung, H., van der Horst, G., and van der Pluijm, G. (2014). Glycogen synthase kinase-3β inhibition depletes the population of prostate cancer stem/progenitor-like cells and attenuates metastatic growth. *Oncotarget* 5, 8986–8994. doi: 10.18632/oncotarget. 1510
- Larrivee, B., Prahst, C., Gordon, E., del Toro, R., Mathivet, T., Duarte, A., et al. (2012). ALK1 signaling inhibits angiogenesis by cooperating with the Notch pathway. *Dev. Cell* 22, 489–500. doi: 10.1016/j.devcel.2012.02.005
- Le Magnen, C., Bubendorf, L., Rentsch, C. A., Mengus, C., Gsponer, J., Zellweger, T., et al. (2013). Characterization and clinical relevance of ALDH bright populations in prostate cancer. *Clin. Cancer Res.* 19, 5361–5371. doi: 10.1158/1078-0432.CCR-12-2857
- Leong, K. G., and Gao, W. Q. (2008). The Notch pathway in prostate development and cancer. *Differentiation* 76, 699–716. doi: 10.1111/j.1432-0436.2008.00288.x
- Li, R., Zhang, W., Cui, J., Shui, W., Yin, L., Wang, Y., et al. (2014). Targeting BMP9-promoted human osteosarcoma growth by inactivation of notch signaling. *Curr. Cancer Drug Targets* 14, 274–285. doi: 10.2174/1568009614666140305105805
- Li, T., Su, Y., Mei, Y., Leng, Q., Leng, B., Liu, Z., et al. (2010). ALDH1A1 is a marker for malignant prostate stem cells and predictor of prostate cancer patients' outcome. *Lab. Invest.* 90, 234–244. doi: 10.1038/labinvest.2009.127

- Liao, J., Wei, Q., Zou, Y., Fan, J., Song, D., Cui, J., et al. (2017). Notch Signaling augments BMP9-induced bone formation by promoting the osteogenesisangiogenesis coupling process in mesenchymal stem cells (MSCs). *Cell. Physiol. Biochem.* 41, 1905–1923. doi: 10.1159/000471945
- Massagué, J. (2008). TGFβ in Cancer. Cell 134, 215–230. doi: 10.1016/j.cell.2008.07.001
- McCulloch, D. R., Opeskin, K., Thompson, E. W., and Williams, E. D. (2005). BM18: a novel androgen-dependent human prostate cancer xenograft model derived from a bone metastasis. *Prostate* 65, 35–43. doi: 10.1002/pros.20255
- Mitchell, D., Pobre, E. G., Mulivor, A. W., Grinberg, A. V., Castonguay, R., Monnell, T. E., et al. (2010). ALK1-Fc inhibits multiple mediators of angiogenesis and suppresses tumor growth. *Mol. Cancer Ther.* 9, 379–388. doi: 10.1158/1535-7163.MCT-09-0650
- Moltzahn, F., and Thalmann, G. N. (2013). Cancer stem cells in prostate cancer. *Transl. Androl. Urol.* 2, 242–253. doi: 10.3978/j.issn.2223-4683.2013.09.06
- Morikawa, M., Koinuma, D., Tsutsumi, S., Vasilaki, E., Kanki, Y., Heldin, C. H., et al. (2011). ChIP-seq reveals cell type-specific binding patterns of BMPspecific Smads and a novel binding motif. *Nucleic Acids Res.* 39, 8712–8727. doi: 10.1093/nar/gkr572
- Olsen, O. E., Wader, K. F., Misund, K., Vatsveen, T. K., Ro, T. B., Mylin, A. K., et al. (2014). Bone morphogenetic protein-9 suppresses growth of myeloma cells by signaling through ALK2 but is inhibited by endoglin. *Blood Cancer J.* 4:e196. doi: 10.1038/bcj.2014.16
- Rafehi, H., Orlowski, C., Georgiadis, G. T., Ververis, K., El-Osta, A., and Karagiannis, T. C. (2011). Clonogenic assay: adherent cells. J. Vis. Exp. e2573. doi: 10.3791/2573
- Ren, W., Liu, Y., Wan, S., Fei, C., Wang, W., Chen, Y., et al. (2014a). BMP9 inhibits proliferation and metastasis of HER2-positive SK-BR-3 breast cancer cells through ERK1/2 and PI3K/AKT pathways. *PLoS ONE* 9:e96816. doi: 10.1371/journal.pone.0096816
- Ren, W., Sun, X., Wang, K., Feng, H., Liu, Y., Fei, C., et al. (2014b). BMP9 inhibits the bone metastasis of breast cancer cells by downregulating CCN2 (connective tissue growth factor, CTGF) expression. *Mol. Biol. Rep.* 41, 1373–1383. doi: 10.1007/s11033-013-2982-8
- Ross, A. E., Marchionni, L., Vuica-Ross, M., Cheadle, C., Fan, J., Berman, D. M., et al. (2011). Gene expression pathways of high grade localized prostate cancer. *Prostate* 71, 1568–1578. doi: 10.1002/pros.21373
- Scharpfenecker, M., van Dinther, M., Liu, Z., van Bezooijen, R. L., Zhao, Q., Pukac, L., et al. (2007). BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. J. Cell Sci. 120, 964–972. doi: 10.1242/jcs.002949
- Schwaninger, R., Rentsch, C. A., Wetterwald, A., van der Horst, G., van Bezooijen, R. L., van der Pluijm, G., et al. (2007). Lack of noggin expression by cancer cells is a determinant of the osteoblast response in bone metastases. *Am. J. Pathol.* 170, 160–175. doi: 10.2353/ajpath.2007.051276
- Seehra, J., Knopf, J., Pearsall, R. S., Grinberg, A., and Kumar, R. (2009). Antagonists of Bmp9, Bmp10, Alk1 and other Alk1 Ligands, and Uses Thereof. International Patent Application WO 2009/139891 A2.
- Shi, S., Hoogaars, W. M., de Gorter, D. J., van Heiningen, S. H., Lin, H. Y., Hong, C. C., et al. (2011). BMP antagonists enhance myogenic differentiation and ameliorate the dystrophic phenotype in a DMD mouse model. *Neurobiol. Dis.* 41, 353–360. doi: 10.1016/j.nbd.2010.10.003
- Shou, J., Ross, S., Koeppen, H., de Sauvage, F. J., and Gao, W. Q. (2001). Dynamics of notch expression during murine prostate development and tumorigenesis. *Cancer Res.* 61, 7291–7297.
- Siegel, P. M., and Massague, J. (2003). Cytostatic and apoptotic actions of TGF
 ß in homeostasis and cancer. Nat. Rev. Cancer 3, 807–820. doi: 10.1038/nrc1208
- Song, J. J., Celeste, A. J., Kong, F. M., Jirtle, R. L., Rosen, V., and Thies, R. S. (1995). Bone morphogenetic protein-9 binds to liver cells and stimulates proliferation. *Endocrinology* 136, 4293–4297. doi: 10.1210/endo.136.10.7664647
- Suzuki, Y., Ohga, N., Morishita, Y., Hida, K., Miyazono, K., and Watabe, T. (2010). BMP-9 induces proliferation of multiple types of endothelial cells *in vitro* and *in vivo. J. Cell Sci.* 123, 1684–1692. doi: 10.1242/jcs.061556
- Urness, L. D., Sorensen, L. K., and Li, D. Y. (2000). Arteriovenous malformations in mice lacking activin receptor-like kinase-1. *Nat. Genet.* 26, 328–331. doi: 10.1038/81634
- van den Hoogen, C., van der Horst, G., Cheung, H., Buijs, J. T., Lippitt, J. M., Guzman-Ramirez, N., et al. (2010). High aldehyde dehydrogenase

activity identifies tumor-initiating and metastasis-initiating cells in human prostate cancer. *Cancer Res.* 70, 5163–5173. doi: 10.1158/0008-5472.CAN-09 -3806

- van Meeteren, L. A., Thorikay, M., Bergqvist, S., Pardali, E., Gallo Stampino, C., Hu-Lowe, D., et al. (2012). Anti-human activin receptor-like kinase 1 (ALK1) antibody attenuates Bone morphogenetic protein 9 (BMP9)-induced ALK1 signaling and interferes with endothelial cell sprouting. *J. Biol. Chem.* 287, 18551–18561. doi: 10.1074/jbc.M111.338103
- Wang, K., Feng, H., Ren, W., Sun, X., Luo, J., Tang, M., et al. (2011). BMP9 inhibits the proliferation and invasiveness of breast cancer cells MDA-MB-231. *J. Cancer Res. Clin. Oncol.* 137, 1687–1696. doi: 10.1007/s00432-011-1047-4
- Wang, T., Zhang, Z., Wang, K., Wang, J., Jiang, Y., Xia, J., et al. (2017). Inhibitory effects of BMP9 on breast cancer cells by regulating their interaction with pre-adipocytes/adipocytes. *Oncotarget* 8, 35890–35901. doi: 10.18632/oncotarget.16271
- Wang, Z., Li, Y., Banerjee, S., Kong, D., Ahmad, A., Nogueira, V., et al. (2010). Down-regulation of Notch-1 and Jagged-1 inhibits prostate cancer cell growth, migration and invasion, and induces apoptosis via inactivation of Akt, mTOR, and NF-κB signaling pathways. J. Cell. Biochem. 109, 726–736. doi: 10.1002/jcb.22451
- Ye, L., and Jiang, W. G. (2016). Bone morphogenetic proteins in tumour associated angiogenesis and implication in cancer therapies. *Cancer Lett.* 380, 586–597. doi: 10.1016/j.canlet.2015.10.036
- Ye, L., Kynaston, H., and Jiang, W. G. (2008). Bone morphogenetic protein-9 induces apoptosis in prostate cancer cells, the role of prostate apoptosis response-4. *Mol. Cancer Res.* 6, 1594–1606. doi: 10.1158/1541-7786.MCR-08-0171
- Ye, L., Lewis-Russell, J. M., Kyanaston, H. G., and Jiang, W. G. (2007). Bone morphogenetic proteins and their receptor signaling in prostate cancer. *Histol. Histopathol.* 22, 1129–1147. doi: 10.14670/HH-22.1129

- Zhang, L., Sha, J., Yang, G., Huang, X., Bo, J., and Huang, Y. (2017). Activation of Notch pathway is linked with epithelial-mesenchymal transition in prostate cancer cells. *Cell Cycle* 16, 999–1007. doi: 10.1080/15384101.2017.1312237
- Zhang, Y., Wang, Z., Ahmed, F., Banerjee, S., Li, Y., and Sarkar, F. H. (2006). Down-regulation of Jagged-1 induces cell growth inhibition and S phase arrest in prostate cancer cells. *Int. J. Cancer* 119, 2071–2077. doi: 10.1002/ijc.22077
- Zhao, D., Mo, Y., Li, M.-T., Zou, S.-W., Cheng, Z.-L., Sun, Y.-P., et al. (2014). NOTCH-induced aldehyde dehydrogenase 1A1 deacetylation promotes breast cancer stem cells. J. Clin. Invest. 124, 5453–5465. doi: 10.1172/JCI76611
- Zoni, E., Chen, L., Karkampouna, S., Granchi, Z., Verhoef, E. I., La Manna, F., et al. (2017). CRIPTO and its signaling partner GRP78 drive the metastatic phenotype in human osteotropic prostate cancer. *Oncogene* 36, 4739–4749. doi: 10.1038/onc.2017.87
- Zoni, E., van der Horst, G., van de Merbel, A. F., Chen, L., Rane, J. K., Pelger, R. C., et al. (2015). miR-25 modulates invasiveness and dissemination of human prostate cancer cells via regulation of α v- and α 6-integrin expression. *Cancer Res.* 75, 2326–2336. doi: 10.1158/0008-5472.CAN-14-2155

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Long Non-coding RNAs in Prostate Cancer with Emphasis on Second Chromosome Locus Associated with Prostate-1 Expression

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OPEN ACCESS

Edited by:

Caterina Nardella, University of Trento, Italy

Reviewed by:

Jun Yan, Nanjing University, China Chandi C. Mandal, Central University of Rajasthan, India

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Specialty section:

This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: 06 July 2017 Accepted: 27 November 2017 Published: 12 December 2017

Citation:

Cimadamore A, Gasparrini S, Mazzucchelli R, Doria A, Cheng L, Lopez-Beltran A, Santoni M, Scarpelli M and Montironi R (2017) Long Non-coding RNAs in Prostate Cancer with Emphasis on Second Chromosome Locus Associated with Prostate-1 Expression. Front. Oncol. 7:305. doi: 10.3389/fonc.2017.00305 Long non-coding RNAs (IncRNAs) are a class of RNA with transcripts longer than 200 nucleotides that lack functional open reading frames. They play various roles in human carcinoma, such as dysregulating gene expression in prostate cancer (PCa), which results in cancer initiation, development, and progression. The non-coding RNA SChLAP1 (second chromosome locus associated with prostate-1) is highly expressed in approximately 25% of PCas with higher prevalence in metastatic compared to localized PCa. Its expression is detectable non-invasively in PCa patient urine samples. Experimental data suggest that targeting SChLAP1 may represent a novel therapeutic application in PCa. This contribution focuses on the role of IncRNAs SChLAP1 expression in PCa diagnosis and prognosis.

Keywords: second chromosome locus associated with prostate-1, metastatic prostate cancer, long non-coding RNA, lethal prostate cancer, prognostic biomarker, marker of aggressiveness

INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed cancer and the second major cause of cancer death in man (1). It is characterized by a wide and heterogeneous spectrum of clinical behaviors, ranging from indolent to aggressive forms. The clinical and morphological methods and features currently used in the routine show a low-predictive value concerning the definition of its level of aggressiveness (2, 3). Predictive and prognostic markers can be determined with clinical and pathological parameters, such as serum prostate-specific antigen (PSA), Gleason score (GS), and TNM stage. Due to PCa heterogeneity, patients with similar TNM stage, GS, and PSA could show opposite outcomes (4).

Additional predictive and prognostic markers are needed to distinguish high-risk from low-risk PCa patients. To this end, genetic and epigenetic investigations have been made to understand the complex genomic landscape of PCa in order to improve its diagnosis and prognosis and to define the potential role of new therapeutical targets (5). Long non-coding RNAs (lncRNAs), a class of RNA with transcripts longer than 200 nucleotides without functional open reading frames, play various roles in human carcinoma (6, 7).

This contribution focuses on the role of lncRNAs second chromosome locus associated with prostate-1 (SChLAP1) expression in PCa diagnosis and prognosis.

LONG NON-CODING RNAs

Long non-coding RNAs are a class of RNA with transcripts longer than 200 nucleotides that lack functional open reading frames (6). Based on their locations in the genome relative to proteincoding genes, lncRNAs have been subdivided into intergenic and intragenic. Intragenic lncRNAs can be further subclassified as exonic, intronic, and overlapping lncRNAs (8). Since proteincoding genes have been the focus of most research, the functional role of lncRNAs has been either underestimated or neglected (9).

As shown by Rinn and Chang, "More than 90% of human genome transcripts, including lncRNAs, do not code for proteins" (10). However, accumulating evidence suggests that lncRNAs play a role in the development of various types of cancers, such as PCa, hepatocellular carcinoma, non-small cell lung cancer, leukemia, colon carcinoma, and breast cancer (11–16).

While the mechanism of many lncRNAs remains to be elucidated, it has become clear that lncRNAs contribute to dysregulation of gene expression in PCa, thus resulting in cancer initiation, development, and progression (17).

IncRNAs IN PROSTATE CANCER

Elucidating the roles of lncRNAs in PCa holds great promise for early detection, prevention, and treatment. A well-known example is prostate cancer antigen3 (PCA3), also known as DD3, initially discovered *via* expression profiling of prostate sample (18). PCA3 has been extensively studied as a PCa-specific biomarker in body fluids. PCA3 urine RNA assay predicts biopsy status and histopathological characteristics (19). However, it does not predict outcomes such as recurrence and metastasis.

Another lncRNA investigated in PCa is metastasis-associated lung adenocarcinoma transcript-1 (MALAT-1), originally known to be overexpressed in patients at high risk for nonsmall cell lung cancer metastasis, as its name implies (20, 21). Its expression is found in many other human solid tumors having close correlation with invasiveness and metastasis (22-26). Ren and colleagues found that MALAT-1 is overexpressed in PCa compared to adjacent normal tissue (20). MALAT-1 expression is significantly higher in castration-resistant PCa (CRPCa) than in primary prostate tumor. Its expression increases from hormone sensitive to CRPCa. The same group of authors showed that this lncRNA could be a promising therapeutic target in patients with CRPCa. The intratumoral administration of therapeutic MALAT-1 siRNA suppressed CRPCa growth and metastasis in vivo, and prolonged the survival of tumor bearing mice (20).

It has also been shown that urine MALAT-1 is an independent predictor of PCa, more accurate than routine PSA. Its use would prevent one-third of unnecessary biopsies in PSA 4–10 ng/ml cohorts, without missing any high-grade PCa (27). Furthermore, in 192 plasma samples, MALAT-1 achieved high diagnostic accuracy in predicting prostate biopsy outcomes and, therefore, it might also be utilized as a plasma-based biomarker for PCa detection (28).

Recently, Zhao et al. investigated the expression profile of FALEC, another lncRNA, in PCa. Like other lncRNAs, its expression is significantly higher in PCa than adjacent normal parenchyma. Its downregulation inhibits cell proliferation, migration, and invasion (29).

Similar results were obtained with CCAT2, a lncRNA involved in proliferation, migration, and invasion of PCa cells. In particular, it was demonstrated that silencing of CCAT2 was able to inhibit N-cadherin, vimentin expression, and improve the expression level of E-cadherin, thus leading to the stimulation of epithelial-mesenchymal transition. High expression level of CCAT2 correlates with poor overall survival and progression-free survival and could be considered an independent prognostic factor in patients with PCa (30). Another promising lncRNA is LOC400891 which showed high expression in patients with an advanced PCa and a shorter biochemical recurrence-free survival time (31).

An interesting feature of lncRNAs is that many of them are not PCa specific. For example, we can observe overexpression of lncRNA-ATB in gastric cancer (32), hepatocellular carcinoma (33), osteosarcoma (34), and other tumors. Its tissue overexpression is directly proportional with the histological grade, high preoperative PSA level, pathological stage, high GS, lymph node metastasis, angiolymphatic invasion, and biochemical recurrence in PCa patients (35).

IncRNA-Based Signature

Signatures, based on microarray lncRNA expression profiling, have been recently developed and widely used in prediction of a series of tumor characteristics and outcomes in various cancer type (36, 37). However, due to its low expression characteristics, a single lncRNA analysis might be associated with false-positive result. To overcome this problem, Huang and colleagues have developed a risk score based on lncRNA expression profile (38). They found four lncRNAs are significantly associated with BCRfree survival. Among the four lncRNAs, two (RP11-108P20.4 and RP11-757G1.6) were positively associated with BCR-free survival, while the remaining two (RP11-347I19.8 and LINC01123) were negatively associated with BCR-free survival. They estimated a risk score for each patient and then divided patients into a highrisk group and a low-risk group by using the median risk score as the cutoff point. The four-lncRNA signature has been shown to be a powerful prognostic factor, independent of age, tumor and lymph node status, GS, margin status, and adjuvant postoperative radiotherapy (38) in (Table 1).

SECOND CHROMOSOME LOCUS ASSOCIATED WITH PROSTATE-1

About 1,800 lncRNAs were identified by Presner et al. through the application of RNA sequencing techniques (i.e., transcriptome sequencing) on a consistent number of tissue samples. Of these 1,800 lncRNAs, 121 resulted transcriptionally dysregulated in PCa (39). Such 121 Prostate Cancer-Associated Transcripts

TABLE 1 | Other IncRNAs associated with prostate cancer.

PCA3	Urine marker useful to predict biopsy status and bistonathological characteristics (10)
MALAT-1	Its expression increases from hormone sensitive to CRPCa (20). Useful plasma biomarker for PCa
	detection (28)
FALEC	Its inhibition decreases cell proliferation, migration, and invasion (29)
CCAT2	Its high expression levels correlates with poor overall surviva and progression-free survival (30)
LOC400891	Its high expression correlates with shorter BCR-free survival time (31)
ATB	Its high expression correlates with preoperative PSA levels, pathological stage, GS, lymph node metastasis, angiolymphatic invasion, and BCR (35)
RP11-108P20.4	Positively associated with BCR-free survival
RP11-757G1.6	Part of the Four-IncRNA signature (38)
RP11-347l19.8	Negatively associated with BCR-free survival
LINC01123	Part of the Four-IncRNA signature (38)

CRPCa, castration resistant; PCa, prostate cancer; BCR, biochemical recurrence; PSA, prostate-specific antigen; GS, Gleason score; IncRNAs, long non-coding RNAs.

represent an unbiased list of potentially functional lncRNAs associated with PCa. By performing a cancer outlier profile analysis to identify intergenic lncRNAs selectively upregulated in a subset of cancers, they found two lncRNAs, PCAT-109 and PCAT-114, both located on Chromosome 2q31.3 in a "gene desert," a region of the genome that are lacking of protein-coding genes. Both genes showed "outlier profiles and ranked among the best outliers in PCa" (40). In particular, PCAT-114 was found to be overexpressed in prostate cell lines. It was named SChLAP1 after its genomic location (40). Schlap1 gene has a transcript length of 24,484 Kb. The complete gene is composed of 7 exons and 1,675 nucleotides. The primary transcript (isoform 1) is composed of 5 exons with a length of 1,436 nucleotides. As a result of a spicing process, a total of 8 isoforms were found, with isoform 1, isoform 2, and 3 accounting for >90% of transcripts (41). RNA-seq, performed on 27 different tissue samples from 95 human individuals, showed that SChLAP1 expression was highly specific for prostate tissue, being present at minor levels in bladder, kidney, and testis samples (42).

SChLAP1's Working Mechanisms: Interaction with SWI/SNF Complex and miR-198

In vitro and *in vivo* gain-of-function and loss-of-function experiments have shown that SChLAP1 plays a crucial role in cancer cell invasiveness and metastasis, antagonizing the activity of the SWI/SNF chromatin-modifying complex, a multiprotein system able to move nucleosomes at gene promoters. In particular, such experiments showed that the inactivation of SWI/SNF complex promoted cancer progression and that multiple SWI/SNF components were somatically inactivated in cancer (41, 43). As shown by Prensner, even though "other lncRNAs, such as HOTAIR and HOTTIP, are known to assist epigenetic complexes such as PRC2 and MLL by facilitating their genomic binding and enhancing their functions, SChLAP1 is the first lncRNA that impairs a major epigenetic complex with well-documented tumor suppressor function" (41, 44).

In vivo, SChLAP1 has been shown to be implicated in tumor cell proliferation and metastasization, as evidenced by both the reduction of tumor growth kinetics and the decreased number and dimensions of metastatic sites as a consequence of the intracardiac injection of 22Rv1 cells with SChLAP1 knockdown in CB-17 SCID mice (41).

Recent studies have shown interaction between SChLAP1 and miR-198. MiR-198 is downregulated in many cancers, such as gastric cancer, lung cancer, and hepatocellular carcinoma (45-47). miR-198 suppress the proliferation and invasion of colorectal carcinoma (48). miR-198 might exert its anticancer effect through inhibition of MAPKs signaling pathway (49). In PCa tissue, a low expression of miR-198 was found. As shown by Li et al., "knockdown of SChLAP1 significantly increased the expression of miR-198 and SChLAP1 overexpression markedly decreased it. Thus, SChLAP1 acted as a negative regulator in the expression of miR-198" and subsequently modulated the MAPK1 signaling pathway in PCa (49) (Figure 1). Transfecting PCa cells with a designed-specific siRNA to knockdown SChLAP1 expression, investigators have obtained, as expected, a significantly reduction in cell proliferation together with an increase in apoptosis-associated proteins. Furthermore, SChLAP1 knockdown determined a decrease of MMP-9, MMP-14, and VEGF expressions both in vitro and in vivo, confirming its involvement in cancer invasiveness and metastasis (49). All such findings show multiple interactions between SChLAP1 and factors involved in oncogenesis and cancer progression and explain the mechanism



FIGURE 1 | Working model of second chromosome locus associated with prostate-1 (SChLAP1) in prostate cancer (PCa). There are two pathways. In Pathway 1, loss of SWI/SNF functionality promotes cancer progression (41). In Pathway 2, miR-198 might exert its anticancer effect through inhibition of MAPKs signaling pathway. SChLAP1 acts as a negative regulator in the expression of miR-198 and subsequently modulates the MAPK1 signaling pathway in PCa (49).

through which SChLAP1 promotes migration and invasion of PCa (50). Understanding this molecular pathway is essential for exploring new potential strategies for early diagnosis and therapy.

SChLAP1: Predictor of Aggressive PCa

Second chromosome locus associated with prostate-1 is highly expressed in approximately 25% of PCa, expression being higher in metastatic compared to localized prostate cancers. It was associated with ETS gene fusions (41). Multivariate and univariate regression analyses have demonstrated that SChLAP1 expression is an independent predictor of PCa aggressiveness with highly significant hazard ratios for predicting BCR, clinical progression to systemic disease, and PCa-specific mortality, compared to other clinical factors such as advanced clinical stage and the GS (41). Moreover, SChLAP1 expression was significantly associated with extracapsular extension, seminal vesicle invasion, and positive surgical margin status (40).

Validation in three-independent cohorts has confirmed the prognostic value of SChLAP1 for metastasis. On multivariate modeling, SChLAP1 expression independently predicted metastasis within 10 years, death within 10 years, and biochemical recurrence within 5 years with odds ratios, comparable to GS. Among all known genes, the lncRNA SChLAP1 ranked first for elevated expression in patients with metastatic progression by receiver-operator-curve area-under-the-curve analyses and was the only prostate-specific gene, ideal for development as a non-invasive biomarker (40).

Similar results have been obtained by Mehra and colleagues using a novel RNA *in situ* hybridization (ISH) assay for detection of SChLAP1 in formalin-fixed, paraffin-embedded tissue (51). They found that high SChLAP1 expression independently predicts biochemical PCa recurrence after radical prostatectomy in patients with clinically localized PCa and that it is associated with the development of lethal PCa.

Interestingly, high SChLAP1 expression is associated with lethal PCa among patients with non-advanced clinical tumor stage. Similarly, high SChLAP1 expression is associated with lethal PCa among patients with low grade tumors (GS \leq 7). However, using SChLAP1 as prognostic test, investigators have obtained a sensitivity around 24% and a specificity of 94% in the group of non-advanced clinical tumor stage and in the group with a 6-7 GS (52). Considering the low sensitivity of the test in the identification of an aggressive disease in contrast with low-risk morphological features, the evaluation of SChLAP1expression alone does not seem to improve treatment decision. In conjunction with other prognostic tools, SChLAP1 has been shown to improve upon established clinical algorithms for the risk stratification of PCa patients, specifically the CAPRA-S score (53, 54), i.e., one of the best clinic-pathological models to date. SChLAP1 further improves prediction upon both the Decipher test 50 and the CCP gene signature (55, 56).

Recently, Chua et al. have investigated SChLAP1 expression in subsets of PCa characterized by cribriform architecture (CA) and intraductal carcinoma (IDC), features both associated with increased risks of biochemical relapse and metastasis. Besides the histological presentation, IDC/CA+ cancers harbor an increased percentage of genome aberration (PGA). This is in agreement with the observation that tumors with IDC or genomic instability have a greater metastatic potential. Using mRNA abundance analyses and assessing >25,000 genes, they found that SChLAP1 was surprisingly the only gene with more than threefold higher expression in IDC/CA+ compared to IDC/CA- cancers (57).

The association of SChLAP1 expression within IDC/CA+ tumors has also been further demonstrated by SChLAP1 RNA-ISH in prostatectomy TMA-cores. The SChLAP1+, IDC/CA+ subgroup has shown a significant increase of biochemical relapse, independent of PGA. Combining histology features of cribriform architecture and intraductal carcinoma with genomic instability or SChLAP1 expression can stratify patients for recurrence more accurately than any parameter alone. Interestingly, SChLAP1 RNA-ISH diffuse expression has been observed in the cribriform architecture and intraductal carcinoma and in the adjacent invasive adenocarcinoma. This further supports a field defect and a common clonal ancestor to both histopathologies (58).

SChLAP1 in Urine Sediments

Second chromosome locus associated with prostate-1 expression is detectable non-invasively in PCa patient in urine samples. Its expression is both more frequent and more highly elevated in GS 7 compared to GS 6 patients even if it is less sensitive than PCA3 and TMPRSS-ERG gene fusion (40). "SChLAP1 expression may complement existing urine diagnostic assays, including PCA3 and TMPRSS2-ERG, and that clinical application of a SChLAP1 urine test would be most effective in conjunction with these, and potentially other, urine assays" (59).

SChLAP1 As a Potential Drug Target

RNA interference (RNAi) technology using short interfering RNA (si-RNA) has shown great potential in the treatment of cancers through silencing of specific genes. *In vitro* and *in vivo* experiments have demonstrated that SChLAP1-knockdown promoted apoptosis and inhibited cell proliferation and invasion (41, 49). SChLAP1 is also overexpressed in bladder cancer compared to paired normal bladder tissues. Cell transfected with SChLAP1 siRNA showed growth arrest, apoptosis, and migration inhibition, suggesting oncogenic roles in bladder cancer and a potential therapeutic target (**Table 2**) (60). Such results might be the starting point to investigate the therapeutical potential of antagonizing SChLAP1 oncogenic functions (60).

IncRNA IN DIAGNOSIS, PROGNOSIS, AND TREATMENT: PROS AND CONS

There are *Pros* and *Cons* with the use of lncRNA in the diagnosis and treatment of PCa patients. Indeed, lncRNA may represent a useful biomarker that can give to clinicians fundamental information on tumor biological behavior and aggressiveness, leading to the possibility of designing personalized and tailored strategies for a single PCa patient. This may also allow an optimization of patients' outcome and to avoid useful costs and consequences of not effective therapies for PCa patients.

As far as the *Cons*, tumor aggressiveness is the result of a complex process that involves lncRNA and a variety of driver

TABLE 2 | SChLAP1 expression in prostate cancer.

Reference	Result	Method	No. specimens
Prensner (39)	 121 novel IncRNA loci (out of >1,800) were aberrantly expressed in PCa tissues Only two, PCAT-109 and PCAT-114, showed striking outlier profiles and ranked among the best outliers in PCa 	RNA-Seq; COPA	102 PCa tissue samples and cell lines
Prensner (41)	 SChLAP1 expression is an independent predictor of PCa aggressiveness with highly significant hazard ratios for predicting BCR, CP, and PCSM SChLAP1 antagonizes tumor-suppressive functions of the SWI/SNF complex 	qPCR	235 RP localized PCa
Prensner (40)	 SChLAP1 expression independently predicted metastasis, PCa-specific death, and BCR with OR comparable to GS SChLAP1 expression was detectable non-invasively in urine samples and associated with higher-risk patients 	RNA extraction, microarray hybridization; qPCR	1,008 patients. Three independent cohorts; 230 urine sediment samples
Mehra (51)	 SChLAP1 expression is enriched in samples from tumors with high GSs (≥8) compared to tumors with lower GSs High SChLAP1 expression independently predicts BCR (PSA relapse) after RP 	ISH	160 clinically localized PCa
Mehra (52)	 High SChLAP1 expression is significantly associated with a higher risk of lethal PCa and PCa-specific death independent of age at diagnosis, GS, and pathologic stage High SChLAP1 expression is associated with lethal PCa among patients with non-advanced clinical tumor stage, but not among patients with advanced clinical tumor stage 	ISH	937 PCa patients
Zhang (60)	 SChLAP1 was overexpressed in bladder cancer tissues compared to paired normal bladder tissues Cell growth arrest, apoptosis induction, and migration inhibition were also observed in bladder cancer cells after transfection with SChLAP1 siRNA 	qPCR; CCK-8 assay, flow cytometry analysis, and wound healing assay	Bladder cancer T24 and 5,637 cells
Chua (57)	 SChLAP1 was the only gene expressed at >3-fold higher in intraductal carcinoma (IDC) and cribriform architecture (CA) PCa than in IDC/CA - tumors 	Profiling of mRNA abundance, ISH	1,325 localized PCa
Li (49)	 Knockdown of SChLAP1 promoted apoptosis and inhibited cell proliferation and invasion <i>in vitro</i> and <i>in vivo</i> SChLAP1 acted as a negative regulator in the expression of miR-198 and accelerates the proliferation and metastasis of PCa promoting the MAPK1 pathway 	Not available	Not available

RNA-Seq, next generation transcriptome sequencing; COPA, cancer outlier profile analysis; PCa, prostate cancer; qPCR, quantitative Polymerase Chain Reaction; FFPE, formalin-fixed paraffin embedded; BCR, biochemical recurrence; CP, clinical progression; PCSM, prostate cancer-specific mortality; GS, Gleason score; OR, odds ratio; RP, radical prostatectomy; ISH, in situ hybridization; SChLAP1, second chromosome locus associated with prostate-1; IncRNAs, long non-coding RNAs.

genes, leading to the necessity for uropathologists to test and validate not a single driver gene, but a panel of genes with direct consequences on the relative costs of these procedures.

CONCLUSION

In conclusion, research on lncRNAs in PCa is at its onset. However, as shown in this review:

• The first set of data has revealed central roles with clinical significance for lncRNAs in different stages of the disease.

REFERENCES

- Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin (2016) 66:7–30. doi:10.3322/caac.21332
- Klotz L. Prostate cancer overdiagnosis and overtreatment. *Curr Opin Endocrinol Diabetes Obes* (2013) 20:204–9. doi:10.1097/MED.0b013e328360332a
- Alcover J, Filella X. Identification of candidates for active surveillance: should we change the current paradigm? *Clin Genitourin Cancer* (2015) 13:499–504. doi:10.1016/j.clgc.2015.06.001

- There is evidence that lncRNAs, including SChLAP1, are critical in PCa development and progression.
- Concerning future perspective, mainly based on experimental data, targeting SChLAP1 may become a novel therapeutic application in PCa.

AUTHOR CONTRIBUTIONS

RM: conception and design. AC and SG: drafting the manuscript. AD and RMa: acquisition of data. LC and AL-B: critical revision of the manuscript. MS: supervision.

- Fraser M, Berlin A, Bristow RG, van der Kwast T. Genomic, pathological, and clinical heterogeneity as drivers of personalized medicine in prostate cancer. Urol Oncol (2015) 33:85–94. doi:10.1016/j.urolonc.2013. 10.020
- Prensner JR, Rubin MA, Wei JT, Chinnaiyan AM. Beyond PSA: the next generation of prostate cancer biomarkers. *Sci Transl Med* (2012) 4:127rv3. doi:10.1126/scitranslmed.3003180
- Guttman M, Rinn JL. Modular regulatory principles of large non-coding RNAs. Nature (2012) 482:339–46. doi:10.1038/nature10887

- Diederichs S, Bartsch L, Berkmann JC, Fröse K, Heitmann J, Hoppe C, et al. The dark matter of the cancer genome: aberrations in regulatory elements, untranslated regions, splice sites, non-coding RNA and synonymous mutations. *EMBO Mol Med* (2016) 8:442–57. doi:10.15252/emmm.201506055
- Derrien T, Johnson R, Bussotti G, Tanzer A, Djebali S, Tilgner H, et al. The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. *Genome Res* (2012) 22:1775–89. doi:10.1101/gr.132159.111
- 9. Gibb EA, Brown CJ, Lam WL. The functional role of long non-coding RNA in human carcinomas. *Mol Cancer* (2011) 10:38. doi:10.1186/1476-4598-10-38
- Rinn JL, Chang HY. Genome regulation by long noncoding RNAs. Annu Rev Biochem (2012) 81:145–66. doi:10.1146/annurev-biochem-051410-092902
- Smolle MA, Bauernhofer T, Pummer K, Calin GA, Pichler M. Current insights into Long Non-Coding RNAs (lncRNAs) in prostate cancer. *Int J Mol Sci* (2017) 18:E473. doi:10.3390/ijms18020473
- Heery R, Finn SP, Cuffe S, Gray SG. Long non-coding RNAs: key regulators of epithelial-mesenchymal transition, tumour drug resistance and cancer stem cells. *Cancers* (2017) 9:E38. doi:10.3390/cancers9040038
- Lin R, Maeda S, Liu C, Karin M, Edgington TS. A large noncoding RNA is a marker for murine hepatocellular carcinomas and a spectrum of human carcinomas. *Oncogene* (2007) 26:851–8. doi:10.1038/sj.onc.1209846
- Calin GA, Liu CG, Ferracin M, Hyslop T, Spizzo R, Sevignani C, et al. Ultraconserved regions encoding ncRNAs are altered in human leukemias and carcinomas. *Cancer Cell* (2007) 12:215–29. doi:10.1016/j.ccr.2007.07.027
- Pibouin L, Villaudy J, Ferbus D, Muleris M, Prospéri MT, Remvikos Y, et al. Cloning of the mRNA of overexpression in colon carcinoma-1: a sequence overexpressed in a subset of colon carcinomas. *Cancer Genet Cytogenet* (2002) 133:55–60. doi:10.1016/S0165-4608(01)00634-3
- Zhang C, Wang X, Li X, Zhao N, Wang Y, Han X, et al. The landscape of DNA methylation-mediated regulation of long non-coding RNAs in breast cancer. *Oncotarget* (2017) 8(31):51134–50. doi:10.18632/oncotarget.17705
- Prensner JR, Chinnaiyan AM. The emergence of lncRNAs in cancer biology. Cancer Discov (2011) 1:391–407. doi:10.1158/2159-8290.CD-11-0209
- Bussemakers MJ, van Bokhoven A, Verhaegh GW, Smit FP, Karthaus HF, Schalken JA, et al. DD3: a new prostate-specific gene, highly overexpressed in prostate cancer. *Cancer Res* (1999) 59:5975–9.
- Nakanishi H, Groskopf J, Fritsche HA, Bhadkamkar V, Blase A, Kumar SV, et al. PCA3 molecular urine assay correlates with prostate cancer tumor volume: implication in selecting candidates for active surveillance. *J Urol* (2008) 179:1804–9. doi:10.1016/j.juro.2008.01.013
- Ren S, Liu Y, Xu W, Sun Y, Lu J, Wang F, et al. Long noncoding RNA MALAT-1 is a new potential therapeutic target for castration resistant prostate cancer. *J Urol* (2013) 190:2278–87. doi:10.1016/j.juro.2013.07.001
- Ji P, Diederichs S, Wang W, Böing S, Metzger R, Schneider PM, et al. MALAT-1, a novel noncoding RNA, and thymosin beta4 predict metastasis and survival in early-stage nonsmall cell lung cancer. *Oncogene* (2003) 22:8031. doi:10.1038/sj.onc.1206928
- Lai MC, Yang Z, Zhou L, Zhu QQ, Xie HY, Zhang F, et al. Long non-coding RNA MALAT-1 overexpression predicts tumor recurrence of hepatocellular carcinoma after liver transplantation. *Med Oncol* (2012) 29:1810. doi:10.1007/ s12032-011-0004-z
- Tano K, Mizuno R, Okada T, Rakwal R, Shibato J, Masuo Y, et al. MALAT-1 enhances cell motility of lung adenocarcinoma cells by influencing the expression of motility-related genes. *FEBS Lett* (2010) 584:4575. doi:10.1016/j. febslet.2010.10.008
- Yamada K, Kano J, Tsunoda H, Yoshikawa H, Okubo C, Ishiyama T, et al. Phenotypic characterization of endometrial stromal sarcoma of the uterus. *Cancer Sci* (2006) 97:106. doi:10.1111/j.1349-7006.2006.00147.x
- Xu C, Yang M, Tian J, Wang X, Li Z. MALAT-1: a long noncoding RNA and its important 30 end functional motif in colorectal cancer metastasis. *Int J Oncol* (2011) 39:169. doi:10.3892/ijo.2011.1007
- Chen D, Liu L, Wang K, Yu H, Wang Y, Liu J, et al. The role of MALAT-1 in the invasion and metastasis of gastric cancer. *Scand J Gastroenterol* (2017) 52:790–6. doi:10.1080/00365521.2017.1280531
- Wang F, Ren S, Chen R, Lu J, Shi X, Zhu Y, et al. Development and prospective multicenter evaluation of the long noncoding RNA MALAT-1 as a diagnostic urinary biomarker for prostate cancer. *Oncotarget* (2014) 5:11091–102. doi:10.18632/oncotarget.2691

- Ren S, Wang F, Shen J, Sun Y, Xu W, Lu J, et al. Long non-coding RNA metastasis associated in lung adenocarcinoma transcript 1 derived miniRNA as a novel plasma-based biomarker for diagnosing prostate cancer. *Eur J Cancer* (2013) 49:2949–59. doi:10.1016/j.ejca.2013.04.026
- Zhao R, Sun F, Bei X, Wang X, Zhu Y, Jiang C, et al. Upregulation of the long non-coding RNA FALEC promotes proliferation and migration of prostate cancer cell lines and predicts prognosis of PCa patients. *Prostate* (2017) 77(10):1107–17. doi:10.1002/pros.23367
- Zheng J, Zhao S, He X, Zheng Z, Bai W, Duan Y, et al. The up-regulation of long non-coding RNA CCAT2 indicates a poor prognosis for prostate cancer and promotes metastasis by affecting epithelial-mesenchymal transition. *Biochem Biophys Res Commun* (2016) 480(4):508–14. doi:10.1016/j.bbrc.2016.08.120
- Wang J, Cheng G, Li X, Pan Y, Qin C, Yang H, et al. Overexpression of long non-coding RNA LOC400891 promotes tumor progression and poor prognosis in prostate cancer. *Tumour Biol* (2016) 37:9603–13. doi:10.1007/ s13277-016-4847-y
- Saito T, Kurashige J, Nambara S, Komatsu H, Hirata H, Ueda M, et al. A Long non-coding RNA activated by transforming growth Factor-β is an independent prognostic marker of gastric cancer. *Ann Surg Oncol* (2015) 22(Suppl 3):S915–22. doi:10.1245/s10434-015-4554-8
- Yuan JH, Yang F, Wang F, Ma JZ, Guo YJ, Tao QF, et al. A long noncoding RNA activated by TGF-β promotes the invasion-metastasis cascade in hepatocellular carcinoma. *Cancer Cell* (2014) 25:666–81. doi:10.1016/j.ccr.2014.03.010
- Han F, Wang C, Wang Y, Zhang L. Long noncoding RNA ATB promotes osteosarcoma cell proliferation, migration and invasion by suppressing miR-200s. *Am J Cancer Res* (2017) 7:770–83.
- Xu S, Yi XM, Tang CP, Ge JP, Zhang ZY, Zhou WQ. Long non-coding RNA ATB promotes growth and epithelial-mesenchymal transition and predicts poor prognosis in human prostate carcinoma. *Oncol Rep* (2016) 36:10–22. doi:10.3892/or.2016.4791
- Zhu X, Tian X, Yu C, Shen C, Yan T, Hong J, et al. A long non-coding RNA signature to improve prognosis prediction of gastric cancer. *Mol Cancer* (2016) 15:60. doi:10.1186/s12943-016-0544-0
- Sun J, Chen X, Wang Z, Guo M, Shi H, Wang X, et al. A potential prognostic long non-coding RNA signature to predict metastasis-free survival of breast cancer patients. *Sci Rep* (2015) 5:16553. doi:10.1038/srep16553
- Huang TB, Dong CP, Zhou GC, Lu SM, Luan Y, Gu X, et al. A potential panel of four-long noncoding RNA signature in prostate cancer predicts biochemical recurrence-free survival and disease-free survival. *Int Urol Nephrol* (2017) 49:825–35. doi:10.1007/s11255-017-1536-8
- Prensner JR, Iyer MK, Balbin OA, Dhanasekaran SM, Cao Q, Brenner JC, et al. Transcriptome sequencing across a prostate cancer cohort identifies PCAT-1, an unannotated lincRNA implicated in disease progression. *Nat Biotechnol* (2011) 29:742–9. doi:10.1038/nbt.1914
- Prensner JR, Zhao S, Erho N, Schipper M, Iyer MK, Dhanasekaran SM, et al. Nomination and validation of the long noncoding RNA SChLAP1 as a risk factor for metastatic prostate cancer progression: a multi-institutional high-throughput analysis. *Lancet Oncol* (2014) 15:1469–80. doi:10.1016/ S1470-2045(14)71113-1
- Prensner JR, Iyer MK, Sahu A, Asangani IA, Cao Q, Patel L, et al. The long noncoding RNA SChLAP1 promotes aggressive prostate cancer and antagonizes the SWI/SNF complex. *Nat Genet* (2013) 45:1392–8. doi:10.1038/ng.2771
- Fagerberg L, Hallström BM, Oksvold P, Kampf C, Djureinovic D, Odeberg J, et al. Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. *Mol Cell Proteomics* (2014) 13:397–406. doi:10.1074/mcp.M113.035600
- Roberts CW, Orkin SH. The SWI/SNF complex chromatin and cancer. Nat Rev Cancer (2004) 4:133–42. doi:10.1038/nrc1273
- Atala A. Re: The long noncoding RNA SChLAP1 promotes aggressive prostate cancer and antagonizes the SWI/SNF complex. J Urol (2014) 192:613. doi:10.1016/j.juro.2014.05.031
- 45. Huang WT, Wang HL, Yang H, Ren FH, Luo YH, Huang CQ, et al. Lower expressed miR-198 and its potential targets in hepatocellular carcinoma: a clinicopathological and in silico study. *Onco Targets Ther* (2016) 22:5163–80. doi:10.2147/OTT.S108828
- Wu S, Zhang G, Li P, Chen S, Zhang F, Li J, et al. miR-198 targets SHMT1 to inhibit cell proliferation and enhance cell apoptosis in lung adenocarcinoma. *Tumour Biol* (2016) 37:5193–202. doi:10.1007/s13277-015-4369-z

- Cui Z, Zheng X, Kong D. Decreased miR-198 expression and its prognostic significance in human gastric cancer. World J Surg Oncol (2016) 6:33. doi:10.1186/s12957-016-0784-x
- Wang M, Wang J, Kong X, Chen H, Wang Y, Qin M, et al. MiR-198 represses tumor growth and metastasis in colorectal cancer by targeting fucosyl transferase 8. *Sci Rep* (2014) 1:6145. doi:10.1038/srep06145
- Li Y, Luo H, Xiao N, Duan J, Wang Z, Wang S. Long noncoding RNA SChLAP1 accelerates the proliferation and metastasis of prostate cancer via targeting miR-198 and promoting the MAPK1 pathway. *Oncol Res* (2017). doi:10.3727 /096504017X14944585873631
- Ma W, Chen X, Ding L, Ma J, Jing W, Lan T, et al. The prognostic value of long noncoding RNAs in prostate cancer: a systematic review and meta-analysis. *Oncotarget* (2017) 8(34):57755–65. doi:10.18632/oncotarget.17645
- Mehra R, Shi Y, Udager AM, Prensner JR, Sahu A, Iyer MK, et al. A novel RNA in situ hybridization assay for the long noncoding RNA SChLAP1 predicts poor clinical outcome after radical prostatectomy in clinically localized prostate cancer. *Neoplasia* (2014) 16:1121–7. doi:10.1016/j.neo. 2014.11.006
- Mehra R, Udager AM, Ahearn TU, Cao X, Feng FY, Loda M, et al. Overexpression of the long non-coding RNA SChLAP1 independently predicts lethal prostate cancer. *Eur Urol* (2016) 70:549–52. doi:10.1016/j. eururo.2015.12.003
- Cooperberg MR, Hilton JF, Carroll PR. The CAPRA-S score: a straightforward tool for improved prediction of outcomes after radical prostatectomy. *Cancer* (2011) 117:5039–46. doi:10.1002/cncr.26169
- Cooperberg MR, Davicioni E, Crisan A, Jenkins RB, Ghadessi M, Karnes RJ. Combined value of validated clinical and genomic risk stratification tools for predicting prostate cancer mortality in a high-risk prostatectomy cohort. *Eur Urol* (2015) 67:326–33. doi:10.1016/j.eururo.2014.05.039
- Glinsky GV, Glinskii AB, Stephenson AJ, Hoffman RM, Gerald WL. Gene expression profiling predicts clinical outcome of prostate cancer. *J Clin Invest* (2004) 113:913–23. doi:10.1172/JCI20032

- Cuzick J, Swanson GP, Fisher G, Brothman AR, Berney DM, Reid JE, et al. Prognostic value of an RNA expression signature derived from cell cycle proliferation genes in patients with prostate cancer: a retrospective study. *Lancet* Oncol (2011) 12:245–55. doi:10.1016/S1470-2045(10)70295-3
- Chua MLK, Lo W, Pintilie M, Murgic J, Lalonde E, Bhandari V, et al. A prostate cancer "nimbosus": genomic instability and SChLAP1 dysregulation underpin aggression of intraductal and cribriform subpathologies. *Eur Urol* (2017) 72(5):665–74. doi:10.1016/j.eururo.2017.04.034
- Spratt DE. Convergence of genomic instability and SChLAP1: weathering the storm of intraductal carcinoma of the prostate. *Eur Urol* (2017) 72(5):675–6. doi:10.1016/j.eururo.2017.05.015
- Whitman EJ, Groskopf J, Ali A, Chen Y, Blase A, Furusato B, et al. PCA3 score before radical prostatectomy predicts extracapsular extension and tumor volume. J Urol (2008) 180:1975–8. doi:10.1016/j.juro.2008.07.060
- Zhang J, Shi Z, Nan Y, Li M. Inhibiting malignant phenotypes of the bladder cancer cells by silencing long noncoding RNA SChLAP1. *Int Urol Nephrol* (2016) 48:711–6. doi:10.1007/s11255-016-1230-2

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microRNAs Make the Call in Cancer Personalized Medicine

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Since their discovery and the advent of RNA interference, microRNAs have drawn enormous attention because of their ubiquitous involvement in cellular pathways from life to death, from metabolism to communication. It is also widely accepted that they possess an undeniable role in cancer both as tumor suppressors and tumor promoters modulating cell proliferation and migration, epithelial-mesenchymal transition and tumor cell invasion and metastasis. Moreover, microRNAs can even affect the tumor surrounding environment influencing angiogenesis and immune system activation and recruitment. The tight association of microRNAs with several cancer-related processes makes them undoubtedly connected to the effect of specific cancer drugs inducing either resistance or sensitization. In this context, personalized medicine through microRNAs arose recently with the discovery of single nucleotide polymorphisms in the target binding sites, in the sequence of the microRNA itself or in microRNA biogenesis related genes, increasing risk, susceptibility and progression of multiple types of cancer in different sets of the population. The depicted scenario implies that the overall variation displayed by these small non-coding RNAs have an impact on patient-specific pharmacokinetics and pharmacodynamics of cancer drugs, pushing on a rising need of personalized treatment. Indeed, microRNAs from either tissues or liquid biopsies are also extensively studied as valuable biomarkers for disease early recognition, progression and prognosis. Despite microRNAs being intensively studied in recent years, a comprehensive review describing these topics all in one is missing. Here we report an up-to-date and critical summary of microRNAs as tools for better understanding personalized cancer biogenesis, evolution, diagnosis and treatment.

OPEN ACCESS

Edited by:

Simona Pisanti, University of Salerno, Italy

Reviewed by:

Chandi C Mandal, Central University of Rajasthan, India Botao Zhao, Shanghai University, China

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Specialty section:

This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: 30 June 2017 Accepted: 08 September 2017 Published: 22 September 2017

Citation:

Detassis S, Grasso M, Del Vescovo V and Denti MA (2017) microRNAs Make the Call in Cancer Personalized Medicine. Front. Cell Dev. Biol. 5:86. doi: 10.3389/fcell.2017.00086 Keywords: microRNAs, personalized medicine, cancer, MiR-SNP, biomarker

MICRORNA BIOGENESIS

microRNAs are small non-coding RNAs described for the first time in 1993 (Lee et al., 1993). They are found in plants (Jones-Rhoades et al., 2006), animals and viruses (Grundhoff and Sullivan, 2011), with functions in RNA silencing and post-transcriptional regulation of gene expression. They also have a role in pathological processes including neurodegenerative diseases (Molasy et al., 2016; Reddy et al., 2016) and cancer (da Silva Oliveira et al., 2016; Mohammadi et al., 2016). microRNAs transcriptional units are present both in introns or exons of other genes and as independent ones (Godnic et al., 2013). They are transcribed mainly by RNA Polymerase II, capped and polyadenylated forming primary microRNAs (pri-microRNAs). A small group is generated by RNA Polymerase III. The pri-microRNA is processed in a precursor microRNA (pre-microRNA)—about 70 nt—by RNase III Drosha and RNA-binding protein DGCR8 (Lee et al., 2003). Subsequently, the pre-microRNA is transported out of the nucleus via exportin-GTPase

RAN system, where is further processed by Dicer producing the double-stranded microRNA of 22nt (Wilson et al., 2015). A complex made of AGO proteins is able to bind it and form the miRISC. Only one strand of the microRNA is loaded in the RISC complex, while the other (the passenger strand) is thought to be degraded. The RISC complex has an important post-transcriptional role in gene expression, regulating stability and turnover of mRNAs. The loaded microRNA can target mRNAs, exploiting its sequence complementarity. If the match is perfect the system leads to the mRNA degradation (Yekta et al., 2004), otherwise it impedes its translation (Ipsaro and Joshua-Tor, 2015). Because of their short length, microRNAs, which usually bind the 3'UTR of target mRNAs, are able to target several distinct mRNAs and, on the other hand, any given mRNA may present many binding sites for different microRNAs (Bartel et al., 2009).

MICRORNA AND CANCER

It has been widely reported that microRNAs are involved in many aspects related to cancer (**Figure 1**). Following the "hallmarks" of cancer (Hanahan and Weinberg, 2011) we can find many articles in which microRNAs play a role in each of these steps on the road of cancer biogenesis and progression. Here we describe some examples (**Table 1**).

- 1. Sustaining the proliferative signal. miR-27a-3p was shown to be associated with progression of nasopharyngeal cancer from patient samples and to be increased compared to healthy tissues. *In vitro* it promotes 5–8 F cell proliferation, migration and invasion targeting MAPK10 (Li and Luo, 2017). On the contrary, miR-545 was found decreased in colorectal cancer (CRC) in comparison to normal tissues and thus, its over-expression led to diminished proliferation and colony formation capacity (Huang and Lu, 2017). Luciferase and western blot assay confirmed the *in-silico* prediction of miR-545 targeting EGFR in CRC cell lines.
- 2. *Evading tumor suppressors*. Liu and colleagues (Liu Y. et al., 2017) showed how miR-19a binds directly the 3'UTR of TIA1 mRNA, involved in stress granuli formation and in the apoptotic pathway, promoting cell proliferation and migration in CRC cells, boosting also tumor growth in xenograft mice.
- 3. *Resistance to cell death.* It has been reported that miR-29 is an endogenous regulator of MCL-1 protein expression, an antiapoptotic molecule, and it has been found down-regulated in cholangiocarcinoma cell lines (Mott et al., 2007). Similarly, miR-15a and miR-16-1, found deleted or down-regulated in the majority of chronic lymphocytic leukemias (CLLs), can directly negatively regulate BCL-2 in CLL. Their expression was described as inversely correlated to BCL2 expression in CLL and their over-expression may induce apoptosis in a leukemic cell line model through BCL2 repression (Cimmino et al., 2005).
- 4. *Enabling replicative immortality.* miR-130b~301b cluster is hypermethylated in prostate cancer cells and it was demonstrated how its expression restoration can replace

senescence mechanisms reducing the malignant phenotype of prostate cancer cells (Chen et al., 2015; Ramalho-Carvalho et al., 2017). Similarly, miR-137 levels are significantly reduced in human pancreatic cancer leading to a defective senescence response. This small non-coding RNA targets KDM4A which expression contributes to avoid miR-137-induced senescence. Therefore, restoration of miR-137 expression it has been reported to promote senescence and dampen proliferation of pancreatic cancer cells (Neault et al., 2016).

- 5. Inducing angiogenesis. miR-135a is generally decreased in gastric cancer tissues compared to normal samples. It targets FAK which is an important regulator and effector of VEGF in tumor angiogenesis. It has been described that upon miR-135a over-expression, the protein levels of FAK in gastric cancer cell lines decrease significantly. Therefore, it has been proposed that miR-135 inhibits tumor growth, migration, invasion and angiogenesis by targeting focal adhesion kinase (FAK) pathway (Cheng et al., 2017). Differently, miR-23 in lung cancer cells under hypoxic conditions is up-regulated in the secretome and directly targets prolyl hydroxylase 1 and 2, enhancing the accumulation of the hypoxiainducible factor-1a. Consequently, hypoxic lung cancer cells enhanced angiogenesis. In addition, it has been shown how secreted miR-23a also inhibits tight junction protein ZO-1, thereby increasing vascular permeability and cancer trans-endothelial migration. Moreover, inhibition of miR-23a dampened angiogenesis and tumor growth in mice and miR-23a found in sera of lung cancer patients positively correlated with proangiogenic activities (Hsu et al., 2017).
- 6. Activation of invasion and metastasis. Daugaard and colleagues demonstrated via RNA-seq analysis of formalinfixed paraffin embedded (FFPE) lung adenocarcinomas from patients with and without detectable metastasis disease, that down-regulation of miR-30a-3p and up-regulation of miR-210-3p were significantly associated with the presence of distant metastases (Kumarswamy et al., 2012; Daugaard et al., 2017). Microarray analysis and quantitative PCR by the Law laboratory identified and validated up-regulated miR-885-5p in liver metastases when compared to primary CRCs. Furthermore, over-expression of miR-885-5p in vitro led to cell migration, invasion and in vivo development of liver and lung metastases. miR-885-5p targets directly the 3'UTR of CPEB2 which negatively regulates TWIST1, a well-known player in epithelial-mesenchymal transition (EMT) (Siu-Chi Lam et al., 2017). Alike, miR-9 may promote ovarian cancer metastasis targeting E-CADHERIN and upregulating N-CADHERIN and VIMENTIN, mesenchymal markers (Zhou et al., 2017).
- 7. *Reprogramming energy metabolism.* It is well-known that cancer cells are able to modify its metabolism favoring survival and proliferation. miR-7 has been demonstrated to decrease the usually up-regulated metabolic autophagy in pancreatic cancer cells via affecting LKB1-AMPK-mTOR signaling (Gu et al., 2017). Another tumor suppressor microRNA is miR-1 which has been described to be down-regulated in CRC cell lines compared to normal colon epithelial cells. Moreover, over-expression of miR-1 decreases cancer cell proliferation



dampening aerobic glycolysis, lactate production and glucose uptake *in vitro* targeting HIF-1 α and impacting SMAD3 pathway (Xu et al., 2017). On the contrary, high levels of miR-150 in glioma cells increased the Warburg effect, via the targeting of VHL 3'UTR, facilitating *in vivo* tumor growth (Li et al., 2017).

8. Evading immune destruction. Khorrami and colleagues showed how over-expression of miR-146 in a CRC cell line cocultured with peripheral blood mononuclear cells extracted from healthy donors, increased T_{reg} frequencies and antiinflammatory cytokines like TGF- β and IL-10, leading to an overall immune suppression in the tumor microenvironment (Rusca and Monticelli, 2011; Khorrami et al., 2017). On the other hand, miR-152 was shown to be decreased in gastric cancer cell lines as well as in human gastric cancer tissues. Restoration of its expression leads to enhanced T cells proliferation and effector cytokines production through the inhibition of the B7-H1/PD-1 pathway (Wang Y. et al., 2017).

In this network of complexity, it should be added that one microRNA, in view of its target promiscuity, could have multiple roles in different type of cancers. miR-21 is one of those microRNAs. It has been found to be an antiapoptotic factor in breast cancer (Si et al., 2007) and its suppression increased CASPASE3/7 enzymatic activities in human glioblastoma cells (Chan et al., 2005). Moreover, miR-21 is able to sustain proliferative signal targeting PTEN, a well-known tumor suppressor, in cholangiocarcinoma (He Q. et al., 2013; Wang L.-J. et al., 2015) and human hepatocellular carcinoma (Meng et al., 2007), inhibiting AKT and mTOR pathway which promotes cell survival and proliferation. miR-21 is linked with PI3K/AKT pathway also via the inhibition of FOXO1 in large B-cell lymphoma (Go et al., 2015). It was also shown to target TPM1 which normally is considered a tumor suppressor gene, regulating microfilament formation and anchorage-independent growth in a breast cancer cell line (Zhu et al., 2007). In addition, in breast cancer, miR-21 has been reported to sustain EMT signaling and IL-6 levels

Hallmark	microRNA	De-regulation in cancer	Target	Function	References
Sustaining proliferative signal	miR-27-3p	↑ in nasopharyngeal cancer	MAPK10	Regulation of ERK1 and ERK2 cascade	Li and Luo, 2017
	miR-545	\downarrow in colorectal cancer	EGFR	Signaling pathway	Huang and Lu, 2017
Evading tumor suppressors	miR-19a	↑ in colorectal cancer	TIA1	Major granule associated species	Liu Y. et al., 2017
Resistance to cell death	miR-29	\downarrow in cholangiocarcinoma	MCL-1	Regulation of apoptosis vs. cell survival, and maintenance of viability	Mott et al., 2007
	miR-15a, miR-16-1	↓ in chronic lymphocytic leukemias	BCL-2	Suppresses apoptosis	Cimmino et al., 2005
Enabling replicative immortality	miR- 130b~301b cluster	↓ in prostate cancer	MMP2	Matrix remodeling	Ramalho-Carvalho et al., 2017; Chen et al., 2015
	miR-137	\downarrow in pancreatic cancer	KDM4A	Histone demethylase	Neault et al., 2016
Inducing angiogenesis	miR-135a	↓ in gastric cancer	FAK	Non-receptor protein-tyrosine kinase	Cheng et al., 2017
	miR-23	↑ in lung cancer	PH1; PH2; ZO-1	Alanine-Glyoxylate Aminotransferase; Glyoxylate And Hydroxypyruvate Reductase; Tight Junction Protein	Hsu et al., 2017
Activation of invasion and metastasis	miR-30a- 3p	↓ in lung cancer	SNAI1	Induction of the epithelial to mesenchymal transition, growth arrest, survival and cell migration	Kumarswamy et al., 2012
	miR-885- 5p	↑ in liver cancer	CPEB2	Cell cycle progression	Siu-Chi Lam et al., 2017
	miR-9	↑ in ovarian cancer	E-CADHERIN	Calcium-dependent cell adhesion	Zhou et al., 2017
Reprogramming energy metabolism	miR-7	\downarrow in pancreatic cancer	LKB1	Cell metabolism, cell polarity, apoptosis and DNA damage response	Gu et al., 2017
	miR-1	↓in colorectal cancer	HIF1α	Activation of genes involved in metabolism,angiogenesis,erythropoiesis and glycolysis	Xu et al., 2017
	miR-150	↑ in glioma cells	VHL	Regulates the hypoxia inducible protein HIF in normoxic conditions	Li et al., 2017
Evading immune destruction	miR-146	↑ in colorectal cancer	IRAK1; TRAF6	Initiates innate immune response against foreign pathogens; activation of NFKB by TNFRSFs	Rusca and Monticelli, 2011; Khorrami et al., 2017
	miR-152	\downarrow in gastric cancer	B7-H1	Costimulatory signal, essential for T-cell proliferation and production of IL10 and IFNG	Wang Y. et al., 2017

affecting the tumor immune microenvironment (De Mattos-Arruda et al., 2015). It is also true that some microRNAs may have a dual role in different cancer types, acting as tumor suppressor or onco-miR. miR-181a when overexpressed, was described in human glioma cells to induce apoptosis and dampen cell invasion (Shi et al., 2008) and migration in non-small cell lung cancer (NSCLC) (Cao et al., 2017). Interestingly, in human gastric cancer cells, miR-181a has been reported to be an onco-miR, promoting cell proliferation, wound healing invasion and EMT targeting RASSF6 (Mi et al., 2017). Thus, the complexity of the involvement of microRNAs in cancer is high and disentangling the dense net of RNAs interaction in

order to build a complete and clear scenario will be a real challenge.

MICRORNA AS CANCER BIOMARKERS

As we have mentioned, it has become evident that microRNAs are involved in many aspects of cancer and because of their mechanism of action they control a big network of targets rather than few specific genes. This means that profiling microRNAs may give insights on complex processes hidden in numerous target genes, helping researchers to find new useful biomarkers.

The definition of biomarker evolved with time and is not unique, but it could be summarized as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Strimbu and Tavel, 2010). As a matter of fact, microRNAs possess most of the characteristics of the ideal biomarker, considering analytical criteria and clinical utility. They are specific to the pathology of interest, a reliable indication of the disease before clinical symptoms appear and sensitive to physiological or pathological changes. First demonstrations of the ability of microRNA expression patterns to be classifiers came in the first decade of 2000. Lu and colleagues implemented a bead-based microRNA profiling method in order to assess microRNA expression in normal and tumor tissues. Unexpectedly, they observed that precise pattern of microRNAs expression can, not only distinguish tumor origin, but also the degree of differentiation and classify poorly undifferentiated tumor tissues (Lu et al., 2005). Other evidences came from microRNA signatures that could discriminate between lung tumor tissues and correspondent nontumor tissues. Differential expression was also seen between adenocarcinoma (AD) and squamous cell carcinoma (SCC) tissues and between distinct prognosis (Yanaihara et al., 2006). A wider analysis on 22 different types of tumor tissue, revealed a signature of 48 microRNAs able to reach a classification accuracy >90% (Rosenfeld et al., 2008). It is important to mention how NGS (Next Generation Sequencing) technologies revolutionized this field becoming progressively fundamental tools for personalized medicine (Schweiger et al., 2011). Even in microRNA studies these methods revealed completely new information which probably would not have been unveiled with standard techniques. In view of the big amount of data coming from NGS, new biomarkers have been discovered starting with an agnostic discovery platform methodology (Leidner et al., 2012; Wu et al., 2012) allowing researchers to be unbiased on their findings. There are some limitations that come together with the power of NGS, like costs, time-consuming experiments and management of big amount of data.

Pattern of microRNAs expression may be used to classify subpopulation of patients in order to choose the right strategy in the clinical practice. However, we have to be aware that the microRNA signatures as biomarkers are not always due to a direct biological mechanism, but also to indirect specific consequence of the disease. In the following paragraphs, we report some examples (**Table 2**).

Biomarkers for Cancer Diagnosis and Sub-Typing

Biomarkers can stratify patients upon different aims. One of the first clinical questions could be to understand whether the physician is facing a pathological condition. Therefore, discriminating between tumor tissues and non-tumor tissues is extremely important. The most recurrent example is miR-21 which is over-expressed in many cancer types (Iorio et al., 2005, 2007; Volinia et al., 2006; Markou et al., 2008; Hezova et al., 2015; Kapodistrias et al., 2016; Parafioriti et al., 2016;

Calatayud et al., 2017; Chen et al., 2017; Cui et al., 2017; Nakka et al., 2017). The problem of using such microRNA as a biomarker is the absence of specificity. Therefore, signatures of a pattern of microRNAs are generally preferred to deliver a specific diagnosis. A nine microRNAs signature was able to discriminate between breast cancer tissues and normal cancer tissues collected by TCGA, with a high accuracy value and AUC of 0.995 (Xiong et al., 2017). Another example comes from the He group which found five microRNAs (miR-424, miR-326, miR-511, miR-125b-2 and miR-451) able to provide high diagnostic accuracy of hepatocellular carcinoma starting from microRNA expression profiles of 377 hepatocellular carcinoma patients (Lu et al., 2017). As finding the pathological condition is relevant, the step forward is to understand what type of condition the clinician is facing. It is well-known that each cancer type is composed of several subtypes coming from different cellular origins and each of them has to be treated accordingly. Thus, it is important to discriminate among them and several studies pointed at this aim. A study on muscle-invasive bladder cancer in 2016 revealed a signature of 63 microRNAs able to discriminate between basal and luminal tumors and a 15 microRNAs based signature able to show basal and luminal tumors with apparent fibroblast infiltration (Ochoa et al., 2016). Similarly, Blenkiron and colleagues performed a model-based discriminant analysis for basal-like and luminal A breast tumors finding a set of microRNAs able to discriminate between those groups (Blenkiron et al., 2007). Another approach by the Jang lab exploited the expression of 1,733 microRNAs to build an unsupervised clustering in order to distinguish subtypes of pancreatic tumors. As result, they found 3 subtypes which could be associated with patient prognosis (Namkung et al., 2016). In lung cancer, in the data of the Volante lab, 10 microRNAs were able to distinguish between lung neuroendocrine (NE) tumors histotypes, 9 of which also discriminated between carcinoids and high-grade NE carcinomas (Rapa et al., 2015). In addition, combination of miR-21 and miR-205 was found to be able to distinguish lung AD from SCC (Lebanony et al., 2009) and this can be further improved with the analysis of miR-375 (Patnaik et al., 2015). As a matter of fact, we demonstrated the non-perfect reliability of miR-205 in discriminating AD vs. SCC lung cancer histotypes (Del Vescovo et al., 2011).

Biomarkers for Cancer Progression

Understanding the aggressiveness and progression of cancer via prognosis of the patient is of enormous relevance in clinical practice. microRNAs are able to predict patient prognosis in several types of cancer. Here show some examples from lung cancer. Let7 was found to be down-regulated in lung cancer *in vitro* and *in vivo*. A cohort of 143 lung cancer tissues was analyzed for the expression of let7 which resulted significantly down-regulated compared to normal tissues. Moreover, reduced let7 associates with higher disease stages and poor post-surgery survival and prognosis. Taking into account only the AD samples, these distinctions are maintained (Takamizawa et al., 2004). A wider analysis led to discover a 5 microRNAs signature (miR-221 and let7a protective, while miR-137, miR-372 and miR-182-3p risky) able to discriminate between NSCLC patients with higher

TABLE 2 | (part 1) Examples of microRNAs as biomarkers.

Type of classification	microRNA	Type of cancer	Reference
Cancer VS Healthy	↑ miR-21	Breast cancer; lung cancer; stomach cancer; prostate cancer; colon cancer; pancreatic cancer; ovarian cancer; esophagus cancer; Ewing's sarcoma; liposarcoma; Wilm's tumor; osteosarcoma; oral tongue cancer	lorio et al., 2005, 2007; Volinia et al., 2006; Markou et al., 2008; Hezova et al., 2015; Kapodistrias et al., 2016; Parafioriti et al., 2016; Calatayud et al., 2017; Chen et al., 2017; Cui et al., 2017; Nakka et al., 2017
	↑ miR-21, miR-96, miR-183, miR-182, miR-141, miR-200a, miR-429;	Breast cancer	Xiong et al., 2017
	↓ miR-139 and miR-145 ↓ miR-424, miR-326, miR-511, miR-125b-2, miR-451	Hepatocellular cancer	Lu et al., 2017
Sub-typing	Panel of 63 microRNAs	Basal and luminal muscle-invasive bladder cancer	Ochoa et al., 2016
5	Panel of 137 microBNAs	Basal and luminal A breast cancer	Blenkiron et al., 2007
	Panel of 19 microBNAs	Pancreatic ductal adenocarcinoma	Namkung et al. 2016
	miR-15a, miR-22, miR-141, miR-497, miR-129-5p, miR-185, miR-409-3p, miR-409-5p and miR-431-5p, miR-129	Lung neuroendocrine cancer histotypes	Rapa et al., 2015
	miR-21, miR-205, miR-375	Lung adenocarcinoma and squamous cell carcinoma	Lebanony et al., 2009; Del Vescovo et al., 2011; Patnaik et al., 2015
Cancer progression	↓ let7	Lung cancer	Takamizawa et al., 2004
	miR-221 and let7a protective, while miR-372 and miR-182-3p risky	Lung cancer	Yu et al., 2008
	Panel of 20 microRNAs	Lung cancer	Yanaihara et al., 2006
	↓ miR-448	Lung cancer	Shan et al., 2017
	↓ miR-383	Lung cancer	Shang et al., 2016
	, ↑ miR-187	Lung cancer	Peng et al., 2016
	↓ miR-187	Renal cell carcinoma	Zhao et al., 2013
	, ↓ miR-187	Ovarian cancer	Chao et al., 2012
Cancer Therapy	↑ miR-21	Colon cancer (poor fluorouracil based adjuvant chemotherapy outcome)	Schetter et al., 2008, 2009
	↑ miR-21	Pancreatic cancer (poor fluorouracil-based adjuvant chemotherapy outcome)	Hwang et al., 2010
	↑ miR-21	Lung cancer (poor platinum-based chemotherapy outcome)	Gao et al., 2012
	↑ miR-448	Lung cancer (cisplatin resistance)	Fang et al., 2016, 2017
	↓ miR-138	Lung cancer (cisplatin resistance)	Wang et al., 2011
	↓ miR-10b	Pancreatic cancer (highly predictive response to gemtabicine-based multimodality neoadjuvant therapy)	Preis et al., 2011
	↓ miR-148	Colorectal cancer (poor fluorouracil and oxaliplatin-based therapy outcome)	Takahashi et al., 2012
	miR-221, miR-222, miR-331, miR-451, miR-28, miR-151, miR-148a, miR-93, miR-491	Diffuse large B-cell lymphoma (prediction of OS and PFS after rituximab and chemotherapy treatment)	Montes-Moreno et al., 2011
	↑ miR-31-3p	Colorectal cancer (poor anti-EGFRmAb therapy outcome)	Mosakhani et al., 2012
	↓ miR-592		
	↑ signature of let7c, miR-99a, miR-125b	Colorectal cancer (good cetuximab and panitumumab outcome)	Cappuzzo et al., 2014
	↑ miR-31-3p, miR-31-5p	Colorectal cancer (lower PFS after anti-EGFRmAb therapy)	lgarashi et al., 2015; Mlcochova et al., 2015
	↑ miR-200c	Lung cancer (good of EGFR-TKIs therapy outcome)	Li et al., 2014b
	A panel of 29 microRNAs	Renal cell carcinoma (TKIs therapy outcome)	Garcìa-Donas et al., 2016

(Continued)

TABL	E 2	Continued

miR-181a-5p, miR-339-5p	Hepatocellular carcinoma (prediction of sorafenib therapy outcome)	Nishida et al., 2017
↑ miR-183	Renal cancer (less efficacious cancer cytotoxicity by natural killer cells)	Zhang et al., 2015
↑ miR-6826, miR-6875	Colorectal cancer (poor vaccine therapy outcome)	Kijima et al., 2016

From diagnosis to the choice of therapeutic intervention.

or lower median overall survival (OS) independently from stage or histology. However, this signature is able to predict patient survival within histological type AD or SCC (Yu et al., 2008). With a similar strategy, a pattern of unique 15 microRNAs was able to discriminate between lung SCC and normal tissues, while a signature of 20 microRNAs was able to predict the OS (Raponi et al., 2009). Some of these microRNAs were more significant, like miR-146b which had the highest prediction score within 3 years, and some had already been linked to lung cancer in other studies like let-7 and miR-155 (Yanaihara et al., 2006). Interestingly, in all these studies, the different isoforms of let-7 found, were down-regulated in patients with poor prognosis. More recent data show that low expression of miR-448 associates with lung SCC progression and poor patients overall survival (Shan et al., 2017). Reduced expression of miR-383 was found in NSCLC tumor tissues compared to adjacent non-tumorous samples and moreover, low miR-383 expression associated with poor post-operative prognosis (Shang et al., 2016). miR-448 and miR-383 are down-regulated, acting like tumor-suppressors, also in ovarian cancer (Lv et al., 2015), hepatocellular carcinoma (Zhu et al., 2015; Chen et al., 2016), colorectal cancer (Li et al., 2016), breast cancer (Li et al., 2011), Hodgkin lymphoma (Paydas et al., 2016), glioma (He Z. et al., 2013; Xu et al., 2014) testicular carcinoma (Lian et al., 2010; Huang et al., 2014) and medulloblastoma (Li et al., 2013). Another study revealed that miR-187 expression was significantly increased in NSCLC tissue samples compared to adjacent non-lung tumor tissues and that this condition associated with TNM classification and shorter OS (Peng et al., 2016). Interestingly, miR-187 has been found down-regulated in clear cell renal cell carcinoma cells (Zhao et al., 2013) but up-regulated in ovarian cancer cells (Chao et al., 2012). However, in both cases the studies agree with what occurs in lung cancer, where low miR-187 level of expression is associated with poor patient survival.

Biomarkers for Cancer Therapy

As a consequence of the intricacy of underlying driving mechanisms of cancer, therapeutic efficacy of a single treatment can change depending on the patient and its type of cancer. microRNAs have been associated to, and also predictive of, therapeutic outcome. Here we report cases of some of the main standard cancer treatments.

miR-21 seems to be a general signal for chemotherapy resistance. In 2008, Schetter and colleagues found that miR-21 expression, in typical colon AD from patients treated with fluorouracil based adjuvant chemotherapy, is higher in patients with a poor therapy outcome (Schetter et al., 2008, 2009). Similar results were obtained for pancreatic cancer (Hwang

et al., 2010). Even in lung cancer, high-expression of miR-21 was associated with chemotherapy resistance in tissues of patients who had undergone platinum-based chemotherapy treatment (Gao et al., 2012). It was shown that A549/DDP lung AD cell line has a lower expression of eIF3a compared to its parental cell line, and it displays chemoresistance to cisplatin. miR-488 targets the 3'UTR of eIF3a transcript enhancing sensitivity to the treatment and inhibiting cell proliferation, migration and invasion (Fang et al., 2016, 2017). Another study reported an increased sensitivity of A549/DDP cells to cisplatin after up-regulation of miR-138. The excision repair cross-complementation group 1 (ERCC1) was targeted by miR-138 and the result was the down-regulation of the protein correlating with increased levels of miR-138 in A549/DDP cells (Wang et al., 2011). In another study on pancreatic ductal AD, patients with resectable or locally advanced disease showed relative low miR-10b expression associated with highly predictive response to gemtabicine based multimodality neoadjuvant chemoradiotherapy. Moreover, by logistic regression, low miR-10b expression was able to predict surgery efficacy. miR-10b levels demonstrated significant ability in survival prediction (Preis et al., 2011). In CRC, miR-148 expression had a potential for predicting therapeutic efficacy of 5-fluorouracil and oxaliplatin in patients with stage IV colorectal cancer, as low levels of this microRNA associated with bad therapeutic response (Takahashi et al., 2012). Sensitivity to cisplatin treatment is, at least partially, regulated by miR-488 which targets eIF3a.

Besides chemotherapy, targeted therapy is an important standard of care for several tumors. Even in this field, microRNAs may be helpful. For diffuse large B-cell lymphoma (DLBCL), combination between chemotherapy and immunotherapy with rituximab has become a standard treatment. A 9 microRNAs signature was able to predict both OS and progression free survival (PFS) in DLBCL patients (Montes-Moreno et al., 2011). In a cohort of metastatic colorectal cancer patients wild type for KRAS and BRAF, a miR-31-3p up-regulation and miR-592 down-regulation were found associated with poor response to anti-EGFRmAb (Mosakhani et al., 2012). An Italian study reported a signature of three microRNAs (miR-let7c, miR-99a, and miR125b) able to predict EGFR monoclonal antibody therapy outcome in colorectal cancer patients. Indeed, highlevel of signature expression showed a good discrimination capacity for patients which were more responsive to cetuximab or panitumumab compared to low responsive patients (Cappuzzo et al., 2014). In two independent studies, miR-31 was found to be associated with PFS after administration of anti-EGFRmAb in metastatic colorectal cancer patients. Mlchocova and colleagues found both miR-31-5p and—3p, while Shinomura group only miR-31-5p, to be higher in patients with lower PFS compared to those with low levels of the microRNA (Igarashi et al., 2015; Mlcochova et al., 2015). microRNAs have been discovered to be predictive also of kinase inhibitors efficacy in hepatocellular carcinoma, renal cell carcinoma and NSCLC (Li et al., 2014b; Garcia-Donas et al., 2016; Nishida et al., 2017).

An emerging field in cancer treatment is immunotherapy. Some studies describe microRNAs as biomarkers of immunotherapy efficacy. In a report on 82 renal cancer patients and 19 healthy individuals, miR-183 has been found up-regulated in sera associated to less efficacious cancer cytotoxicity by natural killer cells, which are the effectors of the IL-2 immunotherapy (Zhang et al., 2015). Nagano group described that miR-6826 and miR-6875 can be good predictor of vaccine treatment efficacy in metastatic CRC, where high expression in plasma of two microRNAs was associated with poorer prognosis (Kijima et al., 2016).

CIRCULATING MICRORNAS

Over the last two decades, it has been demonstrated that a substantial number of microRNAs are present outside cells in blood and other body fluids, the so-called "circulating microRNAs" (c-microRNAs). C-microRNAs have been reported to be very stable under harsh conditions and able to survive high temperatures, extreme pH, and RNase activity. As reviewed by Makarova and colleagues (Makarova et al., 2016) they are often found in association with small membranous particles (extracellular vesicles) and mostly with RNA-binding proteins (Ago2, HDL, etc.). The extracellular vesicles (EV) are represented by a various population of membranous particles with different origins and sizes. Microvesicles originate through the budding of the plasma membrane and have a size around 100-1,000 nm. Exosomes, around 40-100 nm in size, are generated after the fusion of multivesicular bodies with the plasma membrane. The presence of these different carrier options leads researchers to think of selective microRNA sorting and secretion processes, not excluding stochastic (non-selective) ones. Moreover, the pool composition of the microRNAs is different intra- and extracellularly. Unfortunately, the exact mechanisms underlying these processes have to be discovered yet. Even though extracellular vesicles biogenesis is varied, only ceramide-dependent mechanism has been reported as one of the responsible for microRNA secretion so far (Kosaka et al., 2010). About sorting, it has been suggested that the affinity between the RNA and the raft-like membrane regions of the multivesicular bodies (MVBs) can guide it (Janas et al., 2015). In another study, Squadrito et al. (2014) reported that sorting of microRNAs to exosomes is partially regulated by the changes in expression of the targets inside the cell. The finding of a different microRNA sorting in exosomes depending on the KRAS status (Cha et al., 2015), adds concrete value on the selective sorting hypothesis. In 2013, Sànchez-Madrid group (Villarroya-Beltri et al., 2013) performed several microarrays analyses of activation-induced changes in the microRNA and mRNA profiles among T-lymphoblasts and their exosomes. They obtained a discordance between intracellular and extracellular microRNA and mRNA pool composition, demonstrating once again that the sorting into exosomes is not-at least completely-passive. Interestingly, they reported a short sequence motif (GGAG) enriched in exosomal microRNAs. Among the many heterogeneous nuclear ribonucleoproteins (hnRNPs) that precipitated with intracellular and exosomal microRNAs, only hnRNPA1 and hnRNP2B1 seemed to bind exclusively the latter. Another study demonstrated that Vps4A, a key regulator of exosomes biogenesis, seemed to regulate the sorting of oncogenic and oncosuppressive microRNAs in exosomes, favoring the inclusion of the first ones (Wei et al., 2015). Furthermore, it has been described how, in B cells, 3'end adenylated microRNAs appear to be enriched in cells compared to 3' end uridylated isoforms which are more present in exosomes (Koppers-Lalic et al., 2014). What remains really unclear is the mechanism of sorting, if present, and of secretion of AGO-microRNA complexes. To date, the study reported by Turchinovich and colleagues, leads to think that the majority of these complexes is freed in a non-selective manner, because of the positive correlation between the content of c-microRNA in culture media and the increase of cell death (Turchinovich et al., 2011). Moreover, the Cayota group described via RNAseq analysis, how expression values of individual microRNAs in intracellular fractions of MCF-7 cells after a certain threshold, correlated directly with extracellular values, suggesting a passing mechanism of release also for extracellular vesicles related microRNAs (Tosar et al., 2015). However, these conclusions do not wipe out at all the possibilities of a parallel selective secretion.

The presence of putative precise processes underlying cmicroRNAs suggests that they could have an intriguing role in cell-cell communication. For instance, it was demonstrated that microRNAs enriched in extracellular vesicles derived from bone marrow mesenchymal stem cells can be absorbed by tubular epithelial cells resulting in the inhibition of expression of the known targets (Collino et al., 2010). Furthermore, as it has been reviewed (Neviani and Fabbri, 2015), c-microRNAs can influence cancer cells and their surrounding environment both targeting mRNAs and functioning as receptor-like systems. Much of the data supporting this way of cell-cell communication is done through in vitro systems, pushing for new validating studies in vivo which may confirm this hypothesis. One of the unclear point which can be argued is whether the actual amount of c-microRNAs is enough to drive expression changes in recipient cells in vivo. Some studies report that the average amount of microRNAs in exosomes is about 1 unit per exosome (Chevillet et al., 2014; Guzman et al., 2015). This very low amount may lead to some skepticism around the role of cmicroRNAs in cell-cell communication. However, extracellular vesicles associated microRNAs are a small percentage of the total pool of c-microRNAs (Arroyo et al., 2011) and in addition, this semi-quantitative reasoning is far too simplistic, not taking into account, for example, the accumulation of microRNAs in recipient cells and, being a median measure, doesn't consider the content heterogeneity of extracellular vesicles.
Not only microRNAs from tissue can be used to create pattern of signatures able to classify group of patients but also c-microRNAs from liquid biopsies are becoming an increasing source of information (Chen et al., 2008). c-microRNAs as biomarkers have some advantages like great stability, resistance to ribonucleases and to severe physicochemical conditions in body fluids, increasing the feasibility of their use in clinical applications (Mitchell et al., 2008). Another important aspect is the compliance of the patients. Indeed, c-microRNAs are extracted from several body fluids coming from liquid biopsies, which are much less invasive and painful for the patients compared to the standard methods. Moreover, the cost and time for the processing is lower than non-liquid samples, promising big step toward the implementation of personalized medicine. Thus, the interest of the scientific community has grown intensively as demonstrated by the number of articles published in recent years at the entry "circulating microRNAs cancer biomarker" on PubMed (2012 → 86; 2013 → 148; 2014 → 188; 2015 → 239; 2016 → 201). Moreover, there are some reviews which try to collect as much as possible the huge amount of information on circulating microRNAs as cancer biomarkers (Del Vescovo and Denti, 2015; Armand-Labit and Pradines, 2017; Matsuzaki and Ochiya, 2017; Zhao et al., 2017).

MIRSNP

microRNAs exert their function through an interaction with seed sequence on either 3'UTR, 5'UTR or the coding sequence of a target mRNA. The hybridization between the two RNAs follows the Watson-Crick base pairing rules and thus it is guided by the formation of a stabilized double strand structure. Thus, when even single nucleotide changes occur in the sequence of either of the two interactors (miRSNP), the stability of the contact is affected and so is the functional outcome. Indeed, single nucleotide polymorphisms (SNPs) can affect microRNA expression and function and they can be present in the sequence of the microRNA or on its target genes (Figure 2). SNPs may be present also in the sequence of genes involved in the biogenesis of microRNAs, thus affecting their level of expression. These changes can influence the abovementioned patterns of microRNAs, creating completely new classes of patients based on association to risk of cancer or prediction to therapy. Moreover, as reviewed by Del Favero group, it should be considered that SNP density is higher in the flanking region of the microRNA sequence compared to microRNA genes themselves and mature form of microRNAs has lower SNP density than the pre-microRNA. Interestingly, the seed sequence has the lowest SNP density, highlighting their evolutionary and functional importance (Cammaerts et al., 2015).

In literature, there are numerous reports describing cancer risk association with SNPs related to microRNAs life. In a meta-analysis by Liu and colleagues (Liu H. et al., 2017), conducted on ten studies with 6,000 cases and 7,664 controls, a significant association of miR-608 rs4919510 polymorphism with decreased cancer risk via recessive model (CC vs. GG + GC) was found. Interestingly, the same polymorphism has been already described to predict the clinical outcome in patients with different cancer types (Lin et al., 2012; Zheng et al., 2013; Pardini et al., 2015). As mentioned above, SNPs associated with microRNAs can alter several of their usual processes. One of the most common alteration is the degree of target suppression. rs73239138 polymorphism in miR-1269 was associated with increased susceptibility to hepatocellular carcinoma (HCC) and HBV-related HCC in a positive dominant model where genotypes with the A allele increased the risk to cancer (Min et al., 2017). It was shown that in HCC cell lines the over-expression of the miR-1269 variant led to a decreased inhibition of cell growth compared to the over-expression of the wild type microRNA. The authors confirmed the biological outcome demonstrating that the polymorphism on miR-1269 produced a dampened suppression of pErK1/2, SPATS2L and LRP6 compared to the wild type variant, where the last two genes showed to have a 3'UTR binding site for miR-1269. In a study on an Indian population (Sibin et al., 2017), miR-196a2 expression varied with age, tumor grade and tumor type among glioma patients' tissues but not with different genotype of the microRNA. However, they found a significant difference in the expression of its mRNA target HOXC8 depending on different genotypes where CC and TT showed decreased and increased expression, respectively. Remarkably, out of 72 sample pairs of tumor tissues and blood samples, 19,44% showed different genotype of miR-196a2 in the tissue compared to the blood suggesting a critical role in tumorigenesis and in changing of tumor grade. Other evidences pointed out the importance of polymorphisms altering the ability of the microRNAs to post-transcriptionally inhibit gene expression of their target in colorectal (Liu Y. et al., 2016) and gastric cancer (Liu C. et al., 2016). Along with a negative effect of a SNP on microRNA sequence, this variation may lead also to positive effects, increasing binding capacity of the microRNA to its target (Gong et al., 2012). To the best of our knowledge, there are no reports of this scenario in cancer yet, neither in vitro nor in vivo.

Undoubtedly, variations in the sequence of microRNAs can also affect their maturation. Bioinformatic analysis of Gibbs free energy on the structure of the miR-146a stem loop showed that the G allele of the rs2910164 polymorphism increased the stability factor of the overall structure, suggesting that, the association between the C carrier allele in the Iranian population under study may correlate with lower expression of miR-146a and thus higher incidence of the presence of its target Her2 in breast cancer (Meshkat et al., 2016). Indeed, miR-146 is significantly higher in triple-negative breast cancer compared to non-triple-negative breast cancer (Garcia et al., 2011). The already mentioned polymorphism on miR-196a2 was associated in other cancer studies to the microRNA maturation, changing the miR-196a2 expression (Hu et al., 2008, Hoffman et al., 2009). Another common scenario is the presence of a polymorphism in the microRNA binding site of an mRNA target. In a study on 325 colorectal cancer (CRC) patients and 977 normal individuals, the polymorphism rs7930 in the 3'UTR of TOMM20 was found to be associated with CRC susceptibility and the G allele described as the risk allele. Via in silico target analyses, miR-4273-5p was



FIGURE 2 | miRSNP can affect microRNA biogenesis and activity. SNPs may be present on the microRNA decreasing (A) or increasing (B) its binding affinity for the target mRNA. SNPs may be present in the binding site of a target mRNA decreasing (C) or increasing (D) binding affinity (or creating new binding sites). In this last scenario are represented also SNPs in genes of the microRNA biogenesis machinery. These SNPs usually affect the regulation of the genes increasing or decreasing binding affinity of post-transcriptional regulators like microRNAs. SNPs may also affect the secondary structure of premature forms of the microRNAs decreasing (E) or increasing (F) their maturation.

predicted to interact with rs7930. Validation by luciferase assay in human CRC cell lines demonstrated that the G allele plasmid did not have any effect on the reporter compared to a stronger effect of the A allele plasmid. Moreover, cell lines with the AA genotype showed a considerably stronger dampen in TOMM20 levels than those with the AG genotype (Lee et al., 2016). On two stages study made of 2347 cases and 3390 controls in total, Ke and colleagues found that the polymorphism rs1062044 on the sequence of LAMC1 produces an increased risk of colorectal cancer in the GA genotype compared to GG. Moreover, it decreases the ability miR-423-5p to bind LAMC1 in CRC cancer cell lines (Ke et al., 2017). The presence of a polymorphism on the binding site could also lead to a positive effect, thereby creating a new illegitimate binding site. Bartel group found that polymorphism SNP34091 in the 3'-UTR of MDM4 creates a new binding site for miR-191 in ovarian cancer (Wynendaele et al., 2010).

Polymorphisms on genes involved in the microRNA biogenesis can also have an impact on cancer progression. Mullany and colleagues, through RNA-seq and GWAS analysis of colon cancer tissues, found 24 microRNAs which were deregulated in the presence of SNPs significantly associated with altered mRNA expression or cancer risk. In particular, rs2740349 (GEMIN4) and rs235768 (BMP2) were shown to be associated with microRNA expression variation, with upregulation corresponding to the variant genotypes (Mullany et al., 2016). Interestingly, this up-regulation is associated with a down-regulation of the mRNAs of biogenesis genes, implicating new roles for these genes or other mechanisms of microRNA expression influence. In addition, Rotunno and colleagues found that RNASEN/rs640831, present in the GTACCT haplotype was associated with variation in expression of 56 microRNAs, both up- and down-regulated (Rotunno et al., 2010). One mechanism of action of these SNPs is the change in binding affinity of a regulator of the transcript like a microRNA. Jiang and colleagues genotyped 24 SNPs in a cohort of 878 breast cancer patients and 900 controls. They found that polymorphism rs417309 is associated with higher breast cancer risk (Jiang et al., 2013). Moreover, this SNP is placed on the 3'UTR of the DGCR8 mRNA, affecting the binding ability of miR-106b and miR-579. However, the imperfect relationship between up-/down-regulation of microRNA biogenesis-related genes and up-/down-regulation of microRNAs, suggests a series of effects which are far to be completely clear and understood.

Behavioral changes of microRNAs upon SNPs can also affect the performance of cancer drugs. Pharmacogenomics studies how single genome or transcriptome variations can affect pharmacokinetics (PK) and dynamics (PD). A lot of attention has been drawn to microRNAs as possible players in this area. The contribution of microRNAs to PK and PD has been studied also via bioinformatic tools. Some genes are specific to PK, some to PD, whereas others to both. *In silico* data hint to a higher relevance of post-transcriptional regulation by microRNAs on PD unique genes compared to PK unique genes (Rukov et al., 2011). As a matter of fact, the latter show on average a shorter 3'UTR with a less dense presence of predicted target binding site for microRNAs, compared to the former. Among some genes coding for drug metabolizing enzymes (DMEs), transporters and nuclear receptors, CYP1A2, CYP2B6, CYP2D6, CYP3A4, NR112, and UGT2B7 were sequenced for their 3'UTR in a population of 30 South Africans (Swart and Dandara, 2014). 40 out of 52 SNPs detected were predicted to potentially create or abolish microRNA binding sites, thus affecting regulation capacity and expression of those genes. Despite the low number of patients enrolled, this study highlights once more the engagement of microRNAs in pharmacogenomics. Therefore, an increasing interest in creating web tools to analyze miRSNP and drugs has grown.

Mir2Drug is a database able to calculate the influence of miRSNP in drug efficiency. It considers the sequence 30 bp upand downstream a known SNP in the 3'UTR of target genes, and calculates all the predicted binding sites for microRNAs in that region, analyzing the change in free energy from wild type to variant genotype. Upon significant differences, Mir2Drug associates these SNPs as either direct or indirect drug targets. Therefore, it provides comprehensive annotation information on miRSNP belonging to drug target genes (Wang X. et al., 2017).

SMiR-NBI is another bioinformatic tool available on the web, which provides insights on possible pharmacogenomic biomarkers characterized by microRNAs, comprehending a network connecting small molecules to microRNAs regulation (Li et al., 2014a). This growing interest on miRSNP and pharmacogenomics comes from several works and here we try to review examples from different cancer types. A standard of care therapy for advanced lung cancer patients is the platinumbased chemotherapy. This kind of therapy leads to a spectrum of toxicities with different degree of severity. A study of Fang et al. (2016) found that the polymorphism rs2042553 of miR-5197 significantly associates with severe toxicity after platinumbased treatment. Moreover, miR-605 polymorphism rs2043556 was associated with hepatotoxicity, while miR-27a rs895819 was related to gastrointestinal toxicity. Platinum-based therapies are often combined with other drugs like gemcitabine or paclitaxel. Geng et al. (2016) studied the effect of different regimen of chemotherapy based on cisplatin plus paclitaxel, gemcitabine or Changchun vinorelbine, in a cohort of advanced NSCLC patients. They found that polymorphism rs11077 in XPO5, a transport factor involved in the export of pre-microRNAs from the nucleus to the cytoplasm, is associated in AA genotype to a worse prognosis in a chemotherapy regimen compared to the AC genotype. Human Pregnane X Receptor (PXR) induces expression of DMEs, thus it can potentially influence the efficacy of several anticancer drugs. In a study on 96 Indian breast cancer patients (Revathidevi et al., 2016), genomic DNA from blood samples was sequenced for PXR 3'UTR. Among 12 SNPs already reported in several databases, 5 SNPs were observed and in particular, for SNPs rs3732360 and rs3732359 the proportion of the mutant allele is higher compared to the wild type in the studied population. These two polymorphisms conferred a new binding site for miR-500a-3p and decreased the binding of miR-532-3p which is known to play a role in doxorubicin cardiotoxicity (Wang J.-X. et al., 2015). In fact, the observed SNPs either created new binding sites for microRNAs, or abolished some of them, or strengthen or dampened the binding capacity of others.

Therefore, overall regulation of PXR could be affected impacting on the metabolism of drugs. As a matter of fact, microRNA predicted to be influenced by these SNPs are also involved in treatment efficacy and doxorubicin cardiotoxicity, as pathway analysis revealed. Even in non-solid tumors microRNA variation may impact on treatment efficacy and toxicity. Lòpez-Lòpez and colleagues studied possible associations between miRSNP and adverse reactions after methotrexate administration in Acute Lymphoblastic Leukemia (ALL) (Lopez-Lopez et al., 2014). They showed that SNP rs639174 in DROSHA is associated with vomit during consolidation of methotrexate treatment, as well as rs56103835 in pre-miR-453. Moreover, rs12894467 in pre-miR-300 is associated with hepatic toxicity and hyperbilirubinemia in induction. However, the same group found that none of the miRSNP genotyped in a Spanish population of 152 ALL affected children, is associated with Vincristine-related neurotoxicity (Lopez-Lopez et al., 2016), highlighting how microRNAs are not involved randomly in every process, but they are selectively and directly responsible or indirectly involved in some divergent mechanism.

It is worth mentioning that also big sequence changes like INDELs in microRNAs related genomic regions can have an impact on how drugs are affected by human body and vice versa. Garcia-Ortì et al. (2012) found 19 microRNAs associated with gene copy number variations in genomic regions where they are located, in acute myeloid leukemia (AML) cells. 4 out of 19 had NF1 as a potential target gene but only miR-370 was then validated. Patients analysis showed that NF1 down-regulation by either miR-370 over-expression or NF1 gene deletion is common in AML. Thus, considering that NF1 deficiency leads to RAS activation, patients with over-expression of miR-370 may potentially take advantage from RAS or mTOR inhibitors (Parkin et al., 2010). Another study (Bruhn et al., 2016), pinpointed that different lengths in the 3'UTR ATP binding cassette (ABC) membrane transporter P-gp (ABCB1) may alter the presence of several microRNA binding sites. Actually, imatinib resistant leukemia cell lines expressed shorter 3'UTR potentially losing some regulating sequences. Indeed, the shortening of ABCG2 (another ABC transporter) 3'UTR removes miR-519c binding site, therefore contributing to drug resistance (To et al., 2009).

LIMITATIONS OF MICRORNAS AS TOOLS FOR PERSONALIZED MEDICINE

microRNAs are intensively studied as tools for personalized medicine because they encompass many ideal characteristics for fast and robust analysis, which is needed in clinical practice. As a matter of fact, they are generally stable due to protein based carriers or EVs engulfment. Moreover, the detection is easier so far, considering the low amount required, the hybridization methods criteria which avoids the production of complex probes like antibodies for proteins and the accessibility of the technologies. The ability of microRNAs to fine tune the gene expression enables these markers to be more sensitive in the pathology follow-up. On the contrary, biomarkers like ctDNA (circulating tumor DNA), which is a promising new biomarker for cancer practice, being strictly linked to genomic mutation analysis, suffer from uncertainty in ongoing follow-up (Nadal et al., 2017). However, some problems still limit the use of microRNAs in personalized medicine. The source of microRNAs has to be managed accordingly and the influence on recovery and final outcome may be substantial, especially for RNA extracted from biofluids. Regarding the detection, the short sequence of microRNAs impedes an easy design of probes, limiting also the discrimination between pri-, pre- and mature forms. Moreover, despite the ease of use and accessibility of qRT-PCR, ddPCR, microarrays and NGS as main detection techniques for nucleic acids, they lack strong sensibility and accuracy, especially at single base resolution. Another key point is the normalization of the signal. In fact, as the analysis is about a relative expression, the choice of a good normalizer is fundamental and challenging (Masè et al., 2017). In addition to these analytical problems, the complex biology of microRNAs increases the obstacles toward a full comprehension of these small non-coding RNAs. As a matter of fact, functional studies with microRNAs suffer from absence of physiological conditions, thus when over-expression studies are performed it should be taken into account that microRNAs generally act with low quantities and more than one on a single target.

Indeed, personalized medicine is going through the use of cmicroRNAs instead of tissue-derivatives. Despite the clear high potential of c-microRNAs in the future personalized medicine, technical difficulties to perform robust and comparable profiling of these small nucleic acids have impeded progress to develop an approved clinical diagnostic assay (Jarry et al., 2014).

The problems come through three different steps in the analysis of c-microRNAs: pre-analytical, analytical and postanalytical phase. Therefore, from where and how c-microRNAs are extracted, how we detect them and how we process the data, still leads to great variability among different studies. Pre-analytical variables are those factors which can affect the composition of the sample to be tested: from patient's conditions variability to sample handling. Firstly, it has to be considered that usually, c-microRNAs are in low titer in biofluids compared to microRNAs in sample tissues. Considering the study of Tewari and colleagues, the concentration of microRNAs in plasma can be counted as from 100 to 9,000 copies per uL or, as shown in another study with ddPCR, even up to 23,000 copies/uL (Miotto et al., 2014). Similar results were found for cardiac injury induced microRNAs (Thompson et al., 2016). Moreover, the lack of knowledge about the secretion and sorting of the microRNAs outside the cells, puts some limits on the patient's condition which would ensure reproducibility on the assays. Another challenge is represented by the contaminant microRNAs. It is known that c-microRNAs come from different cellular sources. Tewari group showed that blood cells are the major contributor to c-microRNAs, therefore variations in blood cells counts and hemolysis can affect the interpretation of c-microRNAs signatures. They studied several oncological biomarkers reported in literature: many of them are highly expressed in blood cells. They demonstrated that this kind of c-microRNAs correlates with blood cell counts and that miR-122, which is not expressed in blood cells, doesn't follow this trend. Moreover, in hemolyzed

plasma samples, red blood cell-associated microRNAs vary up to 30-fold compared to non-hemolyzed samples, further proving that c-microRNAs pool is affected by blood cells composition (Pritchard et al., 2012). For these reasons, sample handling and processing become extremely important. Duttagupta and colleagues tried to discriminate between whole blood microRNAs derived from blood cells-"contaminant microRNAs"-and what they called "truly circulating microRNAs". Starting from whole blood samples and collecting different fractions from multiple centrifugation steps (Figure 3) they found that from fraction CS and S1 the content of "contaminant microRNAs" dropped, while the true c-microRNAs content stays more or less unchanged. On top of that, they showed that the variability of expression of marker c-microRNAs among a cohort of males and females decreases after the removal of the cellular contaminants originated from cellular microRNA signatures (Duttagupta et al., 2011). This points out how much the processing of the samples may affect the pool of c-microRNAs. Another study (Cheng et al., 2013) confirmed this variability, reporting that different plasma processing led, for the majority of c-microRNAs, to a variation in their expression levels, mainly due to different platelets and microvesicles content.

From the analytical point of view, it has to be considered that different extraction kits have distinct efficiencies in small RNAs recovery (Monleau et al., 2014). There are several challenges which involve also the detection and quantification of cmicroRNAs. The design of qRT-PCR probes and assays is difficult because of (a) the shortness of microRNAs, (b) their wide range of concentrations, (c) the presence of many precursors and (d) the similarity in sequences. On the post-analytical side, we should mention that most of the detection methods rely on relative quantifications, therefore, an endogenous control is requested. This normalization analysis is needed in order to take into account the biological and technical inter-assay variability. So far, such control for c-microRNAs expression normalization, to be used for every tissue type, treatment and disease stage, has to be discovered yet. The most used in literature are miR-16, snRNA U6 and spiked-in cel-miR-39, but there is no general consensus from the scientific community and a different endogenous control is generally used for different purposes. For instance, among the several transcripts of U6, U6-1 was found to be have high variability and U6-2 was not detectable, in a study on sera from Hepatitis B infected patients and matched controls (Zhu et al., 2012). In two different plasma studies on CRC, miR-16 was found to have quite high stability and little variability between control and case patients (Ng et al., 2009; Huang et al., 2010). In another case, it has been reported, in serum samples from lung cancer patients, miR-16 being inconsistent, choosing to directly normalize the expression levels of target microRNAs to total RNA (Chen et al., 2008). Several authors have concluded that a universal endogenous control is unlikely to be discovered and a suitable reference should be assessed every time considering the different biological conditions of the samples. However, cost and sample requirements needed for the choice of several reference RNAs are not always possible, especially in a clinical or diagnostic setting.

CONCLUSIONS

As described in the previous paragraphs, microRNAs have some advantages as high specificity, sensitivity, and classification power, which can be exploited for cancer personalized medicine. Furthermore, microRNAs are remarkably stable small molecules shown to be well preserved in FFPE as well as in fresh snap-frozen specimens and in biofluids.

microRNAs affect cancer biology being involved in all the hallmarks of cancer both as tumor suppressor and as onco-miR. They can be extracted from different biological sources and used as biomarkers in order to classify cancerous vs. non-cancerous tissues, distinguish different cancer types and also efficient cancer therapies. Thanks to the sensitive signatures, patterns of microRNAs may be able to follow the progression of cancer, being an important tool in clinical practice. In particular, detection of c-microRNAs, obtained by a non-invasive procedure, seems to be a new promising field with the potential of revolutionizing cancer diagnostics, increasing compliance of patients, ease of use and accessibility to these biomarkers. As a result of a growing use of high-throughput techniques, another emerging field in microRNA diagnostics is represented by SNPs analysis. miRSNP can affect microRNA expression and function, being present on microRNAs sequence, on their target genes or also in genes involved in their biogenesis. They can affect cancer susceptibility, prognosis and response to treatment.

In this review, we reported the main concepts on microRNA cancer personalized medicine. However, some issues have to be



considered while going through the huge amount of studies on microRNA function and classification capacity.

Despite the advances obtained in the field, many open questions and challenges still remain to be addressed (Table 3). One limitation in microRNAs detection is represented by the closely similar sequences among microRNAs family members, their ancestral RNAs (pre-microRNAs and pri-microRNAs) and isomiRs (Thomas et al., 2010; Chugh and Dittmer, 2012; Zhou et al., 2012). Moreover, there are many methods which can be used to measure microRNAs including qRT-PCR, microarrays, next generation sequencing (NGS), and more recently, digital droplet PCR (ddPCR). These conventional methods have of course their own drawbacks, mainly time-consuming, expensive material and target modification steps for detection. Concerning c-microRNAs, the main limitations are represented by the small amount of RNA and the corresponding microRNAs extracted from biofluids. Indeed, RNA concentration is often under the detection limits of common spectrophotometric devices and this is the reason why for qRT-PCR, it is recommended to use a fixed volume rather than a fixed RNA amount. Furthermore, the right selection of a suitable normalization method to remove technical variations and increase the accuracy of microRNAs quantification is of enormous relevance. The issue of reference genes is especially critical in the quantification of c-microRNAs, due to the extremely low levels in biofluids of common reference genes (U6, miR-16, 5S rRNA, small nucleolar RNAs).

On the other hand, from a biological point of view, microRNAs biogenesis and function must be further explored in order to better understand mechanisms at the basis of their

TABLE 3 Challenges on microRNA studies from basic microRNA analysis to microRNA functional studies.						
microRNA analysis	Source of preparation: lack of standardized protocols					
	Discrimination between pri-, pre- and mature forms: difficult to distinguish the different forms of microRNA maturation					
	Detection techniques: lack of strong sensitivity and sensibility at single-base level					
	Short sequence for primer design					
	Quantity in biofluids: the low quantity of microRNAs in biofluids demands high-sensitivity techniques					
	Normalization methods: the particular nature of microRNAs and their involvement in post-transcriptional regulation makes difficult to find an universal normalizer					
microRNA functional studies	High biological complexity: their ability to target multiple mRNAs and the presence of multiple different target sites on a single mRNA creates a complex network of regulation difficult to untangle					
	Non-physiological conditions: microRNAs generally act as fine-tuners of gene expression, thus, forced over-expression or inhibition in cellular or non-cellular systems are not representative of the reality					
	Few mechanicistic studies on miRSNP: miRSNP have been studied mainly for their association to cancer risk but only few of these works try to unravel the mechanism underneath the shown effects					

involvement in cancer. The commonly accepted mechanism of microRNA action and targeting involves the interaction between microRNA 5'-end ("seed region") and mainly the mRNA 3'-UTR. However, target sites were found also in the coding sequence (CDS) and in 5' UTR (Lytle et al., 2007; Kloosterman et al., 2016). It has been suggested that this preference could be due to the presence of ribosomes in CDS and translation initiation complexes in 5' UTR which compete with the RISC complex (Bartel, 2004). Regarding the way of action, microRNAs contribute to gene expression regulation by fine-tuning rather than knocking-down their target mRNA. Thus, microRNAs overexpression performed for luciferase assay or western blot, the "gold standard" techniques to analyze their effect on targets, do not represent the real physiological situation. Although microRNAs exert slight effects on mRNAs, they adopt other strategies to potentiate their regulation. Indeed, one microRNA can simultaneously regulate multiple targets and different microRNAs can have a role as post-transcriptional regulators on the same target. In addition, microRNAs are often involved in feedback loops, thereby potentiating their suppression potential. A complex interplay exists also between transcriptional and posttranscriptional regulators (transcription factors and microRNAs) to orchestrate gene expression and signaling. Moreover, some microRNAs are able to regulate gene expression of their own biogenesis and processing factors, as Dicer (Ristori et al., 2015). Lastly, it is important to take into account the tissue specificity of microRNAs action, thus studies on single tissue-type are to be considered carefully. All these interactions and regulation levels lead to highly complex networks of microRNA/target pathways. For this reason, systems biology approach tries to obtain a more comprehensive understanding of miRNA regulatory structure, combining biological data acquisition and integration, network construction, mathematical modeling and experimental validation.

One important point to mention is that, recently, miRNAtarget interaction knowledge has been enriched by the discovery of long non-coding RNAs (lncRNAs) (Yoon et al., 2014) and circular RNAs (circRNAs) (Hansen et al., 2013; Memczak et al., 2013) and circRNAs which could act as miRNA sponges, reducing their regulatory effect on mRNAs. One hypothesis is that all RNA transcripts containing binding sites for microRNAs can compete specifically for shared microRNAs, acting as competing endogenous RNAs (ceRNAs) (reviewed in Thomson and Dinger, 2016). This concept is extremely important not only for having a complete view of the mechanisms of action of the microRNAs, but also for their biomarker employment.

In the future new more accurate and PCR-free single base sensitive platform are needed. Therefore, a device in which sample-preparation steps (e.g., enzymatic steps for PCR-based amplifications) are removed, would represent an improvement in microRNAs detection and quantification. Some tentative approaches are under study like the integration of a dynamic chemistry for "Single Nucleobase Labeling" with a bead-based platform (Luminex[®]) (Venkateswaran et al., 2016), or the use of a power-free microfluidic chip involving a technology based on laminar flow-assisted dendritic amplification (LFDA) (Hasegawa et al., 2017). In the former case the same technology was used to

detect microRNAs from serum samples (Rissin et al., 2017). On top of that, new methods to avoid normalization of signals are requested, like the use of ratio between the Ct of two different miRNAs (Sharova et al., 2016). As the detection techiques are fundamental, even the source of microRNAs affect the biological outcome. In this sense, exosomes are representing the future of biomarkers from liquid biopsies. More effort in studying this vehicles will help elucidate the mechanism for which microRNAs are realeased in biofluids, thus affecting our way to use them as biomarkers. In addition, the main step forward toward a safe and stable use in clinics of these tools will be the standardization of protocols regarding pre- and post analytical factors. Therefore, a standardized method for isolation of tissues of biofluids as well as preservation of the sample will drastically decrease variability of results, enhancing similarity and robustness of studies in literature. This point is still missing and it is of critical relevance.

In conclusion, microRNAs are fundamental regulator of cell life, linking all its biological functions. Although their analysis has some challenges, the above-mentioned advantages reveal microRNAs as important tools for biomarkers investigation. If

REFERENCES

- Armand-Labit, V., and Pradines, A. (2017). Circulating cell-free microRNAs as clinical cancer biomarkers. *Biomol. Concepts* 8, 61–81. doi: 10.1515/bmc-2017-0002
- Arroyo, J. D., Chevillet, J. R., Kroh, E. M., Ruf, I. K., Pritchard, C. C., Gibson, D. F., et al. (2011). Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc. Natl. Acad. Sci.* U.S.A. 108, 5003–5008. doi: 10.1073/pnas.1019055108
- Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281–297. doi: 10.1016/S0092-8674(04)00045-5
- Bartel, D. P., Abbott, A. L., Alvarez-Saavedra, E., Miska, E. A., Lau, N. C., Bartel, D. P., et al. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215–233. doi: 10.1016/j.cell.2009.01.002
- Blenkiron, C., Goldstein, L. D., Thorne, N. P., Spiteri, I., Chin, S.-F., Dunning, M. J., et al. (2007). MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. *Genome Biol.* 8:R214. doi: 10.1186/gb-2007-8-10-r214
- Bruhn, O., Drerup, K., Kaehler, M., Haenisch, S., Röder, C., and Cascorbi, I. (2016). Length variants of the ABCB1 3'-UTR and loss of miRNA binding sites: possible consequences in regulation and pharmacotherapy resistance. *Pharmacogenomics* 17, 327–340. doi: 10.2217/pgs.15.175
- Calatayud, D., Dehlendorff, C., Boisen, M. K., Hasselby, J. P., Schultz, N. A., Werner, J., et al. (2017). Tissue MicroRNA profiles as diagnostic and prognostic biomarkers in patients with resectable pancreatic ductal adenocarcinoma and periampullary cancers. *Biomark. Res.* 5:8. doi: 10.1186/s40364-017-0087-6
- Cammaerts, S., Strazisar, M., De Rijk, P., and Del Favero, J. (2015). Genetic variants in microRNA genes: impact on microRNA expression, function, and disease. *Front. Genet.* 6:186. doi: 10.3389/fgene.2015.00186
- Cao, Y., Zhao, D., Li, P., Wang, L., Qiao, B., Qin, X., et al. (2017). MicroRNA-181a-5p impedes IL-17-induced non-small cell lung cancer proliferation and migration through targeting VCAM-1. *Cell. Physiol. Biochem.* 42, 346–356. doi: 10.1159/000477389
- Cappuzzo, F., Sacconi, A., Landi, L., Ludovini, V., Biagioni, F., D'Incecco, A., et al. (2014). MicroRNA signature in metastatic colorectal cancer patients treated with Anti-EGFR monoclonal antibodies. *Clin. Colorectal Cancer* 13, 37.e4–45.e4. doi: 10.1016/j.clcc.2013.11.006
- Cha, D. J., Franklin, J. L., Dou, Y., Liu, Q., Higginbotham, J. N., Demory Beckler, M., et al. (2015). KRAS-dependent sorting of miRNA to exosomes. *Elife* 4:e07197. doi: 10.7554/eLife.07197

this field will be further pursued, clinicians could be guided by simple tests detecting pattern of microRNAs expression or even single nucleotide variations, making them strongly valid for cancer personalized medicine.

AUTHOR CONTRIBUTIONS

SD designed the work thinking how it should have been organized, drafted and wrote the manuscript, draw the figures and revised the manuscript critically. MG contributed to the conception and design of the work, supporting the revision for important intellectual content. VDV revised the work critically. MD gave overall intellectual critical support and approved the version to be published. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

This work was supported by the EU H2020 MSCA-RISE project miRNA-DisEASY (GA n $^{\circ}$ 690866).

- Chan, J. A., Krichevsky, A. M., and Kosik, K. S. (2005). MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res* 65, 6029–6033. doi: 10.1158/0008-5472.CAN-05-0137
- Chao, A., Lin, C.-Y., Lee, Y.-S., Tsai, C.-L., Wei, P.-C., Hsueh, S., et al. (2012). Regulation of ovarian cancer progression by microRNA-187 through targeting Disabled homolog-2. Oncogene 31, 764–775. doi: 10.1038/onc.2011.269
- Chen, L., Guan, H., Gu, C., Cao, Y., Shao, J., and Wang, F. (2016). miR-383 inhibits hepatocellular carcinoma cell proliferation via targeting APRIL. *Tumor Biol.* 37, 2497–2507. doi: 10.1007/s13277-015-4071-1
- Chen, Q., Zhao, X., Zhang, H., Yuan, H., Zhu, M., Sun, Q., et al. (2015). MiR-130b suppresses prostate cancer metastasis through down-regulation of MMP2. *Mol. Carcinog.* 54, 1292–1300. doi: 10.1002/mc.22204
- Chen, X., Ba, Y., Ma, L., Cai, X., Yin, Y., Wang, K., et al. (2008). Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res.* 18, 997–1006. doi: 10.1038/cr.2008.282
- Chen, Z., Yu, T., Cabay, R. J., Jin, Y., Mahjabeen, I., Luan, X., et al. (2017). miR-486-3p, miR-139-5p, and miR-21 as biomarkers for the detection of oral tongue squamous cell carcinoma. *Biomark. Cancer* 9, 1–8. doi: 10.4137/BIC.S40981
- Cheng, H. H., Yi, H. S., Kim, Y., Kroh, E. M., Chien, J. W., Eaton, K. D., et al. (2013). Plasma processing conditions substantially influence circulating microRNA biomarker levels. *PLoS ONE* 8:e64795. doi: 10.1371/journal.pone.0064795
- Cheng, Z., Liu, F., Zhang, H., Li, X., Li, Y., Li, J., et al. (2017). miR-135a inhibits tumor metastasis and angiogenesis by targeting FAK pathway. Oncotarget 8, 31153–31168. doi: 10.18632/oncotarget.16098
- Chevillet, J. R., Kang, Q., Ruf, I. K., Briggs, H. A., Vojtech, L. N., Hughes, S. M., et al. (2014). Quantitative and stoichiometric analysis of the microRNA content of exosomes. *Proc. Natl. Acad. Sci. U.S.A.* 111, 14888–14893. doi: 10.1073/pnas.1408301111
- Chugh, P., and Dittmer, D. P. (2012). Potential pitfalls in microRNA profiling. Wiley Interdiscip. Rev. RNA 3, 601–616. doi: 10.1002/wrna.1120
- Cimmino, A., Calin, G. A., Fabbri, M., Iorio, M. V., Ferracin, M., Shimizu, M., et al. (2005). miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc. Natl. Acad. Sci. U.S.A.* 102, 13944–13949. doi: 10.1073/pnas.0506654102
- Collino, F., Deregibus, M. C., Bruno, S., Sterpone, L., Aghemo, G., Viltono, L., et al. (2010). Microvesicles derived from adult human bone marrow and tissue specific mesenchymal stem cells shuttle selected pattern of miRNAs. *PLoS ONE* 5:e11803. doi: 10.1371/journal.pone.0011803
- Cui, M., Liu, W., Zhang, L., Guo, F., Liu, Y., Chen, F., et al. (2017). Over-expression of miR-21 and lower PTEN Levels in Wilms' Tumor with aggressive behavior. *Tohoku J. Exp. Med.* 242, 43–52. doi: 10.1620/tjem.242.43

- da Silva Oliveira, K. C., Thomaz Araújo, T. M., Albuquerque, C. I., Barata, G. A., Gigek, C. O., Leal, M. F., et al. (2016). Role of miRNAs and their potential to be useful as diagnostic and prognostic biomarkers in gastric cancer. World J. Gastroenterol. 22, 7951–7962. doi: 10.3748/wjg.v22.i35.7951
- Daugaard, I., Venø, M. T., Yan, Y., Kjeldsen, T. E., Lamy, P., Hager, H., et al. (2017). Small RNA sequencing reveals metastasis-related microRNAs in lung adenocarcinoma. Oncotarget 8, 27047–27061. doi: 10.18632/oncotarget.15968
- Del Vescovo, V., Cantaloni, C., Cucino, A., Girlando, S., Silvestri, M., Bragantini, E., et al. (2011). miR-205 expression levels in non-small cell lung cancerdo not always distinguish adenocarcinomas from squamous cell carcinomas. *Am. J. Surg. Pathol.* 35, 268–275. doi: 10.1097/PAS.0b013e3182068171
- Del Vescovo, V., and Denti, M. A. (2015). "microRNA and Lung Cancer," in *Advances in Experimental Medicine and Biology*, eds I. R. COHEN, A. LAJTHA, J. D. LAMBRIS, R. PAOLETTI (Basel: Springer), 153–177.
- De Mattos-Arruda, L., Bottai, G., Nuciforo, P. G., Di Tommaso, L., Giovannetti, E., Peg, V., et al. (2015). MicroRNA-21 links epithelial-to-mesenchymal transition and inflammatory signals to confer resistance to neoadjuvant trastuzumab and chemotherapy in HER2-positive breast cancer patients. *Oncotarget* 6, 37269–37280. doi: 10.18632/oncotarget.5495
- Duttagupta, R., Jiang, R., Gollub, J., Getts, R. C., and Jones, K. W. (2011). Impact of cellular miRNAs on circulating miRNA biomarker signatures. *PLoS ONE* 6:e20769. doi: 10.1371/journal.pone.0020769
- Fang, C., Chen, Y.-X., Wu, N.-Y., Yin, J.-Y., Li, X.-P., Huang, H.-S., et al. (2017). MiR-488 inhibits proliferation and cisplatin sensibility in non-small-cell lung cancer (NSCLC) cells by activating the eIF3a-mediated NER signaling pathway. *Sci. Rep.* 7, 40384. doi: 10.1038/srep40384
- Fang, C., Li, X.-P., Gong, W.-J., Wu, N.-Y., Tang, J., Yin, J.-Y., et al. (2016). Age-related common miRNA polymorphism associated with severe toxicity in lung cancer patients treated with platinum-based chemotherapy. *Clin. Exp. Pharmacol. Physiol.* doi: 10.1111/1440-1681.12704. [Epub ahead of print].
- Neviani, P., and Fabbri, M. (2015). Exosomic microRNAs in the tumor microenvironment. *Front. Med.* 2:47. doi: 10.3389/fmed.2015.00047
- Gao, W., Lu, X., Liu, L., Xu, J.i., Feng, D., and Shu, Y. (2012). MiRNA-21. Cancer Biol. Ther. 13, 330–340. doi: 10.4161/cbt.19073
- Garcia, A. I., Buisson, M., Bertrand, P., Rimokh, R., Rouleau, E., Lopez, B. S., et al. (2011). Down-regulation of BRCA1 expression by miR-146a and miR-146b-5p in triple negative sporadic breast cancers. *EMBO Mol. Med.* 3, 279–290. doi: 10.1002/emmm.201100136
- Garcia-Donas, J., Beuselinck, B., Inglada-Pérez, L., Graña, O., Schöffski, P., Wozniak, A., et al. (2016). Deep sequencing reveals microRNAs predictive of antiangiogenic drug response. *JCI Insight* 1:e86051. doi: 10.1172/jci.insight.86051
- Garcia-Ortì, L., Cristóbal, I., Cirauqui, C., Guruceaga, E., Marcotegui, N., Calasanz, M. J., et al. (2012). Integration of SNP and mRNA Arrays with MicroRNA profiling reveals that MiR-370 is upregulated and targets NF1 in acute myeloid leukemia. *PLoS ONE* 7:e47717. doi: 10.1371/journal.pone.0047717
- Geng, J.-Q., Wang, X.-C., Li, L.-F., Zhao, J., Wu, S., Yu, G.-P., et al. (2016). MicroRNA-related single-nucleotide polymorphism of XPO5 is strongly correlated with the prognosis and chemotherapy response in advanced non-small-cell lung cancer patients. *Tumor Biol.* 37, 2257–2265. doi: 10.1007/s13277-015-3980-3
- Go, H., Jang, J.-Y., Kim, P.-J., Kim, Y.-G., Nam, S. J., Paik, J. H., et al. (2015). MicroRNA-21 plays an oncogenic role by targeting FOXO1 and activating the PI3K/AKT pathway in diffuse large B-cell lymphoma. *Oncotarget* 6, 15035–15049. doi: 10.18632/oncotarget.3729
- Godnic, I., Zorc, M., Jevsinek Skok, D., Calin, G. A., Horvat, S., Dovc, P., et al. (2013). Genome-wide and species-wide *in silico* screening for intragenic micrornas in human, mouse and chicken. *PLoS ONE* 8:e65165. doi: 10.1371/journal.pone.0065165
- Gong, J., Tong, Y., Zhang, H.-M., Wang, K., Hu, T., Shan, G., et al. (2012). Genome-wide identification of SNPs in microRNA genes and the SNP effects on microRNA target binding and biogenesis. *Hum. Mutat.* 33, 254–263. doi: 10.1002/humu.21641
- Grundhoff, A., and Sullivan, C. S. (2011). Virus-encoded microRNAs. *Virology* 411, 325–343. doi: 10.1016/j.virol.2011.01.002
- Gu, D., Jiang, M., Mei, Z., Dai, J., Dai, C., Fang, C., et al. (2017). microRNA-7 impairs autophagy-derived pools of glucose to suppress pancreatic cancer progression. *Cancer Lett.* 400, 69–78. doi: 10.1016/j.canlet.2017.04.020

- Guzman, N., Agarwal, K., Asthagiri, D., Yu, L., Saji, M., Ringel, M. D., et al. (2015). Breast cancer–specific miR signature unique to extracellular vesicles includes "microRNA-like" tRNA fragments. *Mol. Cancer Res.* 13, 891–901. doi: 10.1158/1541-7786.MCR-14-0533
- Hanahan, D., and Weinberg, R. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646–674. doi: 10.1016/j.cell.2011.02.013
- Hansen, T. B., Jensen, T. I., Clausen, B. H., Bramsen, J. B., Finsen, B., Damgaard, C. K., et al. (2013). Natural RNA circles function as efficient microRNA sponges. *Nature* 495, 384–388. doi: 10.1038/nature11993
- Hasegawa, K., Negishi, R., Matsumoto, M., Yohda, M., Hosokawa, K., and Maeda, M. (2017). Specificity of MicroRNA Detection on a power-free microfluidic chip with laminar flow-assisted dendritic amplification. *Anal. Sci.* 33, 171–177. doi: 10.2116/analsci.33.171
- He, Q., Cai, L., Shuai, L., Li, D., Wang, C., Liu, Y., et al. (2013). Ars2 is overexpressed in human cholangiocarcinomas and its depletion increases PTEN and PDCD4 by decreasing microRNA-21. *Mol. Carcinog.* 52, 286–296. doi: 10.1002/mc.21859
- He, Z., Cen, D., Luo, X., Li, D., Li, P., Liang, L., et al. (2013). Downregulation of miR-383 promotes glioma cell invasion by targeting insulin-like growth factor 1 receptor. *Med. Oncol.* 30:557. doi: 10.1007/s12032-013-0557-0
- Hezova, R., Kovarikova, A., Srovnal, J., Zemanova, M., Harustiak, T., Ehrmann, J., et al. (2015). Diagnostic and prognostic potential of miR-21, miR-29c, miR-148 and miR-203 in adenocarcinoma and squamous cell carcinoma of esophagus. *Diagn. Pathol.* 10:42. doi: 10.1186/s13000-015-0280-6
- Hoffman, A. E., Zheng, T., Yi, C., Leaderer, D., Weidhaas, J., Slack, F., et al. (2009). microRNA miR-196a-2 and breast cancer: a genetic and epigenetic association study and functional analysis. *Cancer Res.* 69, 5970–5977. doi: 10.1158/0008-5472.CAN-09-0236
- Hsu, Y.-L., Hung, J.-Y., Chang, W.-A., Lin, Y.-S., Pan, Y.-C., Tsai, P.-H., et al. (2017). Hypoxic lung cancer-secreted exosomal miR-23a increased angiogenesis and vascular permeability by targeting prolyl hydroxylase and tight junction protein ZO-1. Oncogene 36, 4929–4942. doi: 10.1038/onc.2017.105
- Hu, Z., Chen, J., Tian, T., Zhou, X., Gu, H., Xu, L., et al. (2008). Genetic variants of miRNA sequences and non-small cell lung cancer survival. J. Clin. Invest. 118, 2600–2608. doi: 10.1172/JCI34934
- Huang, H., Tian, H., Duan, Z., Cao, Y., Zhang, X.-S., and Sun, F. (2014). microRNA-383 impairs phosphorylation of H2AX by targeting PNUTS and inducing cell cycle arrest in testicular embryonal carcinoma cells. *Cell. Signal.* 26, 903–911. doi: 10.1016/j.cellsig.2014.01.016
- Huang, X., and Lu, S. (2017). MicroR-545 mediates colorectal cancer cells proliferation through up-regulating epidermal growth factor receptor expression in HOTAIR long non-coding RNA dependent. *Mol. Cell. Biochem.* 431, 45–54. doi: 10.1007/s11010-017-2974-4
- Huang, Z., Huang, D., Ni, S., Peng, Z., Sheng, W., and Du, X. (2010). Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. *Int. J. Cancer* 127, 118–126. doi: 10.1002/ijc.25007
- Hwang, J.-H., Voortman, J., Giovannetti, E., Steinberg, S. M., Leon, L. G., Kim, Y.-T., et al. (2010). Identification of MicroRNA-21 as a biomarker for chemoresistance and clinical outcome following adjuvant therapy in resectable pancreatic cancer. *PLoS ONE* 5:e10630. doi: 10.1371/journal.pone.0010630
- Igarashi, H., Kurihara, H., Mitsuhashi, K., Ito, M., Okuda, H., Kanno, S., et al. (2015). Association of MicroRNA-31-5p with clinical efficacy of anti-EGFR therapy in patients with metastatic colorectal cancer. *Ann. Surg. Oncol.* 22, 2640–2648. doi: 10.1245/s10434-014-4264-7
- Iorio, M. V., Ferracin, M., Liu, C.-G., Veronese, A., Spizzo, R., Sabbioni, S., et al. (2005). MicroRNA gene expression deregulation in human breast cancer. *Cancer Res.* 65, 7065–7070. doi: 10.1158/0008-5472.CAN-05-1783
- Iorio, M. V., Visone, R., Di Leva, G., Donati, V., Petrocca, F., Casalini, P., et al. (2007). MicroRNA signatures in human ovarian cancer. *Cancer Res.* 67, 8699–8707. doi: 10.1158/0008-5472.CAN-07-1936
- Ipsaro, J. J., and Joshua-Tor, L. (2015). From guide to target: molecular insights into eukaryotic RNA-interference machinery. *Nat. Struct. Mol. Biol.* 22, 20–28. doi: 10.1038/nsmb.2931
- Janas, T., Janas, M. M., SapońK., and Janas, T. (2015). Mechanisms of RNA loading into exosomes. FEBS Lett. 589, 1391–1398. doi: 10.1016/j.febslet.2015.04.036
- Jarry, J., Schadendorf, D., Greenwood, C., Spatz, A., and van Kempen, L. C. (2014). The validity of circulating microRNAs in oncology: five years of challenges

and contradictions. Mol. Oncol. 8, 819-829. doi: 10.1016/j.molonc.2014. 02.009

- Jiang, Y., Chen, J., Wu, J., Hu, Z., Qin, Z., Liu, X., et al. (2013). Evaluation of genetic variants in microRNA biosynthesis genes and risk of breast cancer in Chinese women. *Int. J. Cancer* 133, 2216–2224. doi: 10.1002/ijc.28237
- Jones-Rhoades, M. W., Bartel, D. P., and Bartel, B. (2006). MicroRNAS and their regulatory roles in plants. *Annu. Rev. Plant Biol.* 57, 19–53. doi: 10.1146/annurev.arplant.57.032905.105218
- Kapodistrias, N., Mavridis, K., Batistatou, A., Gogou, P., Karavasilis, V., Sainis, I., et al. (2016). Assessing the clinical value of microRNAs in formalin-fixed paraffin-embedded liposarcoma tissues: overexpressed miR-155 is an indicator of poor prognosis. Oncotarget 8, 6896–6913. doi: 10.18632/oncotarget.14320
- Ke, J., Tian, J., Li, J., Gong, Y., Yang, Y., Zhu, Y., et al. (2017). Identification of a functional polymorphism affecting microRNA binding in the susceptibility locus 1q25.3 for colorectal cancer. *Mol. Carcinog.* 56, 2014–2021. doi: 10.1002/mc.22649
- Khorrami, S., Zavaran Hosseini, A., Mowla, S. J., Soleimani, M., Rakhshani, N., and Malekzadeh, R. (2017). MicroRNA-146a induces immune suppression and drug-resistant colorectal cancer cells. *Tumor Biol.* 39:101042831769836. doi: 10.1177/1010428317698365
- Kijima, T., Hazama, S., Tsunedomi, R., Tanaka, H., Takenouchi, H., Kanekiyo, S., et al. (2016). MicroRNA-6826 and—6875 in plasma are valuable non-invasive biomarkers that predict the efficacy of vaccine treatment against metastatic colorectal cancer. Oncol. Rep. 37, 23–30. doi: 10.3892/or.2016.5267
- Kloosterman, W. P., Wienholds, E., Ketting, R. F., and Plasterk, R. H. A. (2016). Substrate requirements for let-7 function in the developing zebrafish embryo. *Nucleic Acids Res.* 44, 5993–5993. doi: 10.1093/nar/gkw173
- Koppers-Lalic, D., Hackenberg, M., Bijnsdorp, I. V., van Eijndhoven, M. A. J., Sadek, P., Sie, D., et al. (2014). Nontemplated nucleotide additions distinguish the small RNA composition in cells from exosomes. *Cell Rep.* 8, 1649–1658. doi: 10.1016/j.celrep.2014.08.027
- Kosaka, N., Iguchi, H., Yoshioka, Y., Takeshita, F., Matsuki, Y., and Ochiya, T. (2010). Secretory mechanisms and intercellular transfer of microRNAs in living cells. J. Biol. Chem. 285, 17442–17452. doi: 10.1074/jbc.M110.107821
- Kumarswamy, R., Mudduluru, G., Ceppi, P., Muppala, S., Kozlowski, M., Niklinski, J., et al. (2012). MicroRNA-30a inhibits epithelial-to-mesenchymal transition by targeting Snai1 and is downregulated in non-small cell lung cancer. *Int. J. Cancer* 130, 2044–2053. doi: 10.1002/ijc.26218
- Lebanony, D., Benjamin, H., Gilad, S., Ezagouri, M., Dov, A., Ashkenazi, K., et al. (2009). Diagnostic assay based on hsa-miR-205 expression distinguishes squamous from nonsquamous non-small-cell lung carcinoma. *J. Clin. Oncol.* 27, 2030–2037. doi: 10.1200/JCO.2008.19.4134
- Lee, A.-R., Park, J., Jung, K. J., Jee, S. H., and Kim-Yoon, S. (2016). Genetic variation rs7930 in the miR-4273-5p target site is associated with a risk of colorectal cancer. *Onco. Targets. Ther.* 9, 6885–6895. doi: 10.2147/OTT.S108787
- Lee, R. C., Feinbaum, R. L., Ambros, V., Arasu, P., Ruvkun, G., Staden, R., et al. (1993). The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 75, 843–854. doi: 10.1016/0092-8674(93)90529-Y
- Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., et al. (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425, 415–419. doi: 10.1038/nature01957
- Leidner, R. S., Ravi, L., Leahy, P., Chen, Y., Bednarchik, B., Streppel, M., et al. (2012). The microRNAs, MiR-31 and MiR-375, as candidate markers in Barrett's esophageal carcinogenesis. *Genes, Chromosom. Cancer* 51, 473–479. doi: 10.1002/gcc.21934
- Li, B., Ge, L., Li, M., Wang, L., and Li, Z. (2016). miR-448 suppresses proliferation and invasion by regulating IGF1R in colorectal cancer cells. *Am. J. Transl. Res.* 8, 3013–3022.
- Li, J., Lei, K., Wu, Z., Li, W., Liu, G., Liu, J., et al. (2014a). Network-based identification of microRNAs as potential pharmacogenomic biomarkers for anticancer drugs. Oncotarget 7, 45584–45596. doi: 10.18632/oncotarget.10052
- Li, J., Li, X., Ren, S., Chen, X., Zhang, Y., Zhou, F., et al. (2014b). miR-200c overexpression is associated with better efficacy of EGFR-TKIs in non-small cell lung cancer patients with EGFR wild-type. *Oncotarget* 5, 7902–7916. doi: 10.18632/oncotarget.2302

- Li, K. K.-W., Pang, J. C.-S., Lau, K.-M., Zhou, L., Mao, Y., Wang, Y., et al. (2013). MiR-383 is downregulated in medulloblastoma and targets peroxiredoxin 3 (PRDX3). *Brain Pathol.* 23, 413–425. doi: 10.1111/bpa.12014
- Li, L., and Luo, Z. (2017). Dysregulated miR-27a-3p promotes nasopharyngeal carcinoma cell proliferation and migration by targeting Mapk10. Oncol. Rep. 37, 2679–2687. doi: 10.3892/or.2017.5544
- Li, Q.-Q., Chen, Z.-Q., Cao, X.-X., Xu, J.-D., Xu, J.-W., Chen, Y.-Y., et al. (2011). Involvement of NF-κB/miR-448 regulatory feedback loop in chemotherapyinduced epithelial?mesenchymal transition of breast cancer cells. *Cell Death Differ.* 18, 16–25. doi: 10.1038/cdd.2010.103
- Li, S.-J., Liu, H.-L., Tang, S.-L., Li, X.-J., and Wang, X.-Y. (2017). MicroRNA-150 regulates glycolysis by targeting von Hippel-Lindau in glioma cells. Am. J. Transl. Res. 9, 1058–1066.
- Lian, J., Tian, H., Liu, L., Zhang, X.-S., Li, W.-Q., Deng, Y.-M., et al. (2010). Downregulation of microRNA-383 is associated with male infertility and promotes testicular embryonal carcinoma cell proliferation by targeting IRF1. *Cell Death Dis.* 1:e94. doi: 10.1038/cddis.2010.70
- Lin, M., Gu, J., Eng, C., Ellis, L. M., Hildebrandt, M. A., Lin, J., et al. (2012). Genetic polymorphisms in MicroRNA-related genes as predictors of clinical outcomes in colorectal adenocarcinoma patients. *Clin. Cancer Res.* 18, 3982–3991. doi: 10.1158/1078-0432.CCR-11-2951
- Liu, C., Zhang, Y., Chen, H., Jiang, L., and Xiao, D. (2016). Function analysis of rs9589207 polymorphism in miR-92a in gastric cancer. *Tumor Biol.* 37, 4439–4444. doi: 10.1007/s13277-015-4288-z
- Liu, H., Zhou, Y., Liu, Q., Xiao, G., Wang, B., Li, W., et al. (2017). Association of miR-608 rs4919510 polymorphism and cancer risk: a meta-analysis based on 13,664 subjects. *Oncotarget* 8, 37023–37031. doi: 10.18632/oncotarget.9509
- Liu, Y., Chai, Y., Zhang, J., and Tang, J. (2016). A function variant at miR-501 alters susceptibility to hepatocellular carcinoma in a chinese han population. *Cell. Physiol. Biochem.* 38, 2500–2508. doi: 10.1159/000445600
- Liu, Y., Liu, R., Yang, F., Cheng, R., Chen, X., Cui, S., et al. (2017). miR-19a promotes colorectal cancer proliferation and migration by targeting TIA1. *Mol. Cancer* 16:53. doi: 10.1186/s12943-017-0625-8
- Lopez-Lopez, E., Gutierrez-Camino, A., Astigarraga, I., Navajas, A., Echebarria-Barona, A., Garcia-Miguel, P., et al. (2016). Vincristine pharmacokinetics pathway and neurotoxicity during early phases of treatment in pediatric acute lymphoblastic leukemia. *Pharmacogenomics* 17, 731–741. doi: 10.2217/pgs-2016-0001
- Lopez-Lopez, E., Gutiérrez-Camino, Á., Piñán, M. Á., Sánchez-Toledo, J., Uriz, J. J., Ballesteros, J., et al. (2014). Pharmacogenetics of microRNAs and microRNAs biogenesis machinery in pediatric acute lymphoblastic leukemia. *PLoS ONE* 9:e91261. doi: 10.1371/journal.pone.0091261
- Lu, J., Getz, G., Miska, E. A., Alvarez-Saavedra, E., Lamb, J., Peck, D., et al. (2005). microRNA expression profiles classify human cancers. *Nature* 435, 834–838. doi: 10.1038/nature03702
- Lu, M., Kong, X., Wang, H., Huang, G., Ye, C., and He, Z. (2017). A novel microRNAs expression signature for hepatocellular carcinoma diagnosis and prognosis. *Oncotarget* 8, 8775–8784. doi: 10.18632/oncotarget.14452
- Lv, Y., Lei, Y., Hu, Y., Ding, W., Zhang, C., and Fang, C. (2015). miR-448 negatively regulates ovarian cancer cell growth and metastasis by targeting CXCL12. *Clin. Transl. Oncol.* 17, 903–909. doi: 10.1007/s12094-015-1325-8
- Lytle, J. R., Yario, T. A., and Steitz, J. A. (2007). Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. *Proc. Natl. Acad. Sci. U.S.A.* 104, 9667–9672. doi: 10.1073/pnas.0703820104
- Makarova, J. A., Shkurnikov, M. U., Wicklein, D., Lange, T., Samatov, T. R., Turchinovich, A. A., et al. (2016). Intracellular and extracellular microRNA: an update on localization and biological role. *Prog. Histochem. Cytochem.* 51, 33–49. doi: 10.1016/j.proghi.2016.06.001
- Markou, A., Tsaroucha, E. G., Kaklamanis, L., Fotinou, M., Georgoulias, V., and Lianidou, E. S. (2008). Prognostic value of mature MicroRNA-21 and MicroRNA-205 overexpression in non-small cell lung cancer by quantitative real-time RT-PCR. *Clin. Chem.* 54, 1696–1704. doi: 10.1373/clinchem.2007.101741
- Masè, M., Grasso, M., Avogaro, L., D'Amato, E., Tessarolo, F., Graffigna, A., et al. (2017). Selection of reference genes is critical for miRNA expression analysis in human cardiac tissue. A focus on atrial fibrillation. *Sci. Rep.* 7:41127. doi: 10.1038/srep41127

- Matsuzaki, J., and Ochiya, T. (2017). Circulating microRNAs and extracellular vesicles as potential cancer biomarkers: a systematic review. *Int. J. Clin. Oncol.* 22, 413–420. doi: 10.1007/s10147-017-1104-3
- Memczak, S., Jens, M., Elefsinioti, A., Torti, F., Krueger, J., Rybak, A., et al. (2013). Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 495, 333–338. doi: 10.1038/nature11928
- Meng, F., Henson, R., Wehbe-Janek, H., Ghoshal, K., Jacob, S. T., and Patel, T. (2007). MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology* 133, 647–658. doi: 10.1053/j.gastro.2007.05.022
- Meshkat, M., Tanha, H. M., Naeini, M. M., Ghaedi, K., Sanati, M. H., Meshkat, M., et al. (2016). Functional SNP in stem of mir-146a affects Her2 status and breast cancer survival. *Cancer Biomarkers* 17, 213–222. doi: 10.3233/CBM-160633
- Mi, Y., Zhang, D., Jiang, W., Weng, J., Zhou, C., Huang, K., et al. (2017). miR-181a-5p promotes the progression of gastric cancer via RASSF6-mediated MAPK signalling activation. *Cancer Lett.* 389, 11–22. doi: 10.1016/j.canlet.2016.12.033
- Min, P., Li, W., Zeng, D., Ma, Y., Xu, D., Zheng, W., et al. (2017). A single nucleotide variant in microRNA-1269a promotes the occurrence and process of hepatocellular carcinoma by targeting to oncogenes SPATS2L and LRP6. *Bull. Cancer* 104, 311–320. doi: 10.1016/j.bulcan.2016.11.021
- Miotto, E., Saccenti, E., Lupini, L., Callegari, E., Negrini, M., and Ferracin, M. (2014). Quantification of Circulating miRNAs by Droplet Digital PCR: comparison of EvaGreen- and TaqMan-Based Chemistries. *Cancer Epidemiol. Prev. Biomarkers* 23. 2638–2642. doi: 10.1158/1055-9965
- Mitchell, P. S., Parkin, R. K., Kroh, E. M., Fritz, B. R., Wyman, S. K., Pogosova-Agadjanyan, E. L., et al. (2008). Circulating microRNAs as stable blood-based markers for cancer detection. *Proc. Natl. Acad. Sci. U.S.A.* 105, 10513–10518. doi: 10.1073/pnas.0804549105
- Mlcochova, J., Faltejskova-Vychytilova, P., Ferracin, M., Zagatti, B., Radova, L., Svoboda, M., et al. (2015). MicroRNA expression profiling identifies miR-31-5p/3p as associated with time to progression in wild-type RAS metastatic colorectal cancer treated with cetuximab. *Oncotarget* 6, 38695–38704. doi: 10.18632/oncotarget.5735
- Mohammadi, A., Mansoori, B., and Baradaran, B. (2016). The role of microRNAs in colorectal cancer. *Biomed. Pharmacother.* 84, 705–713. doi: 10.1016/j.biopha.2016.09.099
- Molasy, M., Walczak, A., Szaflik, J., Szaflik, J. P., and Majsterek, I. (2016). MicroRNAs in glaucoma and neurodegenerative diseases. J. Hum. Genet. 62, 105–112. doi: 10.1038/jhg.2016.91
- Monleau, M., Bonnel, S., Gostan, T., Blanchard, D., Courgnaud, V., Lecellier, C.-H., et al. (2014). Comparison of different extraction techniques to profile microRNAs from human sera and peripheral blood mononuclear cells. *BMC Genomics* 15:395. doi: 10.1186/1471-2164-15-395
- Montes-Moreno, S., Martinez, N., Sanchez-Espiridión, B., Díaz Uriarte, R., Rodriguez, M. E., Saez, A., et al. (2011). miRNA expression in diffuse large B-cell lymphoma treated with chemoimmunotherapy. *Blood* 118. 1034–1040. doi: 10.1182/blood-2010-11-321554
- Mosakhani, N., Lahti, L., Borze, I., Karjalainen-Lindsberg, M.-L., Sundström, J., Ristamäki, R., et al. (2012). MicroRNA profiling predicts survival in anti-EGFR treated chemorefractory metastatic colorectal cancer patients with wild-type KRAS and BRAF. *Cancer Genet.* 205, 545–551. doi: 10.1016/j.cancergen.2012.08.003
- Mott, J. L., Kobayashi, S., Bronk, S. F., and Gores, G. J. (2007). mir-29 regulates Mcl-1 protein expression and apoptosis. *Oncogene* 26, 6133–6140. doi: 10.1038/sj.onc.1210436
- Mullany, L. E., Herrick, J. S., Wolff, R. K., Buas, M. F., and Slattery, M. L. (2016). Impact of polymorphisms in microRNA biogenesis genes on colon cancer risk and microRNA expression levels: a population-based, case-control study. *BMC Med. Genomics* 9:21. doi: 10.1186/s12920-016-0181-x
- Nadal, C., Winder, T., Gerger, A., and Tougeron, D. (2017). Future perspectives of circulating tumor DNA in colorectal cancer. *Tumor Biol.* 39:101042831770574. doi: 10.1177/1010428317705749
- Nakka, M., Allen-Rhoades, W., Li, Y., Kelly, A. J., Shen, J., Taylor, A. M., et al. (2017). Biomarker significance of plasma and tumor miR-21, miR-221, and miR-106a in osteosarcoma. *Oncotarget* doi: 10.18632/oncotarget.18236
- Namkung, J., Kwon, W., Choi, Y., Yi, S. G., Han, S., Kang, M. J., et al. (2016). Molecular subtypes of pancreatic cancer based on miRNA expression profiles

have independent prognostic value. J. Gastroenterol. Hepatol. 31, 1160–1167. doi: 10.1111/jgh.13253

- Neault, M., Mallette, F. A., and Richard, S. (2016). miR-137 modulates a tumor suppressor network-inducing senescence in pancreatic cancer cells. *Cell Rep.* 14, 1966–1978. doi: 10.1016/j.celrep.2016.01.068
- Ng, E. K. O., Chong, W. W. S., Jin, H., Lam, E. K. Y., Shin, V. Y., Yu, J., et al. (2009). Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. *Gut* 58, 1375–1381. doi: 10.1136/gut.2008.167817
- Nishida, N., Arizumi, T., Hagiwara, S., Ida, H., Sakurai, T., and Kudo, M. (2017). MicroRNAs for the prediction of early response to sorafenib treatment in human hepatocellular carcinoma. *Liver Cancer* 6, 113–125. doi: 10.1159/000449475
- Ochoa, A. E., Choi, W., Su, X., Siefker-Radtke, A., Czerniak, B., Dinney, C., et al. (2016). Specific micro-RNA expression patterns distinguish the basal and luminal subtypes of muscle-invasive bladder cancer. *Oncotarget* 7, 80164–80174. doi: 10.18632/oncotarget.13284
- Parafioriti, A., Bason, C., Armiraglio, E., Calciano, L., Daolio, P. A., Berardocco, M., et al. (2016). Ewing's sarcoma: an analysis of mirna expression profiles and target genes in paraffin-embedded primary tumor tissue. *Int. J. Mol. Sci.* 17, 656. doi: 10.3390/ijms17050656
- Pardini, B., Rosa, F., Naccarati, A., Vymetalkova, V., Ye, Y., Wu, X., et al. (2015). Polymorphisms in microRNA genes as predictors of clinical outcomes in colorectal cancer patients. *Carcinogenesis* 36, 82–86. doi: 10.1093/carcin/bgu224
- Parkin, B., Ouillette, P., Wang, Y., Liu, Y., Wright, W., Roulston, D., et al. (2010). NF1 inactivation in adult acute myelogenous leukemia. *Clin. Cancer Res.* 16, 4135–4147. doi: 10.1158/1078-0432.CCR-09-2639
- Patnaik, S., Mallick, R., Kannisto, E., Sharma, R., Bshara, W., Yendamuri, S., et al. (2015). MiR-205 and MiR-375 microRNA assays to distinguish squamous cell carcinoma from adenocarcinoma in lung cancer biopsies. *J. Thorac. Oncol.* 10, 446–453. doi: 10.1097/JTO.00000000000023
- Paydas, S., Acikalin, A., Ergin, M., Celik, H., Yavuz, B., and Tanriverdi, K. (2016). Micro-RNA (miRNA) profile in Hodgkin lymphoma: association between clinical and pathological variables. *Med. Oncol.* 33:34. doi: 10.1007/s12032-016-0749-5
- Peng, J., Liu, H.-Z., Zhong, J., Deng, Z.-F., Tie, C.-R., Rao, Q., et al. (2016). MicroRNA-187 is an independent prognostic factor in lung cancer and promotes lung cancer cell invasion via targeting of PTRF. Oncol. Rep. 36, 2609–2618. doi: 10.3892/or.2016.5083
- Preis, M., Gardner, T. B., Gordon, S. R., Pipas, J. M., Mackenzie, T. A., Klein, E. E., et al. (2011). MicroRNA-10b expression correlates with response to neoadjuvant therapy and survival in pancreatic ductal adenocarcinoma. *Clin. Cancer Res.* 17, 5812–5821. doi: 10.1158/1078-0432.CCR-11-0695
- Pritchard, C. C., Kroh, E., Wood, B., Arroyo, J. D., Dougherty, K. J., Miyaji, M. M., et al. (2012). Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. *Cancer Prev. Res. (Phila).* 5, 492–497. doi: 10.1158/1940-6207.CAPR-11-0370
- Ramalho-Carvalho, J., Graça, I., Gomez, A., Oliveira, J., Henrique, R., Esteller, M., et al. (2017). Downregulation of miR-130b~301b cluster is mediated by aberrant promoter methylation and impairs cellular senescence in prostate cancer. J. Hematol. Oncol. 10:43. doi: 10.1186/s13045-017-0415-1
- Rapa, I., Votta, A., Felice, B., Righi, L., Giorcelli, J., Scarpa, A., et al. (2015). Identification of MicroRNAs differentially expressed in lung carcinoid subtypes and progression. *Neuroendocrinology* 101, 246–255. doi: 10.1159/ 000381454
- Raponi, M., Dossey, L., Jatkoe, T., Wu, X., Chen, G., Fan, H., et al. (2009). MicroRNA classifiers for predicting prognosis of squamous cell lung cancer. *Cancer Res.* 69, 5776–5783. doi: 10.1158/0008-5472.CAN-09-0587
- Reddy, P. H., Tonk, S., Kumar, S., Vijayan, M., Kandimalla, R., Kuruva, C. S., et al. (2016). A critical evaluation of neuroprotective and neurodegenerative MicroRNAs in Alzheimer's disease. *Biochem. Biophys. Res. Commun.* 483, 1156–1165. doi: 10.1016/j.bbrc.2016.08.067
- Revathidevi, S., Sudesh, R., Vaishnavi, V., Kaliyanasundaram, M., MaryHelen, K. G., Sukanya, G., et al. (2016). Screening for the 3'UTR polymorphism of the PXR gene in South Indian breast cancer patients and its potential role in pharmacogenomics. *Asian Pac. J. Cancer Prev.* 17, 3971–3977.

- Rissin, D. M., López-Longarela, B., Pernagallo, S., Ilyine, H., Vliegenthart, A. D. B., Dear, J. W., et al. (2017). Polymerase-free measurement of microRNA-122 with single base specificity using single molecule arrays: detection of drug-induced liver injury. *PLoS ONE* 12:e0179669. doi: 10.1371/journal.pone. 0179669
- Ristori, E., Lopez-Ramirez, M., Narayanan, A., Hill-Teran, G., Moro, A., Calvo, C.-F., et al. (2015). A Dicer-miR-107 interaction regulates biogenesis of specific miRNAs crucial for neurogenesis. *Dev. Cell* 32, 546–560. doi: 10.1016/j.devcel.2014.12.013
- Rosenfeld, N., Aharonov, R., Meiri, E., Rosenwald, S., Spector, Y., Zepeniuk, M., et al. (2008). MicroRNAs accurately identify cancer tissue origin. *Nat. Biotechnol.* 26, 462–469. doi: 10.1038/nbt1392
- Rotunno, M., Zhao, Y., Bergen, A. W., Koshiol, J., Burdette, L., Rubagotti, M., et al. (2010). Inherited polymorphisms in the RNA-mediated interference machinery affect microRNA expression and lung cancer survival. *Br. J. Cancer* 103, 1870–1874. doi: 10.1038/sj.bjc.6605976
- Rukov, J. L., Vinther, J., and Shomron, N. (2011). Pharmacogenomics genes show varying perceptibility to microRNA regulation. *Pharmacogenet. Genomics* 21, 251–262. doi: 10.1097/FPC.0b013e3283438865
- Rusca, N., and Monticelli, S. (2011). MiR-146a in Immunity and Disease. *Mol. Biol. Int.* 2011:437301. doi: 10.4061/2011/437301
- Schetter, A. J., Leung, S. Y., Sohn, J. J., Zanetti, K. A., Bowman, E. D., Yanaihara, N., et al. (2008). MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA* 299, 425–436. doi: 10.1001/jama.299.4.425
- Schetter, A. J., Nguyen, G. H., Bowman, E. D., Mathé, E. A., Yuen, S. T., Hawkes, J. E., et al. (2009). Association of inflammation-related and microRNA gene expression with cancer-specific mortality of colon adenocarcinoma. *Clin. Cancer Res.* 15, 5878–5887. doi: 10.1158/1078-0432.CCR-09-0627
- Schweiger, M. R., Kerick, M., Timmermann, B., and Isau, M. (2011). The power of NGS technologies to delineate the genome organization in cancer: from mutations to structural variations and epigenetic alterations. *Cancer Metastasis Rev.* 30, 199–210. doi: 10.1007/s10555-011-9278-z
- Shan, C., Fei, F., Li, F., Zhuang, B., Zheng, Y., Wan, Y., et al. (2017). miR-448 is a novel prognostic factor of lung squamous cell carcinoma and regulates cells growth and metastasis by targeting DCLK1. *Biomed. Pharmacother.* 89, 1227–1234. doi: 10.1016/j.biopha.2017.02.017
- Shang, Y., Zang, A., Li, J., Jia, Y., Li, X., Zhang, L., et al. (2016). MicroRNA-383 is a tumor suppressor and potential prognostic biomarker in human non-small cell lung caner. *Biomed. Pharmacother.* 83, 1175–1181. doi: 10.1016/j.biopha.2016.08.006
- Sharova, E., Grassi, A., Marcer, A., Ruggero, K., Pinto, F., Bassi, P., et al. (2016). A circulating miRNA assay as a first-line test for prostate cancer screening. *Br. J. Cancer* 114, 1362–1366. doi: 10.1038/bjc.2016.151
- Shi, L., Cheng, Z., Zhang, J., Li, R., Zhao, P., Fu, Z., et al. (2008). hsa-mir-181a and hsa-mir-181b function as tumor suppressors in human glioma cells. *Brain Res.* 1236, 185–193. doi: 10.1016/j.brainres.2008.07.085
- Si, M.-L., Zhu, S., Wu, H., Lu, Z., Wu, F., and Mo, Y.-Y. (2007). miR-21-mediated tumor growth. *Oncogene* 26, 2799–2803. doi: 10.1038/sj.onc.1210083
- Sibin, M. K., Harshitha, S. M., Narasingarao, K. V. L., Dhananjaya, I. B., Dhaval, P. S., and Chetan, G. K. (2017). Effect of rs11614913 polymorphism on mature miR196a2 expression and its target gene HOXC8 expression in human glioma. *J. Mol. Neurosci.* 61, 144–151. doi: 10.1007/s12031-016-0855-z
- Siu-Chi Lam, C., Ng, L., Ka-Man Chow, A., Ming-Hun Wan, T., Yau, S., Shiu-Man Cheng, N., et al. (2017). Identification of microRNA 885-5p as a novel regulator of tumor metastasis by targeting CPEB2 in colorectal cancer. *Oncotarget* 8, 26858–26870. doi: 10.18632/oncotarget.15844
- Squadrito, M. L., Baer, C., Burdet, F., Maderna, C., Gilfillan, G. D., Lyle, R., et al. (2014). Endogenous RNAs modulate microRNA sorting to exosomes and transfer to acceptor cells. *Cell Rep.* 8, 1432–1446. doi: 10.1016/j.celrep.2014.07.035
- Strimbu, K., and Tavel, J. A. (2010). What are biomarkers? *Curr. Opin. HIV AIDS* 5, 463–466. doi: 10.1097/COH.0b013e32833ed177
- Swart, M., and Dandara, C. (2014). Genetic variation in the 3???-UTR of CYP1A2, CYP2B6, CYP2D6, CYP3A4, NR1I2, and UGT2B7: potential effects on regulation by microRNA and pharmacogenomics relevance. *Front. Genet.* 5:167. doi: 10.3389/fgene.2014.00167

- Takahashi, M., Cuatrecasas, M., Balaguer, F., Hur, K., Toiyama, Y., Castells, A., et al. (2012). The clinical significance of MiR-148a as a predictive biomarker in patients with advanced colorectal cancer. *PLoS ONE* 7:e46684. doi: 10.1371/journal.pone.0046684
- Takamizawa, J., Konishi, H., Yanagisawa, K., Tomida, S., Osada, H., Endoh, H., et al. (2004). Reduced Expression of the let-7 MicroRNAs in human lung cancers in association with shortened post-operative survival. *Cancer Res.* 64, 3753–3756. doi: 10.1158/0008-5472.CAN-04-0637
- Thomas, M., Lieberman, J., and Lal, A. (2010). Desperately seeking microRNA targets. *Nat. Struct. Mol. Biol.* 17, 1169–1174. doi: 10.1038/nsmb.1921
- Thompson, K. L., Boitier, E., Chen, T., Couttet, P., Ellinger-Ziegelbauer, H., Goetschy, M., et al. (2016). Absolute measurement of cardiac injury-induced microRNAs in biofluids across multiple test sites. *Toxicol. Sci.* 154, 115–125. doi: 10.1093/toxsci/kfw143
- Thomson, D. W., and Dinger, M. E. (2016). Endogenous microRNA sponges: evidence and controversy. *Nat. Rev. Genet.* 17, 272–283. doi: 10.1038/nrg.2016.20
- To, K. K. W., Robey, R. W., Knutsen, T., Zhan, Z., Ried, T., and Bates, S. E. (2009). Escape from hsa-miR-519c enables drug-resistant cells to maintain high expression of ABCG2. *Mol. Cancer Ther.* 8, 2959–2968. doi: 10.1158/1535-7163.MCT-09-0292
- Tosar, J. P., Gámbaro, F., Sanguinetti, J., Bonilla, B., Witwer, K. W., and Cayota, A. (2015). Assessment of small RNA sorting into different extracellular fractions revealed by high-throughput sequencing of breast cell lines. *Nucleic Acids Res.* 43, 5601–5616. doi: 10.1093/nar/gkv432
- Turchinovich, A., Weiz, L., Langheinz, A., and Burwinkel, B. (2011). Characterization of extracellular circulating microRNA. *Nucleic Acids Res.* 39, 7223–7233. doi: 10.1093/nar/gkr254
- Venkateswaran, S., Luque-González, M. A., Tabraue-Chávez, M., Fara, M. A., López-Longarela, B., Cano-Cortes, V., et al. (2016). Novel bead-based platform for direct detection of unlabelled nucleic acids through Single Nucleobase Labeling. *Talanta* 161, 489–496. doi: 10.1016/j.talanta.2016. 08.072
- Villarroya-Beltri, C., Gutiérrez-Vázquez, C., Sánchez-Cabo, F., Pérez-Hernández, D., Vázquez, J., Martin-Cofreces, N., et al. (2013). Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. *Nat. Commun.* 4:2980. doi: 10.1038/ncomms3980
- Volinia, S., Calin, G. A., Liu, C.-G., Ambs, S., Cimmino, A., Petrocca, F., et al. (2006). A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc. Natl. Acad. Sci. U.S.A.* 103, 2257–2261. doi: 10.1073/pnas.0510565103
- Wang, J.-X., Zhang, X.-J., Feng, C., Sun, T., Wang, K., Wang, Y., et al. (2015). MicroRNA-532-3p regulates mitochondrial fission through targeting apoptosis repressor with caspase recruitment domain in doxorubicin cardiotoxicity. *Cell Death Dis*. 6:e1677. doi: 10.1038/cddis.2015.41
- Wang, L.-J., He, C.-C., Sui, X., Cai, M.-J., Zhou, C.-Y., Ma, J.-L., et al. (2015). MiR-21 promotes intrahepatic cholangiocarcinoma proliferation and growth *in vitro* and *in vivo* by targeting PTPN14 and PTEN. *Oncotarget* 6, 5932–5946. doi: 10.18632/oncotarget.3465
- Wang, Q., Zhong, M., Liu, W., Li, J., Huang, J., and Zheng, L. (2011). Alterations of microRNAs in Cisplatin-resistant human non-small cell lung cancer cells (A549/DDP). *Exp. Lung Res.* 37, 427–434. doi: 10.3109/01902148.2011. 584263
- Wang, X., Jiang, H., Wu, W., Zhang, R., Wu, L., Chen, H., et al. (2017). An Integrating Approach for Genome-Wide Screening of MicroRNA Polymorphisms Mediated Drug Response Alterations. *Int. J. Genomics* 2017, 1–7. doi: 10.1155/2017/1674827
- Wang, Y., Wang, D., Xie, G., Yin, Y., Zhao, E., Tao, K., et al. (2017). MicroRNA-152 regulates immune response via targeting B7-H1 in gastric carcinoma. *Oncotarget* 8, 28125–28134. doi: 10.18632/oncotarget.15924
- Wei, J., Lv, L., Wan, Y., Cao, Y., Li, G., Lin, H., et al. (2015). Vps4A functions as a tumor suppressor by regulating the secretion and uptake of exosomal microRNAs in human hepatoma cells. *Hepatology* 61, 1284–1294. doi: 10.1002/hep.27660
- Wilson, R. C., Tambe, A., Kidwell, M. A., Noland, C. L., Schneider, C. P., Doudna, J. A., et al. (2015). Dicer-TRBP complex formation ensures accurate mammalian microRNA biogenesis. *Mol. Cell* 57, 397–407. doi: 10.1016/j.molcel.2014.11.030

- Wu, Q., Wang, C., Lu, Z., Guo, L., and Ge, Q. (2012). Analysis of serum genomewide microRNAs for breast cancer detection. *Clin. Chim. Acta* 413, 1058–1065. doi: 10.1016/j.cca.2012.02.016
- Wynendaele, J., Bohnke, A., Leucci, E., Nielsen, S. J., Lambertz, I., Hammer, S., et al. (2010). An Illegitimate microRNA Target Site within the 3' UTR of MDM4 Affects Ovarian Cancer Progression and Chemosensitivity. *Cancer Res.* 70, 9641–9649. doi: 10.1158/0008-5472.CAN-10-0527
- Xiong, D.-D., Lv, J., Wei, K.-L., Feng, Z.-B., Chen, J.-T., Liu, K.-C., et al. (2017). A nine-miRNA signature as a potential diagnostic marker for breast carcinoma: an integrated study of 1,110 cases. *Oncol. Rep.* 37, 3297–3304. doi: 10.3892/or.2017.5600
- Xu, W., Zhang, Z., Zou, K., Cheng, Y., Yang, M., Chen, H., et al. (2017). MiR-1 suppresses tumor cell proliferation in colorectal cancer by inhibition of Smad3mediated tumor glycolysis. *Cell Death Dis.* 8:e2761. doi: 10.1038/cddis.2017.60
- Xu, Z., Zeng, X., Tian, D., Xu, H., Cai, Q., Wang, J., et al. (2014). MicroRNA-383 inhibits anchorage-independent growth and induces cell cycle arrest of glioma cells by targeting CCND1. *Biochem. Biophys. Res. Commun.* 453, 833–838. doi: 10.1016/j.bbrc.2014.10.047
- Yanaihara, N., Caplen, N., Bowman, E., Seike, M., Kumamoto, K., Yi, M., et al. (2006). Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell* 9, 189–198. doi: 10.1016/j.ccr.2006.01.025
- Yekta, S., Shih, I., and Bartel, D. P. (2004). MicroRNA-Directed Cleavage of HOXB8 mRNA. Science 304, 594–596. doi: 10.1126/science.1097434
- Yoon, J.-H., Abdelmohsen, K., and Gorospe, M. (2014). Functional interactions among microRNAs and long noncoding RNAs. Semin. Cell Dev. Biol. 34, 9–14. doi: 10.1016/j.semcdb.2014.05.015
- Yu, S.-L., Chen, H.-Y., Chang, G.-C., Chen, C.-Y., Chen, H.-W., Singh, S., et al. (2008). MicroRNA signature predicts survival and relapse in lung cancer. *Cancer Cell* 13, 48–57. doi: 10.1016/j.ccr.2007.12.008
- Zhang, Q., Di, W., Dong, Y., Lu, G., Yu, J., Li, J., et al. (2015). High serum miR-183 level is associated with poor responsiveness of renal cancer to natural killer cells. *Tumor Biol.* 36, 9245–9249. doi: 10.1007/s13277-015-3604-y
- Zhao, J., Lei, T., Xu, C., Li, H., Ma, W., Yang, Y., et al. (2013). MicroRNA-187, down-regulated in clear cell renal cell carcinoma and associated with lower survival, inhibits cell growth and migration though targeting B7-H3. *Biochem. Biophys. Res. Commun.* 438, 439–444. doi: 10.1016/j.bbrc.2013.07.095

- Zhao, Y., Song, Y., Yao, L., Song, G., and Teng, C. (2017). Circulating microRNAs: promising biomarkers involved in several cancers and other diseases. DNA Cell Biol. 36, 77–94. doi: 10.1089/dna.2016.3426
- Zheng, J., Deng, J., Xiao, M., Yang, L., Zhang, L., You, Y., et al. (2013). A sequence polymorphism in miR-608 predicts recurrence after radiotherapy for nasopharyngeal carcinoma. *Cancer Res.* 73, 5151–5162. doi: 10.1158/0008-5472.CAN-13-0395
- Zhou, B., Xu, H., Xia, M., Sun, C., Li, N., Guo, E., et al. (2017). Overexpressed miR-9 promotes tumor metastasis via targeting E-cadherin in serous ovarian cancer. *Front. Med.* 11, 214–222. doi: 10.1007/s11684-017-0518-7
- Zhou, H., Arcila, M. L., Li, Z., Lee, E. J., Henzler, C., Liu, J., et al. (2012). Deep annotation of mouse iso-miR and iso-moR variation. *Nucleic Acids Res.* 40, 5864–5875. doi: 10.1093/nar/gks247
- Zhu, H.-T., Dong, Q.-Z., Wang, G., Zhou, H.-J., Ren, N., Jia, H.-L., et al. (2012). Identification of suitable reference genes for qRT-PCR analysis of circulating microRNAs in hepatitis B virus-infected patients. *Mol. Biotechnol.* 50, 49–56. doi: 10.1007/s12033-011-9414-6
- Zhu, H., Zhou, X., Ma, C., Chang, H., Li, H., Liu, F., et al. (2015). Low Expression of miR-448 Induces EMT and promotes invasion by regulating ROCK2 in hepatocellular carcinoma. *Cell. Physiol. Biochem.* 36, 487–498. doi: 10.1159/000430114
- Zhu, S., Si, M.-L., Wu, H., and Mo, Y.-Y. (2007). MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). J. Biol. Chem. 282, 14328–14336. doi: 10.1074/jbc.M611393200

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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microRNAs and Acute Myeloid Leukemia Chemoresistance: A Mechanistic Overview

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Up until the early 2000s, a functional role for microRNAs (miRNAs) was yet to be elucidated. With the advent of increasingly high-throughput and precise RNA-sequencing techniques within the last two decades, it has become well established that miRNAs can regulate almost all cellular processes through their ability to post-transcriptionally regulate a majority of protein-coding genes and countless other non-coding genes. In cancer, miRNAs have been demonstrated to play critical roles by modifying or controlling all major hallmarks including cell division, self-renewal, invasion, and DNA damage among others. Before the introduction of anthracyclines and cytarabine in the 1960s, acute myeloid leukemia (AML) was considered a fatal disease. In decades since, prognosis has improved substantially; however, long-term survival with AML remains poor. Resistance to chemotherapy, whether it is present at diagnosis or induced during treatment is a major therapeutic challenge in the treatment of this disease. Certain mechanisms such as DNA damage response and drug targeting, cell cycling, cell death, and drug trafficking pathways have been shown to be further dysregulated in treatment resistant cancers. miRNAs playing key roles in the emergence of these drug resistance phenotypes have recently emerged and replacement or inhibition of these miRNAs may be a viable treatment option. Herein, we describe the roles miRNAs can play in drug resistant AML and we describe miRNA-transcript interactions found within other cancer states which may be present within drug resistant AML. We describe the mechanisms of action of these miRNAs and how they can contribute to a poor overall survival and outcome as well. With the precision of miRNA mimic- or antagomir-based therapies, miRNAs provide an avenue for exquisite targeting in the therapy of drug resistant cancers.

Keywords: microRNA, acute myeloid leukemia, drug resistance, RNA therapy, daunorubicin, cytarabine, chemotherapy

INTRODUCTION

Despite rapid progress in our understanding of the cellular and molecular etiology of cancer and the development of countless new anticancer agents and therapeutic strategies, little has changed in the treatment of many cancers over the last few decades. For instance, the standard of care for acute myeloid leukemia (AML) which consists of combined cytarabine and anthracycline therapy has been fundamentally unchanged for the past 30 years (1). The long-standing presence of this strategy is owed to its effectiveness with a mean response rate up to 70% and a lack of superior strategies for most AML subtypes (2, 3). New targeted therapy strategies including monoclonal

OPEN ACCESS

Edited by:

Angela Re, Istituto Italiano di Tecnologia, Italy

Reviewed by:

Bernd Groner, Georg Speyer Haus, Germany Gabriele Multhoff, Technische Universität München, Germany

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Specialty section:

This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: 17 August 2017 Accepted: 11 October 2017 Published: 30 October 2017

Citation:

Gabra MM and Salmena L (2017) microRNAs and Acute Myeloid Leukemia Chemoresistance: A Mechanistic Overview. Front. Oncol. 7:255. doi: 10.3389/fonc.2017.00255 antibodies and small molecule inhibitors are constantly being developed; however to date, none of these targeted therapies have proven more effective than the standard of care with the exception of the use of all-trans retinoic acid (ATRA) in acute promyelocytic leukemia (APL) which has become nearly curable in the majority of cases (4).

Notwithstanding, drug resistance is a major therapeutic challenge in the treatment of AML. Failure of initial therapy can be observed in up to 40% of AML patients, and even when initial therapy is effective, up to 70% of patients eventually succumb to their disease due to aggressive relapse within 5 years (5–7).

The cause of poor long-term survival is primarily drug resistance, which is either intrinsic in patients that fail initial therapy or acquired after chemotherapy through selection or acquisition of mutations (8). Indeed, relapsed AML is often composed of cells that have distinct molecular and cytogenetic characteristics leading to deficiencies or perturbations in various pathways associated with therapeutic resistance including DNA damage response and drug targeting, cell cycling, cell death, and drug trafficking pathways due to increased or altered drug targets are commonly observed (**Figure 1**) (8–10). Consequently, outcomes of relapsed disease are abysmal, which highlights a desperate need for novel therapeutic approaches with potential to overcome or prevent therapeutic resistance.

Non-Coding RNAs (ncRNAs) in AML Therapy Resistance

Among several emerging functions, ncRNAs can act as modulators of gene expression through roles in epigenetics, transcription, translation, as well as homology-dependent post-translational regulation of mRNA transcripts (11). The most widely recognized class of ncRNAs are the microRNAs (miRNAs), which are small 18–24 bp dsRNAs that use cellular RNA-interference machinery to suppress protein expression levels by both degrading or blocking translation of mRNA transcripts (12, 13). It has been convincingly demonstrated in numerous cancers that miRNAs can (1) promote or suppress the development of cancer, (2) be of value in prediction of treatment responses and disease prognosis, and (3) be perturbed as a response to chemotherapy (14, 15).

This review is focused on the small ncRNAs, the miRNAs, in drug resistance; however, long non-coding RNAs (lncRNAs) which are typically >200 bp in length and comprise a large proportion of cellular transcribed RNA have numerous emerging functions in AML pathogenesis (16). lncRNA dysregulation in AML have been reported to have consequences for various cellular processes such as proliferation, survival, and migration (17-19) and have been associated with poor clinical outcome (20-23). Furthermore, lncRNAs signatures associated with well-defined cancer types (24). For instance, Homeobox (HOX) transcript antisense RNA (HOTAIR) and HOX antisense intergenic RNA myeloid 1 (HOTAIRM1) are substantially upregulated in AML. It was shown in both cell lines and patient samples that the upregulation of HOTAIR is specifically associated with indirect upregulation of c-kit through sponging of miR-193 (20). Recently, doubt has been raised over the prognostic value of HOTAIR by Sayad et al.; however, in case-control samples, there was a trend toward clinical significance of HOTAIR (25). HOTAIRM1, on the other hand, is thought to behave as an endogenous miRNA-sponge for miR20a, miR-20a/106b, and miR-125b and prevents targeting of ULK1, E2F1, and DRAM2 as demonstrated in luciferase reporter assays



(26, 27). In drug resistant AML, however, little is known about the dysregulation of lncRNAs and their respective mechanisms of function.

miRNA Biology

miRNA derive from the transcription of miRNA loci on genomic DNA by RNA polymerases which create a ~80 nt long transcript primary (pri)-miRNA that are then spliced, capped, polyadenylated, and packaged similar to long-stranded transcripts (28). Further splicing and processing by DROSHA and PASHA transform the pri-miRNA into pre-miRNA. When pre-miRNA exits the nucleus through the function of exportin-5, it is folded into a self-bound hairpin secondary structure known as a "stem-loop" (28, 29). At this stage, the 70-100 nt which make up this stem-loop pre-miRNA is cleaved by a cytoplasmic RNase III such as Dicer into a dsRNA dimer which rapidly breaks down into two strands (29). Depending on the stability of the single strand of miRNA either strand can be active (30-32). A functional third miRNA formed from this complex is thought to originate from the loop region, known as loop-miRNA (33, 34). Next, single-stranded mature miRNAs 19-25 nt in length, bind to the argonaute (Ago) proteins which are one member of a complex of proteins collectively known as the RNA-induced silencing complex (RISC) (35, 36).

Guided by miRNAs, Ago and the RISC move to miRNA recognition elements on mRNA which are commonly, but not limited to non-coding 3'-untranslated regions (3'-UTR) (37, 38). Unlike siRNA, miRNA do not require perfect complementary binding; and only binding to the seed-region appears to be a requirement in most cases (39, 40). This comparatively less stringent binding compared to siRNA allows miRNA to regulate the expression levels of multiple RNA transcripts through target promiscuity (39). Once bound to a target, the endonuclease activity of the RISC is activated via the slicer activity of Ago1 (28, 41). Following cleavage, the entire strand is rapidly degraded by endonucleases. Multiple interactions between miRNA and mRNA transcripts are the basis of complex cellular regulatory networks whereby miRNAs control the majority of all protein-coding genes and countless other non-coding genes. In cancer, miRNAs have been demonstrated to play critical roles by modifying or controlling all major hallmarks of cancer including cell division, self-renewal, apoptosis, and DNA damage response among others (42-47).

To date, no comprehensive study examines the role of miRNAs in drug resistant AML. Herein, we describe the miRNAs that have been examined in clinical samples and we highlight miRNA that have been examined mechanistically. Furthermore, we discuss potential miRNA-binding partners of important AML drug resistance machinery found within other cancers to guide future research.

AML CHEMOTHERAPY, DNA DAMAGE, AND miRNA DYSREGULATION

The most common treatment for AML includes an anthracycline like daunorubicin and a nucleoside analog like cytarabine in the (7 + 3) regimen where daunorubicin is administered IV for the

first 3 days concomitantly to the IV infusion of cytarabine for 7 days (48, 49). The 7 + 3 regimen is termed *induction therapy* (because of its intent is to induce remission) and has been in place since the 1960s (50). The aim of induction therapy is achieving complete remission (CR), defined clinically as myeloid blast counts in the bone marrow below 5% or minimum residual disease status (49). Efforts to enhance this regimen by escalating dose or adding a third drug has only resulted in increased toxicity with minimal improvement in patient survival. Upon achieving CR, treatment can be consolidated using high doses of cytarabine. Unfortunately, despite undergoing such aggressive chemotherapy regimen with all the associated toxicities and side effect, many patients still relapse within 5 years (48, 49). This is in part due to lack of targeting of leukemic-initiating cells, selection of rare pre-existing resistant AML clones, or the mutagenic effects of the treatments, all of which increase the probability of generating more aggressive AML.

Fundamentally, drug resistance occurs in cells which can evade or withstand treatment. While tumor heterogeneity may explain selection of a pre-existing clone with a favorable mutation, acquired drug resistance is generally defined as the ability of a cell to resist response to the drug to which it was initially responsive. Acquired resistance may be achieved through multiple dosing of the same drug or through as little as a single dose may be explained by the mechanism of drug action.

For instance, anthracyclines used to treat AML such as daunorubicin, doxorubicin, and idarubicin, intercalate DNA, and stall proper DNA replication events (51). Anthracyclines can also target topoisomerase II which normally binds to the scaffold/matrix-associated protein region (S/MAR) to resolve DNA supercoils (52, 53). By binding to topoisomerase II in its open DNA-bound conformation, a stall occurs which can lead to a double-strand break. These double-strand breaks may be fixed aberrantly through non-homologous end joining which can lead to gene mutation. One common mutation in AML, t4:11, occurs at an S/MAR (54-56). This mutation has also been shown to occur in significant proportions in secondary AML patients as well (56). Loss or translocation of the S/MAR may further modulate various miRNAs. As demonstrated by Chavali et al., protein binding to the S/MAR induces histone acetylation that leads to the increased expression of the miR-17-92 cluster and the miRNAs miR-221, miR-93, miR-17, and let-7b (57). As DNA damage is most likely to occur in these regions due to daunorubicin, it is likely that dysregulation of miRNA expression can be due to daunorubicininduced damage directly.

Cytarabine, on the other hand, is a cytosine analog that terminates translation and replication events. It primarily inhibits cells in S phase (DNA replication) but can also inhibit the progression from G_1 phase into S phase (58, 59). It is known that cytarabine is first metabolized into the triphosphate bound product by deoxycytidine kinase (DCK) and other nucleoside analog enzymes whereby it can then incorporate into the DNA. It is shown that its incorporation can often lead to extensive DNA damage including chromatid breaks (60). Stalled replication forks can also lead to bypass mechanisms such as translesion synthesis (61). This method of DNA replication is more error prone and can lead to mutation events as well. Each of these mechanisms can be demonstrated to have direct or indirect consequences for miRNA function.

As described with both drugs, genotoxic effects can lead to breaks that are then repaired using homologous or non-homologous repair mechanisms leading to miRNA alterations and the upregulation of drug resistance mechanisms. Conversely, miRNA which regulate these associated pathways may also contribute to drug resistance when perturbed by increasing tolerance to DNA damage. For instance, ataxia telangiectasia mutated (ATM) is an important DNA damage sensing and DNA damage response protein that has been demonstrated to contribute to chemoresistance (62). In experiments conducted in leukemic HL60, NB4, and K562 cell lines, it was found that the overexpression of miR-181a leads to increased cell proliferation and increased cell cycling through ATM targeting and downregulation (63). Similarly, miR-128 was reported to affect the propensity for DNA damage in AML cells. In a study conducted in HL60 cells, it was observed that the transfection of miR-128 led to increased apoptosis, drug sensitivity, and the amount of DNA damage tolerated; however, the mechanism is yet to be elucidated (64). miR-128 is thought to be upregulated in various cancers, but its levels are reduced in AML cells carrying NPM1 mutations (Figure 2; Table 1) (65, 66).

Recently, Lai et al. identified a mechanism by which *miR-128* is likely targeting *Rad51* directly and leading to the increased DNA damage response in OCI-AML3 and MV4-11 AML cell lines. In these experiments, *miR-128* led to the sensitization of these cell lines to sapacitabine, a novel oral nucleoside analog prodrug (46). In other cancers, *Rad51* has been shown to be a direct target of other miRNAs such as *miR-506*, *miR-103*, and *miR-107*. Clinical significance in chemoresistant high-grade serous ovarian cancers was established for *miR-506* while a miRNA mimic library screen revealed *miR-103* and *miR-107* as strong drug resistance contributors in the U2OS cell line, a model for osteosarcoma (**Figure 2**; **Table 1**) (45, 47). To date, proteins that are thought to be integral to the activity of anthracyclines and nucleoside analogs such as topoisomerase II and the DNA polymerases are not known to interact with miRNAs. However, topoisomerase II has been demonstrated to be downregulated in drug resistant subtypes of AML (67, 68). miRNA targeting may prove to be a mechanism of topoisomerase II downregulation, but more research is required to establish important links of miRNA-induced dysregulation of DNA repair machinery in drug resistant AML.

miRNA AND CELL CYCLING IN AML RESISTANCE

The cell cycle represents a series of events that require the input of various checkpoint proteins known as cyclins and cyclin-dependent kinases (CDK) to proceed into division (69). These proteins, in turn, receive input from DNA damage sensing proteins such as ATM/ATR and CHK1/2 (70, 71). The majority of rapidly dividing cancer cells can be found in one of two major phases: the interphase; which consists of G1, S phase (DNA replication) followed by G2; and the M phase, where cells undergo mitosis. Cell cycle manipulation can be a drug resistance mechanism as cell cycle arrest at different phases or quiescence can lead to chemotherapy





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Protein	miRNA miRNA status in drug resistance		Sample/cancer	Mechanism	Reference	
ATM	miR-181a	Overexpressed	HL60, NB4, K562/AML, CML	ATM downregulation leads to uninhibited growth	Liu et al. (63)	
Rad51	miR-128	Overexpressed	OCI-AML3, MV4-11/AML	Rad51 downregulation leads to increased DNA damage response	Lai et al. (46)	
Rad51	miR-506	Overexpressed	Patient samples/high grade serous ovarian cancer	Rad51 downregulation leads to increased DNA damage response	Liu et al. (47)	
Rad51 Rad51	miR-103 miR-107	Overexpressed Overexpressed	U2OS/osteosarcoma U2OS/osteosarcoma	Rad51 downregulation leads to increased DNA damage response Rad51 downregulation leads to increased DNA damage response	Huang et al. (45) Huang et al. (45)	

TABLE 1 | miRNAs demonstrated to directly bind to DNA damage regulatory proteins

miRNAs, microRNAs; ATM, ataxia telangiectasia mutated; AML, acute myeloid leukemia.

evasion; however, increased proliferation can also contribute to resistance (72–75).

The process of cell division begins in G1 by the duplication of various proteins, chromatin remodeling, and the verification that the DNA is free of DNA damage. In a healthy cell, if substantial levels of DNA damage are found, ATM/ATR become activated leading to eventual CDK2 inhibition and arrest at the G1/S checkpoint through p21 signaling, where the mechanisms of action of many miRNAs have been elucidated (76). *CDK2* has been found to be inhibited by *miR-638*, where it was demonstrated in HL-60, NB4, and THP-1 that an upregulation of *miR-638* leads to a reduction in cell cycling and a differentiation block in APL (77). The differentiation block was found to occur at the G1/S checkpoint and differentiation inducers like ATRA were found to be more effective in cells with *miR-638* downregulation (77).

CDK2 has been demonstrated to be a target of various miRNAs in cancer including *miR-885-5p*, *miR-372*, and *miR-188* (**Figure 3**; **Table 2**). In contrast to *miR-638* in AML, *miR-885-5p* was demonstrated to play a tumor suppressive role in neuroblastoma by inhibiting *CDK2* and promoting senescence and apoptosis (78). *miR-372* demonstrated targeting of both *CDK2* and cyclin A1, which is highly expressed during S phase. Like *miR-885-5p*, *miR-372* was demonstrated to play a tumor suppressive role as demonstrated in HeLa cells and tissue samples of cervical cancer (79). *miR-188* was demonstrated to directly bind several genes which play a role in cycling such as cyclin D1, cyclin D3, cyclin A2, cyclin E1, CDK2, and CDK4 with varying degrees and it demonstrated modest knockdown of CDK2 relative to the other genes (80). In this study, it was found that the arrest occurs at the G1/S transition and that *miR-188* plays a tumor suppressive role (80).

Other miRNAs such as the *miR-16* family members famously known for downregulation of *BCL2* (**Figure 4**) are also shown to simultaneously directly target several cycling genes such as *cyclin D1, cyclin D3, cyclin E1*, and *CDK6* (**Figure 3**; **Table 2**). As demonstrated in the A549 cell line by Liu et al., this targeting and likely the targeting of downstream effectors leads to the arrest in G1 and at G1/S, a phenomenon observed by others (81, 85, 86). The targeting of *Cyclin E* has since been demonstrated as playing an important role in certain cancers such as cervical cancer and breast cancer (86–89). The *miR-15* and *miR-16* family may be response elements of E2F1 and as such, may be contributing to a feedback mechanism (90).

The transcription factor *E2F* family may also be a target of miRNAs. *E2F7*, a transcriptional response element gene implicated in cell cycling, is downregulated by *miR-26a* in AML (82).

This inhibition in turn reduces *c-myc* transcriptional activation and sequential *miR-17-92* reduced transcription, which has previously been implicated in promoting a differentiation block (82, 91, 92). When active, *miR-17-92* members may be in part directly targeting p21 and promoting cycling, as demonstrated in MLL transformed leukemic cells by Wong et al. (83). The inhibition of *E2F7* may lead to a reduction of miRNAs involved in proliferation such as *miR-25*, *miR-26a*, *miR-27b*, *miR-92a*, and *miR-7* thus behaving as a regulatory mechanism (93).

In other instances, miRNAs can behave as direct inhibitors of their own transcriptional repressor thus behaving as autoregulatory elements. It has been demonstrated by Pulikkan et al. that this is the case for *miR-223* and *E2F1* regulation (84). E2F1, an important response element in G1/S, can repress transcription of *miR-223* which in turn can repress *E2F1* (84, 94, 95). The differentiation block observed in APL may be further exacerbated by miRNAs like *miR-223* (**Figure 3**; **Table 2**). The complexity of interactions within miRNA–mRNA networks demonstrates the need for further analyses elucidating the major pathways of feedback and feedforward signaling.

CELL DEATH AND miRNA

In the majority of blast cells that experience sufficient levels of DNA damage upon chemotherapy, programmed cell death (PCD) will become activated. PCD may take the form of apoptosis or autophagy. Apoptosis is characterized by specific changes in morphology such as cell shrinkage and pyknosis (96). Autophagy, on the other hand, is characterized by cellular degradation and the re-introduction of catabolic products into anabolic processes (97, 98). Autophagy can play both a detrimental and a beneficial role in cancer cells and it can also contribute to the generation of leukemia (98–100). Apoptosis, on the other hand, while it is an essential component of normal cell turnover, only its downregulation will often be a major contributor for aberrant cancer growth and its further suppression can lead to drug resistance.

miRNA and BCL2 Family Members

miRNA-associated dysregulation of apoptosis has been observed in drug resistant AML cells. Given that AML is often characterized by aberrant DNA repair and maintenance, tolerance of these damaged lesions is observed through the downregulation of pro-apoptotic markers and damage sensors, or the upregulation of antiapoptotic factors. Of the apoptosis-related families, the BCL2 protein family is the most well described in miRNA



FIGURE 3 | microRNAs (miRNAs) can dysregulate cell cycling mechanisms by dysregulating several phases of the cell cycle, but the majority of known targeting occurs at the G1 and S phases and at the G1/S transition. The downregulation of the cyclins that would normally signal for cell cycling to proceed can be downregulated. Cyclin D1 and cyclin D3 can be dysregulated by miR-188 and miR-16, cyclin E1 can be knocked down by miR-16 while cyclin E2 can be downregulated by miR-17-92 and finally, cyclin A1 and A2 are downregulated by miR-188 and miR-372, respectively. The cyclin-dependent kinases (CDKs) are also adjustable through miRNA targeting and their targeting reduces cycling as well. CDK2 can be downregulated by miR-638, miR-885-5p, miR-372, and miR-188; CDK4 is downregulated by miR-188, and CDK6 is downregulated by miR-16. Effector proteins such as E2F1, E2F7, and p21 can also be downregulated by miRNAs to lead to differentiation blocks. They can be targeted by miR-223, miR-26a, and miR-17-92, respectively.

TABLE 2 | Cell cycling gene dysregulations induced by miRNA binding.

Protein	miRNA	miRNA status in drug resistance	Sample/cancer	Mechanism	Reference	
CDK2	miR-638	Overexpressed	HI 60 NB4 THP-1/API	CDK2 downregulation prevents G1/S progression	Lin et al. (77)	
CDK2	miR-885-5p	Reduction	Patient samples, SH-EP, KELLY, IMR32, SK-N-BE(2)c, and HDN 33 cell lines/ neuroblastoma	Reduced cycling promotes senescence	Afanasyeva et al. (78)	
CDK2	miR-372	Reduced	HeLa/cervical cancer	Reduced cycling prevents cell growth	Tian et al. (79)	
CDK2	miR-188	Reduced	CNE cells/nasopharyngeal carcinoma	G1/S arrest prevents cell cycling	Wu et al. (80)	
Cyclin A1	miR-372	Reduced	HeLa/cervical cancer	Reduced cycling prevents cell growth	Tian et al. (79)	
Cyclin D1	miR-188	Reduced	CNE cells/nasopharyngeal carcinoma	G1/S arrest prevents cell cycling	Wu et al. (80)	
Cyclin D1	miR-16	Reduced	A549/lung cancer	G1 and G1/S arrest reduces proliferation	Liu et al. (81)	
Cyclin D3	miR-188	Reduced	CNE cells/nasopharyngeal carcinoma	G1/S arrest prevents cell cycling	Wu et al. (80)	
Cyclin D3	miR-16	Reduced	A549/lung cancer	G1 and G1/S arrest reduces proliferation	Liu et al. (81)	
Cyclin A2	miR-188	Reduced	CNE cells/nasopharyngeal carcinoma	G1/S arrest prevents cell cycling	Wu et al. (80)	
Cyclin E2	miR-188	Reduced	CNE cells/nasopharyngeal carcinoma	G1/S arrest prevents cell cycling	Wu et al. (80)	
Cdk4	miR-188	Reduced	CNE cells/nasopharyngeal carcinoma	G1/S arrest prevents cell cycling	Wu et al. (80)	
Cdk6	miR-16	Reduced	A549/lung cancer	G1 and G1/S arrest reduces proliferation	Liu et al. (81)	
Cyclin E1	miR-16	Reduced	A549/lung cancer	miR-16 loss may lead to G1 and G1/S arrest reduces proliferation	Liu et al. (81)	
E2F7	miR-26a	Reduced	Patient samples, HL60, U937/APL	Downregulation of E2F7 reduces progression	Salvatori et al. (82)	
P21	miR-17-92	Overexpressed	MLL transformed cells/AML	Downregulation of p21 promotes non-differentiation	Wong et al. (83)	
E2F1	miR-223	Overexpressed	Patient samples, K562, U937/AML, CML	E2F1 downregulation contributes to non-differentiated cell cycle progression	Pulikkan et al. (84)	

miRNAs, microRNAs; APL, acute promyelocytic leukemia; CDK, cyclin-dependent kinase; AML, acute myeloid leukemia.



proteins part of the intrinsic or extrinsic pathway can respond to minNAs to innibit apoptosis or reduce their regulatory signaling of apoptosis. BoLL2, an antiapoptosis gene, will gain signaling when the associated miRNAs such as miR-156, miR-15a/b, miR-16, miR-125b-5p, and miR-139-5p are lost in the drug resistant cell. The gain of BAK1 miRNA targeting through miR-125b or the gain of BIM targeting through miR-32 will lead to the same effect as well. The Fas-ligand can also be suppressed by miR-149-5p thus ending extrinsic apoptosis signaling. P53 suppression through miR-125b and miR-504 will prevent apoptosis as well. Dysregulating autophagy through increased targeting may increase drug resistance through the binding of miR-125b and miR-101 on Atg4D. miR-30a is known to inversely correlate with Beclin1 and Atg5 in leukemia cell lines, but less is known about the outcome of this interaction.

dysregulation driven in AML. The BCL2 protein itself is commonly considered as a crucial anti-apoptosis gene as it inhibits the mitochondrial pro-apoptotic proteins such as Bak and Bax. While it can be dysregulated or mutated in cancers, it is observed that dysregulation may also occur in the development of drug resistance. Many miRNAs including *miR-15/miR-16*, *miR-125b-5p*, *miR-139-5p*, *miR-145*, and *miR-181a* have been shown to suppress the translation of *BCL2* and decrease the propensity for activation of apoptosis (**Figure 4**; **Table 3**).

Of the BCL2-targeting miRNAs, only miR-181a has been shown to do so in AML cells. In K562 CML cells, it was demonstrated by Li et al. that the drug resistant form had 40% of the miR-181a levels found in the parental cell line. When the parental cells were transfected with a miR-181a inhibitor, resistance developed (101). In a separate study conducted by Bai et al. in cytarabine resistant HL60, it was found that the resistance phenotype can be also be attributed to reduced BCL2 targeting by miR-181a, whereas its ectopic expression sensitizes the cells to treatment to cytarabine (102). Other studies of miR-181a in AML have also demonstrated that it is often downregulated in drug resistant AML, that it can serve as an independent prognostic marker and potentially modulate the interaction with natural killer cells as well (118-122). In molecular poor risk group AML with FLT3-ITD mutations, it was demonstrated that high *miR-181a* also strongly predicted better survival (123).

The *miR-15/16* have been shown to suppress BCL2 in multiple cancers including gastric cancer, breast cancer, and glioma and

the loss of this locus has also been observed in CLL (124–127). Xia et al. demonstrated that *miR-15b* and *miR-16* are lost in vincristine resistant SGC7901 cells, a gastric cell line (103). Cittelly et al. later demonstrated that in a common mutation of the *HER2* gene, *HER* Δ *16*, representative of 30% of HER2 dysregulations in estrogen receptor positive breast cancers, the downregulation of *miR-15a* and *miR-16* is observed (104). In MCF-7 cells ectopically expressing this mutant variant, it was shown that tamoxifen resistance may be in part due to the reduced regulation of *BCL2* by *miR-15a* and *miR-16*, which leads to apoptosis evasion (104). In glioma cells that are resistant to temozolomide, it was demonstrated that the loss of *miR-16* specifically can contribute to resistance in the U251MG/Temozolomide resistant cell line and that the blocking of *miR-16* in the temozolomide sensitive AM38 cell line increased resistance by de-repressing *BCL2* (105).

In a genome-wide gene expression analysis of gallbladder cancer clinical samples, miR-125b-5p was found to be statistically downregulated in cisplatin resistant patients (N = 6). Analyses demonstrated that this miRNA can directly bind to the 3'UTR of *BCL2*, contribute to cisplatin desensitization, and increase tumor formation in mice (106). A similar analysis of patient samples conducted in colorectal cancer demonstrated that miR-139-5p inhibits the epithelial-to-mesenchymal transition and contributes to drug resistance by downregulating *BCL2* (107). Bioinformatic studies also demonstrate binding of other miRNAs to the *BCL2* mRNA as putative mechanisms of miRNA-induced downregulations. For instance, bioinformatic analysis of miR-451

TABLE 3 | The interactions of miRNAs with cell death-related proteins.

Protein miRNA		miRNA status in drug resistance	Sample/cancer	Mechanism	Reference	
BCL2	miR-181a	Reduced	K562/CML HL60/APL	Reduced miR-181a leads to increased apoptosis suppression Cytarabine resistance presents with reduced miR-181a expression and apoptosis suppression	Li et al. (101) Bai et al. (102)	
BCL2	miR-15b	Reduced	SG7901 cells/gastric cancer	SG7901 cells/gastric cancer Reduced miR-15b expression leads to BCL2 overexpression and apoptosis suppression		
BCL2	miR-16	Reduced	SG7901 cells/gastric cancer ER∆16 MCF7/breast cancer U251MG, AM38	G7901 cells/gastric cancer Reduced miR-16 expression leads to BCL2 overexpression and apoptosis suppression J251MG, AM38		
BCL2	miR-15a	Reduced	HER∆16 MCF7/breast cancer	Downregulated miR-15a leads to BCL2 overexpression	Cittelly et al. (104)	
BCL2	miR-125b-5p	Reduced	Patient samples/gallbladder cancer	Downregulation of miR-125b-5p disinhibits BCL2 and leads to anti-apoptosis	Yang et al. (106)	
BCL2	miR-139-5p	Reduced	Colorectal cancer	Downregulation of miR-139-5p leads to BCL2 disinhibition and anti-apoptosis	Li et al. (107)	
BAK1	miR-125b	Overexpressed	HL60, NB4/APL NB4, K562/CML MDA-MB-435, MDA-MB-231/breast cancer HMLE/breast cancer PC-3466C, LNCaP/prostate cancer	1/APL Suppression of Bak1 leads to apoptosis avoidance 2/CML 435, MDA-MB-231/breast ast cancer 2, LNCaP/orostate cancer		
BIM	miR-32	Overexpressed	LNCaP/prostate cancer	Downregulation of <i>BIM</i> leads to apoptosis evasion		
p53	miR-125b	Overexpressed	SH-SY5Y/neuroblastoma	Direct binding to the <i>P</i> 53 by <i>miR-125b</i> leads to further inhibition of apoptosis response	Le et al. (112)	
p53	miR-504	Overexpressed	HCT116 (colorectal carcinoma), H460 (large cell lung cancer), MCF-7 (ER + breast cancer), U2OS (osteosarcoma), A498 (kidney carcinoma)	Direct binding by <i>miR-504</i> reduces the propensity of a cell to enter apoptosis	Hu et al. (113)	
Fas-ligand	miR-149-5p	Overexpressed	THP-1/AML	Downregulation of the Fas-ligand reduces activation of the extrinsic apoptosis pathway	Tian and Yan (114)	
Beclin 1	miR-30a	Unknown	K562/CML	Inverse correlation found	Yu et al. (115)	
ATG5	miR-30a	Unknown	K562/CML	Inverse correlation found	Yu et al. (115)	
ATG4D	miR-125b1	Overexpressed	NB4/APL	Inhibition of autophagy	Zeng et al. (116)	
ATG4D	miR-101	Overexpressed	MCF7/breast cancer	Inhibition of autophagy contributed to tamoxifen resistance	Frankel et al. (117)	

miRNAs, microRNAs; APL, acute promyelocytic leukemial; AML, acute myeloid leukemia.

through miRBase and miRanda identified it as an inhibitor of *BCL2* (128). Similarly, in paclitaxel-resistant breast cancer, it was demonstrated that *miR-451* may also inhibit BCL2.

The BCL2 antagonist/killer 1 (Bak1) protein is upregulated in the progression of apoptosis in normal cells; in drug resistant cancers, however, it is observed that there is *Bak1* suppression through *miR-125b* binding. The binding of *miR-125b* to the *Bak1* transcript was initially examined in the prostate cancer cell lines PC-346C and LNCaP in the context of androgen-independent signaling, but effect on drug resistance was not examined (110). In APL, *miR-125b* was demonstrated to be clinically relevant, in CML mice models, and it was further demonstrated that direct suppression occurs in the cell lines NB4, HL60, and K562 (7, 108). A similar link between *miR-125b* and *Bak1* was established in MDA-MB-435 and MDA-MB-231 where it was demonstrated that *miR-125b* is capable of *Bak1* suppression in Taxol resistant cells (109). The mechanism of *miR-125b* upregulation was further elucidated to be through Wnt signaling and specifically through Snail binding; an upregulation thought to also occur in cancer stem cells (129).

The Bcl-2-like protein 11, also known as, BIM, has been demonstrated to be a direct target of *miR-32* in a previous study in LNCaP prostate cancer cells. This pro-apoptotic protein can be downregulated by *miR-32* and consequently lead to resistance and increased cell proliferation (130). Studies in the AML cell lines HL60 and U937 also demonstrated an inverse correlation between *miR-32* and *BIM* (111).

miRNA and P53 Regulation

The tumor-suppressor protein p53, often referred to as guardian of the genome is dysregulated in 50% of all cancers. In *wild-type* cells, p53 is often suppressed and destabilized by mdm2, mdm4, and mdmx which behave like E3 ligases, marking P53 by ubiquitination for degradation. Phosphorylation of p53 by ATM leads

to its stabilization and release from the mdm protein family. p53 can then behave as a transcription factor by activating apoptosisrelated genes (both intrinsic and extrinsic), cell cycle arrest related genes or DNA repair related genes and it can directly bind to the mitochondria to participate in membrane permeabilization (131, 132).

P53 has been identified as a direct target of miRNA binding by miR-125b and miR-504. miR-125b was shown to directly decrease P53 transcript levels and consequently decrease apoptosis response to irradiation in neuroblastoma cells and in lung fibroblasts (Figure 4; Table 3) (112). miR-504 was first computationally predicted and then demonstrated in various cell lines including HCT116 (colorectal carcinoma), H460 (large cell lung cancer), MCF-7 (ER + breast cancer), U2OS (osteosarcoma), and A498 (kidney carcinoma) cells to directly target the 3'UTR of P53 (113). P53 is also importantly downregulated through indirect ways by miR-34a, which is thought to play a crucial role in P53's pro-apoptotic abilities (133, 134). It has been demonstrated that miR-34a can indirectly increase P53 by inhibiting P53 negative regulators such as SIRT1 in colon cancer as demonstrated by Yamakuchi et al. and likely through binding of mdm4 as well, as predicted bioinformatically (135-137).

Furthermore, it has been demonstrated that P53 transcriptionally activates miR-34a which in turn modulates and fine tunes P53's signal (134). Consequently, the relationship between miR-34a and P53 is context dependent as the mutation status of P53 can influence the response and outcome of miR-34a activity (138). In the study conducted by Rücker et al., it was found that P53 alterations were the most common molecular lesions which coincided with complex karyotypes in AML (138). Low miR-34a and P53 alterations were shown to have the poorest clinical outcome in terms of drug resistance and survival. The low expression was shown to also correlate with a specific gene expression profile consisting of P53-associated proteins. In complex karyotypes that did not have a P53 alteration, high miR-34a predicted a poor overall survival while loss of P53 and high miR-34a predicted better outcome (138). The interplay between miR-34a and P53 demonstrates that the same miRNA can have opposite effects depending on the mutation status of the associated mRNA and highlights the necessity of describing miRNA activity in relation to the activity of associated mRNA.

Other Apoptosis-Related Proteins

For the apoptotic extrinsic pathway, it was reported by Tian et al. that *miR-149-5p* can directly downregulate the *Fas-ligand* and reduce the levels of the apoptosis effector proteins caspase-8, caspase-2, and caspase-3; however, no effect on drug resistance is demonstrated (114). It is possible that *miR-181a* and *miR-21* can suppress the Fas-ligand in cancers as they are shown to interact with the Fas-ligand in bone marrow-derived mesenchymal cells and cardiomyocytes, respectively (139, 140). The binding of miR-NAs to caspases has also not been examined closely in cancers, but in an experiment conducted by Zhang et al. in endothelial cells demonstrated *caspase-3* downregulation due to *let-7g* inhibition. As such, this targeting reduced the progression of apoptosis and lead to higher tolerance of oxidative stress (141).

Autophagy and miRNA

Autophagy is regulated by many autophagy related (ATG) proteins which play various roles in the formation of the autophagosome (100, 142). It has been observed that miRNAs can likely play a role in autophagy and that AML cells can have dysregulated autophagy (97). To date, two miRNAs have been found to associate with autophagy in leukemia: miR-30a and miR-125b1 (115, 116). miR-30a is inversely correlated with Beclin1 and ATG5 in K562, but direct binding and relevance to drug resistance is yet to be demonstrated (115). miR-125b1, on the other hand, can bind RAM2, ATG4D, and UVRAG as demonstrated in NB4 cells (116). The activity of miR-125b1 in this circumstance contributed to inhibition of autophagy through ATG4D. In other cancers, ATG4D was found to be a direct target of miR-101 and its inhibition may contribute to 4-hydroxytamoxifen sensitization in the breast cancer lines MCF7 and T47D (117).

DRUG METABOLISM AND CHEMORESISTANCE

Drug activation and drug clearance can be altered in cells to reduce the effective dose of the drug. These proteins are highly varied, but can largely be characterized into two major classes: the phase I and the phase II class of enzymes. Phase I enzymes typically perform redox reactions or hydrolysis reactions. While they often precede phase II enzyme activity, this is not always required. Phase II enzymes typically increase the polarity of the molecule through the addition of a sub-group such as UDP-glucoronate, sulfate, methane, acetate, or glutathione (143).

Anthracyclines are active drugs that can carry out their genotoxic effects directly. Their metabolism into the semiquinone form, the hydroxyaglycone form, deoxyaglycone form, or the alcohol form will decrease its likelihood of intercalating DNA as it reduces the anthracycline's lipophilicity. It is unclear whether the anthracyclines lose efficacy through metabolism. As demonstrated from cardiotoxicity assays in rat and rabbit, the metabolites may have differing effects depending on the organism in question and the rate of metabolism. In rats, the alcohol form may retain some activity, but the effects of the active drug are more pronounced (143, 144). In rabbits, the alcohol derivative is implicated in the cardiotoxic effects of the anthracyclines (143, 145). It is thought that the enzymes CBR1/3 and AKR1A1/C3 can act on the parent drug to form the alcohol form. The hydroxaglycone and the deoxyaglycone forms can be generated in part by certain cytochrome P450 (CYP) enzymes such as CYP3A4/5, CYP2D6, xanthine dehydrogenase (XDH), and NAD(P)H quinone dehydrogenase 1 (NQO1) (146-150). XDH, NQO1 along with nitric oxide synthase can help in generating the semiquinone form (151-153).

Cytarabine and other nucleoside analogs require phosphorylation through DNA/RNA synthesizing enzymes such as the nucleoside kinases to become candidates for incorporation into nascent DNA. Cytarabine requires activation by several enzymes including deoxycytidine monophosphate kinase, nucleoside





TABLE 4 miRN	IA targeting	proteins	involved	in drug	metabolism
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Protein	miRNA	miRNA status in drug resistance	Sample/cancer	Mechanism	Reference
CYP3A4 miR-27b		Overexpressed	LS-180, PANC 1/colon adenocarcinoma and pancreatic cancer	Inhibition of CYP3A4 lead to reduced activation of cyclophosphamide and reduced sensitivity	Pan et al. (156)
CYP3A4	miR-298	Overexpressed	LS-180, PANC 1/colon adenocarcinoma and pancreatic cancer	Inhibition of CYP3A4 lead to reduced activation of cyclophosphamide and reduced sensitivity	Wei et al. (157)
CYP3A4	miR-577	Overexpressed	HEK 293T/cancer	Inhibition of CYP3A4 lead to reduced activation of cyclophosphamide and reduced sensitivity	Wei et al. (157)
CYP3A4	miR-1	Overexpressed	HEK 293T/cancer	Inhibition of CYP3A4 lead to reduced activation of cyclophosphamide and reduced sensitivity	Wei et al. (157)
CYP3A4	miR-532-3p	Overexpressed	HEK 293T/cancer	Inhibition of CYP3A4 lead to reduced activation of cyclophosphamide and reduced sensitivity	Wei et al. (157)
CYP3A4	miR-627	Overexpressed	HEK 293T/cancer	Inhibition of CYP3A4 lead to reduced activation of cyclophosphamide and reduced sensitivity	Wei et al. (157)
DCK	miR-330	Overexpressed	HEK 293T/cancer	Inverse correlation between miRNA-mRNA suggests interaction	Hodzic et al. (158)

miRNAs, microRNAs; DCK, deoxycytidine kinase.

diphosphate kinase, and the rate limiting DCK (**Figure 5**) (154, 155). It is then metabolized by various enzymes including CYP3A4, 5' nucleotidase, cytidine deaminase, and deoxycyti-dylate deaminase (154, 155).

Currently, there are few publications that highlight the role of miRNAs in anthracycline and cytosine analog metabolizing enzymes in AML. However, certain miRNAs such as *miR-27b* and *miR-298* have demonstrated direct binding of *CYP3A4* in a pancreatic cell line and *miR-577*, *miR-1*, *miR-532-3p*, and *miR-627* were found to target *CYP3A4* in HEK 293T cells (**Figure 5**; **Table 4**) (156, 157). In gencitabine resistant colon and lung cancer cells, Hodzic et al. established a correlation between *miRNA-330* and *DCK* expression levels (158). Further studies interrogating the role of computationally predicted miRNAs and miRNAs discovered in other cancer subtypes may help establish a role for miRNAs in metabolism in drug resistant AML.

DRUG TRAFFICKING AND miRNA IN CHEMORESISTANCE

The trafficking of the anticancer drugs can dramatically modulate treatment response as a reduction in influx or an increase in efflux will reduce the effective intracellular concentration of drug. Due to the lipophilicity of the anthracyclines, they can freely diffuse into the cell, but they can also bind to the SLC22A16 solute pump to enter cells (153, 159–161). While there are some reports that suggest the role of SLC22A16 in bleomycin resistance, the role of this transporter in anthracycline resistance is yet to be explored (162, 163). As such, while there are predicted miRNA-binding sites on this protein, none are yet confirmed.

Cytarabine and other cytosine analogs, on the other hand, necessitate the function of nucleoside transporters to enter the cell. The nucleoside transporters are composed of six major protein families: human equilibrative nucleoside transporters (hENTs) and human concentrative nucleoside transporters (hCNTs), organic anion transporters, organic cation transporters, peptide transporters, and the multidrug resistance protein family (MRP), with the hCNTs and hENTs playing the most major role of cytarabine import (164–166). In childhood leukemia, the hENT protein family has demonstrated to correlate with cytarabine resistance, but miRNA-mediated mechanisms are yet to be confirmed (167, 168).

In contrast, many efflux pumps can confer resistance to diverse and seemingly unrelated drugs and the characterization of several of these transporters has been extensive in AML. These ATP-binding cassette (ABC) proteins can be upregulated in the drug resistant forms of cancers and as such, the downregulation of miRNAs that target efflux pumps can contribute to resistance. Within this class, ABCB1 (P-glycoprotein, MDR1), ABCC1 (MRP1), ABCC2 (MRP2), and ABCG2 (BCRP) have been the most extensively examined out of 48 proteins within this functionally similar class (**Figure 6**) (169, 170). Indeed, previous treatments of drug resistant AML centered on the targeting of P-glycoprotein. It has been clearly demonstrated that the surface expression of P-glycoprotein is inversely proportional to the concentration of intracellular daunorubicin in blast cells and in tissue culture samples; however, blocking of P-glycoprotein did not yield positive results in clinical settings (171).

P-glycoprotein can be targeted by several miRNAs including miR-27a, miR-331-5p, miR-145, miR-298, miR-508-5p, miR-9, and miR-451 (Figure 6; Table 5). In leukemia, only miR-27a and miR-331-5p have been demonstrated to bind to P-glycoprotein in the K562 and HL-60 leukemia cell lines (172). In ovarian and cervix cell lines, it was demonstrated that the downregulation of both miR-27a and miR-451 can lead to downregulation of P-glycoprotein; however, in the case of miR-27a, this contradictory effect on P-glycoprotein is likely in part due to targeting of HPK2 upstream (173). This was further phenotypically demonstrated by the reduced uptake of intracellular dyes and by the response to cisplatin and methotrexate (174). In more recent experiments conducted in hepatocellular carcinoma cells, in addition to direct binding to P-glycoprotein and HPK2 binding, it was demonstrated that the inhibitory effect of miR-27a on *P-glycoprotein* may also be partially attributed to upstream modulation of the β-catenin pathway through direct binding of



FIGURE 6 | microRNAs (miRNAs) have been shown to dysregulate drug efflux mechanisms in both leukemia and other cancer. There are no known miRNA regulators of the drug influx proteins. In leukemia, P-glycoprotein has been demonstrably targeted by miR-27a and miR-331. In other cancers, P-glycoprotein has been shown to be regulated by miR-145, miR-298, miR-451, miR-508-5p, and miR-9. MRP1 has been targeted by miR-1291, miR-873, miR-221, miR-223, and miR-326, while MRP2 has been shown to be targeted my miR-379. The last protein to exhibit miRNA binding in lab setting is BCRP which has been shown to be a target of miR-328 and miR-519c.

TABLE 5 | Drug trafficking gene disinhibitions caused by loss of miRNAs can lead to drug resistance.

Protein	miRNA	miRNA status in drug resistance	Sample/cancer	Mechanism	Reference
ABCB1 (P-glycoprotein/MDR1)	miR-145	Reduced	Caco2 cells, HEK293/colorectal adenocarcinoma	Reduced efflux leads to multidrug resistance	lkemura et al. (177)
ABCB1 (P-glycoprotein/MDR1)	miR-298	Reduced	MDA-MB-231/breast cancer	Reduced efflux leads to multidrug resistance	Bao et al. (178)
ABCB1 (P-glycoprotein/MDR1)	miR-27a	Reduced	K-562, HL60, patient sample/ AML A2780/ovarian cancer A2780, KB-3-1/ovarian cancer	Reduced efflux leads to multidrug resistance	Feng et al. (172) Li et al. (173) Zhu et al. (174)
ABCB1 (P-glycoprotein/MDR1)	miR-331-5p	Reduced	K-562, HL60, patient sample/ AML A2780/ovarian cancer	Reduced efflux leads to multidrug resistance	Feng et al. (172)
ABCB1 (P-glycoprotein/MDR1)	miR-451	Reduced	A2780/ovarian cancer MCF-7 cells/breast cancer	Reduced efflux leads to multidrug resistance	Li et al. (173) Kovalchuk et al. (179)
ABCB1 (P-glycoprotein/MDR1)	miR-508-5p	Reduced	SGC7901/gastric cancer	Direct binding leads to reduced efflux and to multidrug resistance	Shang et al. (175)
ABCB1 (P-glycoprotein/MDR1)	miR-9	Reduced	U87 and T98G/glioblastoma multiforme	Putative or indirect knockdown. Reduced efflux leads to multidrug resistance	Munoz et al. (180)
MRP1	miR-1291	Reduced	PANC1/pancreatic cancer	Loss of binding of MRP1 contributes to doxorubicin resistance	Pan et al. (181)
MRP1	miR-873	Reduced	OVCAR3 and A2780/ovarian cancer	Loss of binding of MRP1 contributes to multidrug resistance	Wu et al. (182)
MRP1	miR-221	Reduced	NCI-H929, RPMI-8226, and U266/multiple myeloma	Loss of binding leads to MRP1-mediated drug resistance	Gullà et al. (183)
MRP1	miR-222	Reduced	NCI-H929, RPMI-8226, and U266/multiple myeloma	Loss of binding leads to MRP1-mediated drug resistance	Gullà et al. (183)
MRP1	miR-326	Reduced	MCF7/breast cancer	Inverse correlation, and likely binding of miRNA	Liang et al. (184)
MRP2	miR-379	Reduced	HepG2/hepatocellular carcinoma	Reduced miR-379 binding leads to MRP2 overexpression and increased efflux	Haenisch et al. (185)
BCRP	miR-328	Reduced	MCF7/breast cancer	Inverse correlation of the miRNA-mRNA pair, suppression of BCRP is possible and it is leading to resistance	Pan et al. (186)
BCRP	miR-519c	Reduced	S1/colon cancer	Transcript variant of BCRP loses miR-519c binding site to lead to resistance	To et al. (187)

miRNAs, microRNAs; AML, acute myeloid leukemia.

SFRP1 and potentially through *FZD7* as well (172, 175). It is possible and likely that P-glycoprotein is involved in processes that are unrelated to drug trafficking as well such as apoptosis which may explain the contradictory expression in different cancers and the varying predisposition of its mutagenicity in certain cancers; however, its actions remain unclear (176).

Direct binding of *miR-451* to *P-glycoprotein* transcripts was demonstrated in MCF-7 cells, where it was demonstrated that it could contribute to doxorubicin resistance; however, this has not yet been demonstrated to be clinically significant in cancer patients (179). In colon cancer cell-derived cell lines and HEK293 cells, it was demonstrated that *miR-145* can play a role in the repression of P-glycoprotein and increase the efflux of rhodamine 123 (177). *miR-298* was demonstrated to directly bind to the transcript in resistant breast cancer cell lines (178). This suggests that it may play a role in patients, but follow-up studies are needed. *miR-508-5p* was demonstrated to directly bind to *P-glycoprotein*

in gastric cancers and its upregulation was found clinically as well (175). It has also been suggested by Munoz et al. that *miR-9* may also target P-glycoprotein and confer resistance to temozolomide in glioblastoma multiforme cells (180). These miRNAs may also prove to be relevant in AML, but no studies have been attempted to date.

While the *MRP1* gene has not demonstrated miRNA binding in AML, it was demonstrated in other cancers that the *MRP1* gene can also be targeted by miRNAs such as *miR-1291*, *miR-873*, *miR-221/222*, and *miR-326* (**Figure 6**; **Table 5**). In an analysis conducted by Pan et al., doxorubicin treatment of pancreatic cancer cells demonstrated that *miR-1291* will become upregulated and target *MRP1* directly (181). *MRP1* downregulation contributes to multidrug resistance as well in other cancers such as ovarian cancer (182). It was recently demonstrated through *in vivo* and *in vitro* studies that *miR-873* can be biologically significant in paclitaxel and cisplatin resistance in ovarian cancer cell lines where it can directly bind to *MRP1* (182). Consequently, *miR-873* is often downregulated in *MRP1*-dependent ovarian cancers. In melphalan-refractory multiple myeloma cells, Gulla et al. demonstrated that *miR-221/222* may be binding and reducing *MRP1* thus contributing to drug resistance (183). Finally, *miR-326* was inversely correlated with *MRP1* in multidrug resistant MCF7 cell lines (184). Less is known about *MRP2* targeting by miRNAs, but in the liver cell line HepG2, *miR-379* was demonstrated to be highly upregulated and to target *MRP2* directly as a response to Rifampicin resistance (185).

BCRP, in contrast, has been shown to be a target of miR-520h, miR-328, and miR-519c and to potentially play a role in the hematopoietic system (Figure 6; Table 5). In CD34+CD38hematopoietic stem cells, it was demonstrated that miR-520h is enriched compared to CD34+ cells alone and that it can directly target BCRP in this fraction (188). An examination of miR-520h in leukemic cells and AML may demonstrate a similar trend of upregulation and a contribution of *miR-520h* to drug resistance, but more experiments are required. In mitoxantrone-resistant MCF-7 cells, Pan et al. showed that the expression of miR-328 is inversely correlated with BCRP and that it is directly suppressing BCRP, leading to resistance (186). To et al. demonstrated that *miR-519c* may play a role in downregulating BCRP in S1 colon cancer cell lines; however, they demonstrated that binding of miR-519c was limited to a longer form of the transcript only found in their parental cell line compared to their mitoxantrone-resistant counterpart (187, 189). This study highlights the importance of splice variants and how they may gain or lose miRNA-binding sites and thereby contribute to resistance.

IMPLICATIONS IN TREATMENT

Drug resistance is only a single aspect of clinical setbacks; however, it is a major contributor to therapy failure. Although treatment has improved substantially in some cancers in the past few decades, many other cancer types continue to demonstrate substantial patient populations that relapse after an initially successful treatment. While we focused on the regulation of drug resistance-associated miRNAs common between different cancers and drug classes, there are likely various miRNA that are specific to different drug treatments and cancers. However, the miRNA dysregulations discussed may have therapeutic value beyond AML. Furthermore, although we describe several drug resistance proteins, our analysis only focused on miRNA specifically implied in drug resistance where they were demonstrated to have direct activity and as such, the list is not exhaustive (190).

There are also many other molecular changes that occur in the development of drug resistance such as copy number variations, aberrant methylation, and aberrant post-transcriptional and post-translational processing (191, 192). The modulation of miRNAs offers a new perspective on drug resistance as miRNA replacement therapy and miRNA inhibition therapy raises the potential of developing new and effective drug therapies. Subtle miRNA changes can lead to significant changes in protein-coding gene expression and can consequently lead to changes in tumor progression and patient outcome. Experimental success *in vitro* and *in vivo* models may point to the likely coming of more miRNA-based clinical trials.

Previously, Mrx34 emerged as a promising therapy for the treatment of unresectable primary liver cancer. Due to multiple immune-related adverse events, this therapy was terminated in phase I although there was evidence of benefit in a subset of patients (193). Its promise came from being a p53-response element that was thought to mediate p53's antitumor effects and consequently affecting downstream signaling in proliferation arrest and induction of apoptosis by targeting *c-MYC*, *CDK6*, and *c-MET* (194). However, recent research now demonstrates that it may not always behave as a tumor-suppressor either and furthermore, p53 may also be a direct target of *miR-34a* (138, 195). In liver cancers with β -catenin mutations, it is demonstrated that LNA-34a, a *miR-34a* inhibitor, displays antitumor effects. This is suggested to occur through blocking HNF-4 α targeting which in turn decreases cyclin D1 and inhibits proliferation (196, 197).

A *miR-16* mimic has also been recently introduced in patients in an open-label phase I clinical trial for mesothelioma and non-small cell lung cancer (NSLC). *miR-16* was shown to be dysregulated in many different cancers (87, 89, 90, 103–105, 124, 198). A directed analysis in mesothelioma showed that *miR-16* is reduced in patient samples and that a knock-in of a *miR-16* mimic is tumor suppressive (198). This observation was repeated in xenografted mice with high success (198). Currently, there are no miRNA-based therapies for drug resistant AML or AML-related diseases.

Currently, there are two miRNA-based therapies intended to treat different cancers that are on-going or with pending results. MesomiR-1, a *miR-16* mimic, was in a multi-center Phase I trial intended to treat mesothelioma and NSLC. This trial has been completed as of January 2017 and the results are currently pending. MRG-106 is a miRNA inhibitor that targets *miR-155* that is currently being examined in cutaneous T-cell lymphoma and mycosis fungoides. Like mesomiR-1, it is also currently in phase I. It is thought to block the action of *miR-155* from targeting tumor suppressors such as $C/EBP\beta$ and altering the TGF- β response (199). This study is currently still recruiting patients. These studies may offer promise of miRNA treatment as therapy and pave the way for future studies similar in nature.

CONCLUDING STATEMENT

Today, the main hurdle for miRNA-based therapies remains to be the method of delivery. Many types of viruses are thought to be potentially useful for treatment and many stabilizing modifications such as phosphorothioate, methyl- and fluorosubstitutions on RNA species may help to overcome this hurdle (200, 201). Given the diverse set of roles that miRNAs play in regular cellular function, it is evident that clear elucidation of specific miRNA mechanisms may be required before their integration into modern cancer therapy (202). In contrast, due to the dependence and overexpression of a few coding mRNA in tumorigenic cells, it is possible that miRNAs may have a higher therapeutic index. miRNAs may prove to be an important addition to treatment in the years to come to treat drug resistant cancers in the future.

AUTHOR CONTRIBUTIONS

MG contributed to the research, figure design, and writing of manuscript. LS contributed to the research, editing, and overall design of manuscript.

REFERENCES

- Döhner H, Weisdorf DJ, Bloomfield CD. Acute myeloid leukemia. N Engl J Med (2015) 373(12):1136–52. doi:10.1056/NEJMra1406184
- Büchner T, Schlenk RF, Schaich M, Döhner K, Krahl R, Krauter J, et al. Acute myeloid leukemia (AML): different treatment strategies versus a common standard arm – combined prospective analysis by the German AML Intergroup. *J Clin Oncol* (2012) 30(29):3604–10. doi:10.1200/ JCO.2012.42.2907
- De Kouchkovsky I, Abdul-Hay M. Acute myeloid leukemia: a comprehensive review and 2016 update. *Blood Cancer J* (2016) 6(7):e441. doi:10.1038/ bcj.2016.50
- Coombs CC, Tavakkoli M, Tallman MS. Acute promyelocytic leukemia: where did we start, where are we now, and the future. *Blood Cancer J* (2015) 5:e304. doi:10.1038/bcj.2015.25
- Crespo-Solis E, Contreras-Cisneros J, Demichelis-Gómez R, Rosas-López A, Vera-Zertuche JM, Aguayo A, et al. Survival and treatment response in adults with acute promyelocytic leukemia treated with a modified international consortium on acute promyelocytic leukemia protocol. *Rev Bras Hematol Hemoter* (2016) 38(4):285–90. doi:10.1016/j.bjhh.2016.08.002
- Verma D, Kantarjian H, Faderl S, O'Brien S, Pierce S, Vu K, et al. Late relapses in acute myeloid leukemia: analysis of characteristics and outcome. *Leuk Lymphoma* (2010) 51(5):778–82. doi:10.3109/10428191003661852
- Zhang H, Luo X-Q, Feng D-D, Zhang X-J, Wu J, Zheng Y-S, et al. Upregulation of microRNA-125b contributes to leukemogenesis and increases drug resistance in pediatric acute promyelocytic leukemia. *Mol Cancer* (2011) 10:108. doi:10.1186/1476-4598-10-108
- 8. Zahreddine H, Borden KLB. Mechanisms and insights into drug resistance in cancer. *Front Pharmacol* (2013) 4:28. doi:10.3389/fphar.2013.00028
- Shaffer BC, Gillet J-P, Patel C, Baer MR, Bates SE, Gottesman MM. Drug resistance: still a daunting challenge to the successful treatment of AML. *Drug Resist Updat* (2012) 15(1–2):62–9. doi:10.1016/j.drup.2012.02.001
- Cree IA, Charlton P. Molecular chess? Hallmarks of anti-cancer drug resistance. BMC Cancer (2017) 17(1):10. doi:10.1186/s12885-016-2999-1
- Cech TR, Steitz JA. The noncoding RNA revolution-trashing old rules to forge new ones. *Cell* (2014) 157(1):77–94. doi:10.1016/j.cell.2014.03.008
- Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, et al. The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans. Nature* (2000) 403(6772):901–6. doi:10.1038/ 35002607
- Ambros V. MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell* (2003) 113(6):673–6. doi:10.1016/S0092-8674(03)00428-8
- Macha MA, Seshacharyulu P, Krishn SR, Pai P, Rachagani S, Jain M, et al. MicroRNAs (miRNAs) as biomarker(s) for prognosis and diagnosis of gastrointestinal (GI) cancers. *Curr Pharm Des* (2014) 20(33):5287–97. doi: 10.2174/1381612820666140128213117
- Grady WM, Tewari M. The next thing in prognostic molecular markers: microRNA signatures of cancer. *Gut* (2010) 59(6):706–8. doi:10.1136/ gut.2009.200022
- Kung JTY, Colognori D, Lee JT. Long noncoding RNAs: past, present, and future. *Genetics* (2013) 193(3):651–69. doi:10.1534/genetics.112.146704
- 17. Huarte M. The emerging role of lncRNAs in cancer. *Nat Med* (2015) 21(11):1253–61. doi:10.1038/nm.3981
- Sun L, Xue H, Jiang C, Zhou H, Gu L, Liu Y, et al. LncRNA DQ786243 contributes to proliferation and metastasis of colorectal cancer both in vitro and in vivo. *Biosci Rep* (2016) 36(3):e00328. doi:10.1042/BSR20160048
- Zhang Z-L, Zhao L-J, Chai L, Zhou S-H, Wang F, Wei Y, et al. Seven lncRNAmRNA based risk score predicts the survival of head and neck squamous cell carcinoma. *Sci Rep* (2017) 7(1):309. doi:10.1038/s41598-017-00252-2
- 20. Xing C, Hu X, Xie F, Yu Z, Li H, Zhou B, et al. Long non-coding RNA HOTAIR modulates c-KIT expression through sponging miR-193a in

ACKNOWLEDGMENTS

LS is a recipient of a Tier II Canada Research Chair. MG is supported by a scholarship from the Centre for Pharmaceutical Oncology at the Leslie Dan Faculty of Pharmacy, University of Toronto.

acute myeloid leukemia. FEBS Lett (2015) 589(15):1981–7. doi:10.1016/j. febslet.2015.04.061

- Wu S, Zheng C, Chen S, Cai X, Shi Y, Lin B, et al. Overexpression of long non-coding RNA HOTAIR predicts a poor prognosis in patients with acute myeloid leukemia. *Oncol Lett* (2015) 10(4):2410–4. doi:10.3892/ol.2015.3552
- 22. Hao S, Shao Z. HOTAIR is upregulated in acute myeloid leukemia and that indicates a poor prognosis. *Int J Clin Exp Pathol* (2015) 8(6):7223–8.
- 23. Díaz-Beyá M, Brunet S, Nomdedéu J, Pratcorona M, Cordeiro A, Gallardo D, et al. The lincRNA HOTAIRM1, located in the HOXA genomic region, is expressed in acute myeloid leukemia, impacts prognosis in patients in the intermediate-risk cytogenetic category, and is associated with a distinctive microRNA signature. *Oncotarget* (2015) 6(31):31613–27. doi:10.18632/ oncotarget.5148
- Garzon R, Volinia S, Papaioannou D, Nicolet D, Kohlschmidt J, Yan PS, et al. Expression and prognostic impact of lncRNAs in acute myeloid leukemia. *Proc Natl Acad Sci U S A* (2014) 111(52):18679–84. doi:10.1073/ pnas.1422050112
- Sayad A, Hajifathali A, Hamidieh AA, Roshandel E, Taheri M. HOTAIR long noncoding RNA is not a biomarker for acute myeloid leukemia (AML) in Iranian patients. Asian Pac J Cancer Prev (2017) 18(6):1581–4. doi:10.22034/ APJCP.2017.18.6.1581
- Chen Z-H, Wang W-T, Huang W, Fang K, Sun Y-M, Liu S-R, et al. The IncRNA HOTAIRM1 regulates the degradation of PML-RARA oncoprotein and myeloid cell differentiation by enhancing the autophagy pathway. *Cell Death Differ* (2017) 24(2):212–24. doi:10.1038/cdd.2016.111
- Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? *Cell* (2011) 146(3):353–8. doi:10.1016/j.cell.2011.07.014
- Macfarlane L-A, Murphy PR. MicroRNA: biogenesis, function and role in cancer. Curr Genomics (2010) 11(7):537–61. doi:10.2174/138920210793175895
- Gunaratne PH, Creighton CJ, Watson M, Tennakoon JB. Large-scale integration of microRNA and gene expression data for identification of enriched microRNA-mRNA associations in biological systems. *Methods Mol Biol* (2010) 667:297–315. doi:10.1007/978-1-60761-811-9_20
- Bail S, Swerdel M, Liu H, Jiao X, Goff LA, Hart RP, et al. Differential regulation of microRNA stability. RNA (2010) 16(5):1032–9. doi:10.1261/rna.1851510
- Okamura K, Liu N, Lai EC. Distinct mechanisms for microRNA strand selection by drosophila argonautes. *Mol Cell* (2009) 36(3):431–44. doi:10.1016/ j.molcel.2009.09.027
- Li S-C, Liao Y-L, Ho M-R, Tsai K-W, Lai C-H, Lin W. miRNA arm selection and isomiR distribution in gastric cancer. *BMC Genomics* (2012) 13 (Suppl 1):S13. doi:10.1186/1471-2164-13-S1-S13
- Winter J, Link S, Witzigmann D, Hildenbrand C, Previti C, Diederichs S. Loop-miRs: active microRNAs generated from single-stranded loop regions. *Nucleic Acids Res* (2013) 41(10):5503–12. doi:10.1093/nar/gkt251
- Okamura K, Ladewig E, Zhou L, Lai EC. Functional small RNAs are generated from select miRNA hairpin loops in flies and mammals. *Genes Dev* (2013) 27(7):778–92. doi:10.1101/gad.211698.112
- Redfern AD, Colley SM, Beveridge DJ, Ikeda N, Epis MR, Li X, et al. RNAinduced silencing complex (RISC) proteins PACT, TRBP, and dicer are SRA binding nuclear receptor coregulators. *Proc Natl Acad Sci U S A* (2013) 110(16):6536–41. doi:10.1073/pnas.1301620110
- Klusmann J-H, Li Z, Böhmer K, Maroz A, Koch ML, Emmrich S, et al. miR-125b-2 is a potential oncomiR on human chromosome 21 in megakaryoblastic leukemia. *Genes Dev* (2010) 24(5):478–90. doi:10.1101/gad.1856210
- Chen K, Rajewsky N. Deep conservation of microRNA-target relationships and 3'UTR motifs in vertebrates, flies, and nematodes. *Cold Spring Harb Symp Quant Biol* (2006) 71:149–56. doi:10.1101/sqb.2006.71.039
- Tay Y, Zhang J, Thomson AM, Lim B, Rigoutsos I. MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. *Nature* (2008) 455(7216):1124–8. doi:10.1038/nature07299

- Lam JKW, Chow MYT, Zhang Y, Leung SWS. siRNA versus miRNA as therapeutics for gene silencing. *Mol Ther Nucleic Acids* (2015) 4:e252. doi:10.1038/ mtna.2015.23
- Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* (2005) 433(7027):769–73. doi:10.1038/ nature03315
- Nakanishi K, Ascano M, Gogakos T, Ishibe-Murakami S, Serganov AA, Briskin D, et al. Eukaryote-specific insertion elements control human ARGONAUTE slicer activity. *Cell Rep* (2013) 3(6):1893–900. doi:10.1016/j. celrep.2013.06.010
- Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) 144(5):646–74. doi:10.1016/j.cell.2011.02.013
- Hatfield SD, Shcherbata HR, Fischer KA, Nakahara K, Carthew RW, Ruohola-Baker H. Stem cell division is regulated by the microRNA pathway. *Nature* (2005) 435(7044):974–8. doi:10.1038/nature03816
- Bitarte N, Bandres E, Boni V, Zarate R, Rodriguez J, Gonzalez-Huarriz M, et al. MicroRNA-451 is involved in the self-renewal, tumorigenicity, and chemoresistance of colorectal cancer stem cells. *Stem Cells* (2011) 29(11):1661–71. doi:10.1002/stem.741
- 45. Huang J-W, Wang Y, Dhillon KK, Calses P, Villegas E, Mitchell PS, et al. Systematic screen identifies miRNAs that target RAD51 and RAD51D to enhance chemosensitivity. *Mol Cancer Res* (2013) 11(12):1564–73. doi:10.1158/1541-7786.MCR-13-0292
- 46. Lai T-H, Ewald B, Zecevic A, Liu C, Sulda M, Papaioannou D, et al. HDAC inhibition induces microRNA-182, which targets RAD51 and impairs HR repair to sensitize cells to sapacitabine in acute myelogenous leukemia. *Clin Cancer Res* (2016) 22(14):3537–49. doi:10.1158/1078-0432.CCR-15-1063
- 47. Liu G, Yang D, Rupaimoole R, Pecot CV, Sun Y, Mangala LS, et al. Augmentation of response to chemotherapy by microRNA-506 through regulation of RAD51 in serous ovarian cancers. *J Natl Cancer Inst* (2015) 107(7):djv108. doi:10.1093/jnci/djv108
- 48. Döhner H, Estey EH, Amadori S, Appelbaum FR, Büchner T, Burnett AK, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European Leukemia Net. *Blood* (2010) 115(3):453–74. doi:10.1182/blood-2009-07-235358
- Estey E. AML in older patients: are we making progress? Best Pract Res Clin Haematol (2009) 22(4):529–36. doi:10.1016/j.beha.2009.08.007
- 50. Wiernik PH. Interpreting data in AML. Leukemia (1996) 10(Suppl 1):S44-5.
- Barone G, Guerra CF, Gambino N, Silvestri A, Lauria A, Almerico AM, et al. Intercalation of daunomycin into stacked DNA base pairs. DFT study of an anticancer drug. *J Biomol Struct Dyn* (2008) 26(1):115–30. doi:10.1080/073 91102.2008.10507229
- D'Ugo E, Bruni R, Argentini C, Giuseppetti R, Rapicetta M. Identification of scaffold/matrix attachment region in recurrent site of woodchuck hepatitis virus integration. DNA Cell Biol (1998) 17(6):519–27. doi:10.1089/ dna.1998.17.519
- 53. Sumer H, Craig JM, Sibson M, Choo KHA. A rapid method of genomic array analysis of scaffold/matrix attachment regions (S/MARs) identifies a 2.5-Mb region of enhanced scaffold/matrix attachment at a human neocentromere. *Genome Res* (2003) 13(7):1737–43. doi:10.1101/gr.1095903
- Heidenreich E, Novotny R, Kneidinger B, Holzmann V, Wintersberger U. Non-homologous end joining as an important mutagenic process in cell cycle-arrested cells. *EMBO J* (2003) 22(9):2274–83. doi:10.1093/emboj/ cdg203
- Hensel JP, Gillert E, Fey GH, Marschalek R. Breakpoints of t(4;11) translocations in the human MLL and AF4 genes in ALL patients are preferentially clustered outside of high-affinity matrix attachment regions. *J Cell Biochem* (2001) 82(2):299–309. doi:10.1002/jcb.1161
- 56. Domer PH, Head DR, Renganathan N, Raimondi SC, Yang E, Atlas M. Molecular analysis of 13 cases of MLL/11q23 secondary acute leukemia and identification of topoisomerase II consensus-binding sequences near the chromosomal breakpoint of a secondary leukemia with the t(4;11). *Leukemia* (1995) 9(8):1305–12.
- Chavali PL, Funa K, Chavali S. Cis-regulation of microRNA expression by scaffold/matrix-attachment regions. *Nucleic Acids Res* (2011) 39(16):6908–18. doi:10.1093/nar/gkr303

- Fathi AT, Karp JE. New agents in acute myeloid leukemia: beyond cytarabine and anthracyclines. *Curr Oncol Rep* (2009) 11(5):346–52. doi:10.1007/ s11912-009-0047-x
- Seedhouse C, Grundy M, Shang S, Ronan J, Pimblett H, Russell N, et al. Impaired S-phase arrest in acute myeloid leukemia cells with a FLT3 internal tandem duplication treated with clofarabine. *Clin Cancer Res* (2009) 15(23):7291–8. doi:10.1158/1078-0432.CCR-09-1222
- Prakasha Gowda AS, Polizzi JM, Eckert KA, Spratt TE. Incorporation of gemcitabine and cytarabine into DNA by DNA polymerase beta and ligase III/XRCC1. *Biochemistry* (2010) 49(23):4833–40. doi:10.1021/bi100200c
- Prakash S, Johnson RE, Prakash L. Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function. *Annu Rev Biochem* (2005) 74:317–53. doi:10.1146/annurev.biochem.74.082803.133250
- Bouwman P, Jonkers J. The effects of deregulated DNA damage signalling on cancer chemotherapy response and resistance. *Nat Rev Cancer* (2012) 12(9):587–98. doi:10.1038/nrc3342
- Liu X, Liao W, Peng H, Luo X, Luo Z, Jiang H, et al. miR-181a promotes G1/S transition and cell proliferation in pediatric acute myeloid leukemia by targeting ATM. J Cancer Res Clin Oncol (2016) 142(1):77–87. doi:10.1007/ s00432-015-1995-1
- 64. Seca H, Lima RT, Almeida GM, Sobrinho-Simoes M, Bergantim R, Guimaraes JE, et al. Effect of miR-128 in DNA damage of HL-60 acute myeloid leukemia cells. *Curr Pharm Biotechnol* (2014) 15(5):492–502. doi:10 .2174/1389201015666140519122524
- Volinia S, Calin GA, Liu C-G, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A* (2006) 103(7):2257–61. doi:10.1073/ pnas.0510565103
- 66. Garzon R, Garofalo M, Martelli MP, Briesewitz R, Wang L, Fernandez-Cymering C, et al. Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. *Proc Natl Acad Sci U S A* (2008) 105(10):3945–50. doi:10.1073/pnas.0800135105
- Chikamori K, Hill JE, Grabowski DR, Zarkhin E, Grozav AG, Vaziri SAJ, et al. Downregulation of topoisomerase IIbeta in myeloid leukemia cell lines leads to activation of apoptosis following all-trans retinoic acid-induced differentiation/growth arrest. *Leukemia* (2006) 20(10):1809–18. doi:10.1038/ sj.leu.2404351
- Hermanson DL, Das SG, Li Y, Xing C. Overexpression of Mcl-1 confers multidrug resistance, whereas topoisomerase IIβ downregulation introduces mitoxantrone-specific drug resistance in acute myeloid leukemia. *Mol Pharmacol* (2013) 84(2):236–43. doi:10.1124/mol.113.086140
- Lim S, Kaldis P. CDKs, cyclins and CKIs: roles beyond cell cycle regulation. Development (2013) 140(15):3079–93. doi:10.1242/dev.091744
- Shiloh Y, Ziv Y. The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. *Nat Rev Mol Cell Biol* (2013) 14(4):197–210. doi:10.1038/nrm3546
- Uziel T, Lerenthal Y, Moyal L, Andegeko Y, Mittelman L, Shiloh Y. Requirement of the MRN complex for ATM activation by DNA damage. *EMBO J* (2003) 22(20):5612–21. doi:10.1093/emboj/cdg541
- Shah MA, Schwartz GK. Cell cycle-mediated drug resistance: an emerging concept in cancer therapy. *Clin Cancer Res* (2001) 7(8):2168–81.
- Beaumont KA, Hill DS, Daignault SM, Lui GYL, Sharp DM, Gabrielli B, et al. Cell cycle phase-specific drug resistance as an escape mechanism of melanoma cells. *J Invest Dermatol* (2016) 136(7):1479–89. doi:10.1016/j. jid.2016.02.805
- Kaaijk P, Kaspers GJL, Van Wering ER, Broekema GJ, Loonen AH, Hählen K, et al. Cell proliferation is related to in vitro drug resistance in childhood acute leukaemia. Br J Cancer (2003) 88(5):775–81. doi:10.1038/sj.bjc.6600787
- Vinogradov S, Wei X. Cancer stem cells and drug resistance: the potential of nanomedicine. *Nanomedicine (Lond)* (2012) 7(4):597–615. doi:10.2217/ nnm.12.22
- Bueno MJ, Malumbres M. MicroRNAs and the cell cycle. Biochim Biophys Acta (2011) 1812(5):592–601. doi:10.1016/j.bbadis.2011.02.002
- Lin Y, Li D, Liang Q, Liu S, Zuo X, Li L, et al. miR-638 regulates differentiation and proliferation in leukemic cells by targeting cyclin-dependent kinase 2. *J Biol Chem* (2015) 290(3):1818–28. doi:10.1074/jbc.M114.599191
- Afanasyeva EA, Mestdagh P, Kumps C, Vandesompele J, Ehemann V, Theissen J, et al. MicroRNA miR-885-5p targets CDK2 and MCM5,

activates p53 and inhibits proliferation and survival. *Cell Death Differ* (2011) 18(6):974–84. doi:10.1038/cdd.2010.164

- 79. Tian R-Q, Wang X-H, Hou L-J, Jia W-H, Yang Q, Li Y-X, et al. MicroRNA-372 is down-regulated and targets cyclin-dependent kinase 2 (CDK2) and cyclin A1 in human cervical cancer, which may contribute to tumorigenesis. *J Biol Chem* (2011) 286(29):25556–63. doi:10.1074/jbc.M111.221564
- Wu J, Lv Q, He J, Zhang H, Mei X, Cui K, et al. MicroRNA-188 suppresses G1/S transition by targeting multiple cyclin/CDK complexes. *Cell Commun Signal* (2014) 12:66. doi:10.1186/s12964-014-0066-6
- Liu Q, Fu H, Sun F, Zhang H, Tie Y, Zhu J, et al. miR-16 family induces cell cycle arrest by regulating multiple cell cycle genes. *Nucleic Acids Res* (2008) 36(16):5391–404. doi:10.1093/nar/gkn522
- Salvatori B, Iosue I, Mangiavacchi A, Loddo G, Padula F, Chiaretti S, et al. The microRNA-26a target E2F7 sustains cell proliferation and inhibits monocytic differentiation of acute myeloid leukemia cells. *Cell Death Dis* (2012) 3:e413. doi:10.1038/cddis.2012.151
- Wong P, Iwasaki M, Somervaille TCP, Ficara F, Carico C, Arnold C, et al. The miR-17-92 microRNA polycistron regulates MLL leukemia stem cell potential by modulating p21 expression. *Cancer Res* (2010) 70(9):3833–42. doi:10.1158/0008-5472.CAN-09-3268
- Pulikkan JA, Dengler V, Peramangalam PS, Peer Zada AA, Müller-Tidow C, Bohlander SK, et al. Cell-cycle regulator E2F1 and microRNA-223 comprise an autoregulatory negative feedback loop in acute myeloid leukemia. *Blood* (2010) 115(9):1768–78. doi:10.1182/blood-2009-08-240101
- Linsley PS, Schelter J, Burchard J, Kibukawa M, Martin MM, Bartz SR, et al. Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression. *Mol Cell Biol* (2007) 27(6):2240–52. doi:10.1128/ MCB.02005-06
- Wang F, Fu X-D, Zhou Y, Zhang Y. Down-regulation of the cyclin E1 oncogene expression by microRNA-16-1 induces cell cycle arrest in human cancer cells. *BMB Rep* (2009) 42(11):725–30. doi:10.5483/BMBRep.2009.42.11.725
- Zubillaga-Guerrero MI, Alarcón-Romero LDC, Illades-Aguiar B, Flores-Alfaro E, Bermúdez-Morales VH, Deas J, et al. MicroRNA miR-16-1 regulates CCNE1 (cyclin E1) gene expression in human cervical cancer cells. *Int J Clin Exp Med* (2015) 8(9):15999–6006.
- Guo X, Connick MC, Vanderhoof J, Ishak M-A, Hartley RS. MicroRNA-16 modulates HuR regulation of cyclin E1 in breast cancer cells. *Int J Mol Sci* (2015) 16(4):7112–32. doi:10.3390/ijms16047112
- Rivas MA, Venturutti L, Huang Y-W, Schillaci R, Huang TH-M, Elizalde PV. Downregulation of the tumor-suppressor miR-16 via progestin-mediated oncogenic signaling contributes to breast cancer development. *Breast Cancer Res* (2012) 14(3):R77. doi:10.1186/bcr3187
- Ofir M, Hacohen D, Ginsberg D. miR-15 and miR-16 are direct transcriptional targets of E2F1 that limit E2F-induced proliferation by targeting cyclin E. *Mol Cancer Res* (2011) 9(4):440–7. doi:10.1158/1541-7786.MCR-10-0344
- Fontana L, Pelosi E, Greco P, Racanicchi S, Testa U, Liuzzi F, et al. MicroRNAs 17-5p-20a-106a control monocytopoiesis through AML1 targeting and M-CSF receptor upregulation. *Nat Cell Biol* (2007) 9(7):775–87. doi:10.1038/ ncb1613
- Pospisil V, Vargova K, Kokavec J, Rybarova J, Savvulidi F, Jonasova A, et al. Epigenetic silencing of the oncogenic miR-17-92 cluster during PU.1-directed macrophage differentiation. *EMBO J* (2011) 30(21):4450–64. doi:10.1038/ emboj.2011.317
- Mitxelena J, Apraiz A, Vallejo-Rodríguez J, Malumbres M, Zubiaga AM. E2F7 regulates transcription and maturation of multiple microRNAs to restrain cell proliferation. *Nucleic Acids Res* (2016) 44(12):5557–70. doi:10.1093/nar/ gkw146
- Eyholzer M, Schmid S, Schardt JA, Haefliger S, Mueller BU, Pabst T. Complexity of miR-223 regulation by CEBPA in human AML. *Leuk Res* (2010) 34(5):672–6. doi:10.1016/j.leukres.2009.11.019
- Rodriguez-Ubreva J, Ciudad L, van Oevelen C, Parra M, Graf T, Ballestar E. C/EBPa-mediated activation of microRNAs 34a and 223 inhibits Lef1 expression to achieve efficient reprogramming into macrophages. *Mol Cell Biol* (2014) 34(6):1145–57. doi:10.1128/MCB.01487-13
- 96. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* (2007) 35(4):495–516. doi:10.1080/01926230701320337
- Watson AS, Riffelmacher T, Stranks A, Williams O, De Boer J, Cain K, et al. Autophagy limits proliferation and glycolytic metabolism in acute myeloid leukemia. *Cell Death Discov* (2015) 1:15008. doi:10.1038/cddiscovery.2015.8

- Evangelisti C, Evangelisti C, Chiarini F, Lonetti A, Buontempo F, Neri LM, et al. Autophagy in acute leukemias: a double-edged sword with important therapeutic implications. *Biochim Biophys Acta* (2015) 1853(1):14–26. doi:10.1016/j.bbamcr.2014.09.023
- Zhang S-P, Niu Y-N, Yuan N, Zhang A-H, Chao D, Xu Q-P, et al. Role of autophagy in acute myeloid leukemia therapy. *Chin J Cancer* (2013) 32(3):130–5. doi:10.5732/cjc.012.10073
- Gozuacik D, Akkoc Y, Ozturk DG, Kocak M. Autophagy-regulating microR-NAs and cancer. Front Oncol (2017) 7:65. doi:10.3389/fonc.2017.00065
- Li H, Hui L, Xu W. miR-181a sensitizes a multidrug-resistant leukemia cell line K562/A02 to daunorubicin by targeting BCL-2. *Chin J Biochem Biophys* (2012) 44(3):269–77. doi:10.1093/abbs/gmr128
- Bai H, Cao Z, Deng C, Zhou L, Wang C. miR-181a sensitizes resistant leukaemia HL-60/Ara-C cells to Ara-C by inducing apoptosis. J Cancer Res Clin Oncol (2012) 138(4):595–602. doi:10.1007/s00432-011-1137-3
- 103. Xia L, Zhang D, Du R, Pan Y, Zhao L, Sun S, et al. miR-15b and miR-16 modulate multidrug resistance by targeting BCL2 in human gastric cancer cells. *Int J Cancer* (2008) 123(2):372–9. doi:10.1002/ijc.23501
- 104. Cittelly DM, Das PM, Salvo VA, Fonseca JP, Burow ME, Jones FE. Oncogenic HER2{Delta}16 suppresses miR-15a/16 and deregulates BCL-2 to promote endocrine resistance of breast tumors. *Carcinogenesis* (2010) 31(12):2049–57. doi:10.1093/carcin/bgq192
- Han J, Chen Q. miR-16 modulate temozolomide resistance by regulating BCL-2 in human glioma cells. *Int J Clin Exp Pathol* (2015) 8(10):12698–707.
- 106. Yang D, Zhan M, Chen T, Chen W, Zhang Y, Xu S, et al. miR-125b-5p enhances chemotherapy sensitivity to cisplatin by down-regulating Bcl2 in gallbladder cancer. *Sci Rep* (2017) 7:43109. doi:10.1038/srep43109
- 107. Li Q, Liang X, Wang Y, Meng X, Xu Y, Cai S, et al. miR-139-5p inhibits the epithelial-mesenchymal transition and enhances the chemotherapeutic sensitivity of colorectal cancer cells by down regulating BCL2. *Sci Rep* (2016) 6:27157. doi:10.1038/srep27157
- 108. Li Q, Wu Y, Zhang Y, Sun H, Lu Z, Du K, et al. miR-125b regulates cell progression in chronic myeloid leukemia via targeting BAK1. Am J Transl Res (2016) 8(2):447–59.
- 109. Zhou M, Liu Z, Zhao Y, Ding Y, Liu H, Xi Y, et al. MicroRNA-125b confers the resistance of breast cancer cells to paclitaxel through suppression of pro-apoptotic Bcl-2 antagonist killer 1 (Bak1) expression. *J Biol Chem* (2010) 285(28):21496–507. doi:10.1074/jbc.M109.083337
- 110. Shi X-B, Xue L, Yang J, Ma A-H, Zhao J, Xu M, et al. An androgen-regulated miRNA suppresses Bak1 expression and induces androgen-independent growth of prostate cancer cells. *Proc Natl Acad Sci U S A* (2007) 104(50):19983–8. doi:10.1073/pnas.0706641104
- 111. Gocek E, Wang X, Liu X, Liu C-G, Studzinski GP. MicroRNA-32 upregulation by 1,25-dihydroxyvitamin D3 in human myeloid leukemia cells leads to Bim targeting and inhibition of AraC-induced apoptosis. *Cancer Res* (2011) 71(19):6230–9. doi:10.1158/0008-5472.CAN-11-1717
- 112. Le MTN, Teh C, Shyh-Chang N, Xie H, Zhou B, Korzh V, et al. MicroRNA-125b is a novel negative regulator of p53. *Genes Dev* (2009) 23(7):862–76. doi:10.1101/gad.1767609
- 113. Hu W, Chan CS, Wu R, Zhang C, Sun Y, Song JS, et al. Negative regulation of tumor suppressor p53 by microRNA miR-504. *Mol Cell* (2010) 38(5):689–99. doi:10.1016/j.molcel.2010.05.027
- 114. Tian P, Yan L. Inhibition of microRNA-149-5p induces apoptosis of acute myeloid leukemia cell line THP-1 by targeting Fas ligand (FASLG). *Med Sci Monit* (2016) 22:5116–23. doi:10.12659/MSM.899114
- 115. Yu Y, Yang L, Zhao M, Zhu S, Kang R, Vernon P, et al. Targeting microR-NA-30a-mediated autophagy enhances imatinib activity against human chronic myeloid leukemia cells. *Leukemia* (2012) 26(8):1752–60. doi:10.1038/ leu.2012.65
- 116. Zeng C-W, Chen Z-H, Zhang X-J, Han B-W, Lin K-Y, Li X-J, et al. MIR125B1 represses the degradation of the PML-RARA oncoprotein by an autophagy-lysosomal pathway in acute promyelocytic leukemia. *Autophagy* (2014) 10(10):1726–37. doi:10.4161/auto.29592
- 117. Frankel LB, Wen J, Lees M, Høyer-Hansen M, Farkas T, Krogh A, et al. MicroRNA-101 is a potent inhibitor of autophagy. *EMBO J* (2011) 30(22):4628–41. doi:10.1038/emboj.2011.331
- 118. Nanbakhsh A, Visentin G, Olive D, Janji B, Mussard E, Dessen P, et al. miR-181a modulates acute myeloid leukemia susceptibility to natural killer cells. Oncoimmunology (2015) 4(12):e996475. doi:10.1080/2162402X.2014.996475

- Mrózek K, Radmacher MD, Bloomfield CD, Marcucci G. Molecular signatures in acute myeloid leukemia. *Curr Opin Hematol* (2009) 16(2):64–9. doi:10.1097/MOH.0b013e3283257b42
- 120. Jongen-Lavrencic M, Sun SM, Dijkstra MK, Valk PJM, Löwenberg B. MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia. *Blood* (2008) 111(10):5078–85. doi:10.1182/ blood-2008-01-133355
- 121. Marcucci G, Maharry K, Radmacher MD, Mrózek K, Vukosavljevic T, Paschka P, et al. Prognostic significance of, and gene and microRNA expression signatures associated with, CEBPA mutations in cytogenetically normal acute myeloid leukemia with high-risk molecular features: a Cancer and Leukemia Group B Study. J Clin Oncol (2008) 26(31):5078–87. doi:10.1200/ JCO.2008.17.5554
- Volinia S, Galasso M, Costinean S, Tagliavini L, Gamberoni G, Drusco A, et al. Reprogramming of miRNA networks in cancer and leukemia. *Genome Res* (2010) 20(5):589–99. doi:10.1101/gr.098046.109
- 123. Schwind S, Maharry K, Radmacher MD, Mrózek K, Holland KB, Margeson D, et al. Prognostic significance of expression of a single microRNA, miR-181a, in cytogenetically normal acute myeloid leukemia: a cancer and leukemia group B study. *J Clin Oncol* (2010) 28(36):5257–64. doi:10.1200/ JCO.2010.29.2953
- 124. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A* (2002) 99(24):15524–9. doi:10.1073/pnas.242606799
- 125. Raveche ES, Salerno E, Scaglione BJ, Manohar V, Abbasi F, Lin Y-C, et al. Abnormal microRNA-16 locus with synteny to human 13q14 linked to CLL in NZB mice. *Blood* (2007) 109(12):5079–86. doi:10.1182/ blood-2007-02-071225
- Balatti V, Pekarky Y, Croce CM. Role of microRNA in chronic lymphocytic leukemia onset and progression. J Hematol Oncol (2015) 8:12. doi:10.1186/ s13045-015-0112-x
- 127. Allegra D, Bilan V, Garding A, Döhner H, Stilgenbauer S, Kuchenbauer F, et al. Defective DROSHA processing contributes to downregulation of miR-15/-16 in chronic lymphocytic leukemia. *Leukemia* (2014) 28(1):98–107. doi:10.1038/leu.2013.246
- Gu X, Li J-Y, Guo J, Li P-S, Zhang W-H. Influence of miR-451 on drug resistances of paclitaxel-resistant breast cancer cell line. *Med Sci Monit* (2015) 21:3291–7. doi:10.12659/MSM.894475
- 129. Liu Z, Liu H, Desai S, Schmitt DC, Zhou M, Khong HT, et al. miR-125b functions as a key mediator for snail-induced stem cell propagation and chemoresistance. *J Biol Chem* (2013) 288(6):4334–45. doi:10.1074/jbc. M112.419168
- 130. Ambs S, Prueitt RL, Yi M, Hudson RS, Howe TM, Petrocca F, et al. Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer. *Cancer Res* (2008) 68(15):6162–70. doi:10.1158/0008-5472.CAN-08-0144
- 131. Jamil S, Lam I, Majd M, Tsai S-H, Duronio V. Etoposide induces cell death via mitochondrial-dependent actions of p53. *Cancer Cell Int* (2015) 15:79. doi:10.1186/s12935-015-0231-z
- Caelles C, Helmberg A, Karin M. p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. *Nature* (1994) 370(6486): 220–3. doi:10.1038/370220a0
- 133. Goeman F, Strano S, Blandino G. Micrornas as key effectors in the p53 network. Int Rev Cell Mol Biol (2017) 333:51–90. doi:10.1016/bs.ircmb.2017.04.003
- 134. Chang T-C, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH, et al. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell* (2007) 26(5):745–52. doi:10.1016/j.molcel.2007.05.010
- 135. He L, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, et al. A microRNA component of the p53 tumour suppressor network. *Nature* (2007) 447(7148):1130–4. doi:10.1038/nature05939
- Hünten S, Siemens H, Kaller M, Hermeking H. The p53/microRNA network in cancer: experimental and bioinformatics approaches. *Adv Exp Med Biol* (2013) 774:77–101. doi:10.1007/978-94-007-5590-1_5
- 137. Yamakuchi M, Ferlito M, Lowenstein CJ. miR-34a repression of SIRT1 regulates apoptosis. Proc Natl Acad Sci U S A (2008) 105(36):13421-6. doi:10.1073/pnas.0801613105

- Rücker FG, Russ AC, Cocciardi S, Kett H, Schlenk RF, Botzenhardt U, et al. Altered miRNA and gene expression in acute myeloid leukemia with complex karyotype identify networks of prognostic relevance. *Leukemia* (2013) 27(2):353–61. doi:10.1038/leu.2012.208
- 139. Shao B, Liao L, Yu Y, Shuai Y, Su X, Jing H, et al. Estrogen preserves Fas ligand levels by inhibiting microRNA-181a in bone marrow-derived mesenchymal stem cells to maintain bone remodeling balance. *FASEB J* (2015) 29(9):3935–44. doi:10.1096/fj.15-272823
- 140. Sayed D, He M, Hong C, Gao S, Rane S, Yang Z, et al. MicroRNA-21 is a downstream effector of AKT that mediates its antiapoptotic effects via suppression of Fas ligand. *J Biol Chem* (2010) 285(26):20281–90. doi:10.1074/ jbc.M110.109207
- 141. Zhang Y, Chen N, Zhang J, Tong Y. Hsa-let-7g miRNA targets caspase-3 and inhibits the apoptosis induced by ox-LDL in endothelial cells. *Int J Mol Sci* (2013) 14(11):22708–20. doi:10.3390/ijms141122708
- 142. Mizushima N, Yoshimori T, Ohsumi Y. The role of Atg proteins in autophagosome formation. Annu Rev Cell Dev Biol (2011) 27:107–32. doi:10.1146/ annurev-cellbio-092910-154005
- 143. Jancova P, Anzenbacher P, Anzenbacherova E. Phase II drug metabolizing enzymes. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub (2010) 154(2):103–16. doi:10.5507/bp.2010.017
- 144. Platel D, Bonoron-Adèle S, Robert J. Role of daunorubicinol in daunorubicin-induced cardiotoxicity as evaluated with the model of isolated perfused rat heart. *Pharmacol Toxicol* (2001) 88(5):250–4. doi:10.1111/j.1600-0773.2001.880505.x
- Cusack BJ, Mushlin PS, Voulelis LD, Li X, Boucek RJ, Olson RD. Daunorubicininduced cardiac injury in the rabbit: a role for daunorubicinol? *Toxicol Appl Pharmacol* (1993) 118(2):177–85. doi:10.1006/taap.1993.1023
- 146. Huang Z, Wang J, Qian J, Li Y, Xu Z, Chen M, et al. Effects of cytochrome P450 family 3 subfamily A member 5 gene polymorphisms on daunorubicin metabolism and adverse reactions in patients with acute leukemia. *Mol Med Rep* (2017) 15(6):3493–8. doi:10.3892/mmr.2017.6470
- Colburn DE, Giles FJ, Oladovich D, Smith JA. In vitro evaluation of cytochrome P450-mediated drug interactions between cytarabine, idarubicin, itraconazole and caspofungin. *Hematology* (2004) 9(3):217–21. doi:10.1080/ 10245330410001701585
- 148. Yee SB, Pritsos CA. Comparison of oxygen radical generation from the reductive activation of doxorubicin, streptonigrin, and menadione by xanthine oxidase and xanthine dehydrogenase. *Arch Biochem Biophys* (1997) 347(2):235–41. doi:10.1006/abbi.1997.0340
- Gustafson DL, Swanson JD, Pritsos CA. Role of xanthine oxidase in the potentiation of doxorubicin-induced cardiotoxicity by mitomycin C. *Cancer Commun* (1991) 3(9):299–304.
- 150. Jamieson D, Cresti N, Bray J, Sludden J, Griffin MJ, Hawsawi NM, et al. Two minor NQO1 and NQO2 alleles predict poor response of breast cancer patients to adjuvant doxorubicin and cyclophosphamide therapy. *Pharmacogenet Genomics* (2011) 21(12):808–19. doi:10.1097/FPC.0b013e32834b6918
- 151. Deng S, Kruger A, Schmidt A, Metzger A, Yan T, Gödtel-Armbrust U, et al. Differential roles of nitric oxide synthase isozymes in cardiotoxicity and mortality following chronic doxorubicin treatment in mice. *Naunyn Schmiedebergs Arch Pharmacol* (2009) 380(1):25–34. doi:10.1007/s00210-009-0407-y
- 152. Oktem G, Bilir A, Selvi N, Yurtseven ME, Vatansever S, Ates U, et al. Chemotherapy influences inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) activity on 3D breast cancer cell line. *Oncol Res* (2006) 16(4):195–203.
- 153. Thorn CF, Oshiro C, Marsh S, Hernandez-Boussard T, McLeod H, Klein TE, et al. Doxorubicin pathways: pharmacodynamics and adverse effects. *Pharmacogenet Genomics* (2011) 21(7):440–6. doi:10.1097/ FPC.0b013e32833ffb56
- Lamba JK. Genetic factors influencing cytarabine therapy. *Pharmacogenomics* (2009) 10(10):1657–74. doi:10.2217/pgs.09.118
- 155. Liu X, Zhou B, Mi S, Xue L, Shih J, Lee J, et al. An increase of cytochrome C oxidase mediated disruption of gemcitabine incorporation into DNA in a resistant KB clone. *Biochem Pharmacol* (2007) 73(12):1927–38. doi:10.1016/j. bcp.2007.03.014
- 156. Pan Y-Z, Gao W, Yu A-M. MicroRNAs regulate CYP3A4 expression via direct and indirect targeting. *Drug Metab Dispos* (2009) 37(10):2112–7. doi:10.1124/dmd.109.027680

- 157. Wei Z, Jiang S, Zhang Y, Wang X, Peng X, Meng C, et al. The effect of microRNAs in the regulation of human CYP3A4: a systematic study using a mathematical model. *Sci Rep* (2014) 4:4283. doi:10.1038/srep04283
- Hodzic J, Giovannetti E, Diosdado B, Adema AD, Peters GJ. Regulation of deoxycytidine kinase expression and sensitivity to gemcitabine by micro-RNA 330 and promoter methylation in cancer cells. *Nucleosides Nucleotides Nucleic Acids* (2011) 30(12):1214–22. doi:10.1080/15257770.2011. 629271
- 159. Armenian SH, Ding Y, Mills G, Sun C, Venkataraman K, Wong FL, et al. Genetic susceptibility to anthracycline-related congestive heart failure in survivors of haematopoietic cell transplantation. Br J Haematol (2013) 163(2):205–13. doi:10.1111/bjh.12516
- 160. Szachowicz-Petelska B, Figaszewski Z, Lewandowski W. Mechanisms of transport across cell membranes of complexes contained in antitumour drugs. *Int J Pharm* (2001) 222(2):169–82. doi:10.1016/S0378-5173(01)00713-X
- El-Kareh AW, Secomb TW. Two-mechanism peak concentration model for cellular pharmacodynamics of doxorubicin. *Neoplasia* (2005) 7(7):705–13. doi:10.1593/neo.05118
- 162. Aouida M, Poulin R, Ramotar D. The human carnitine transporter SLC22A16 mediates high affinity uptake of the anticancer polyamine analogue bleomycin-A5. J Biol Chem (2010) 285(9):6275–84. doi:10.1074/jbc.M109.046151
- 163. Aouida M, Ramotar D. A new twist in cellular resistance to the anticancer drug bleomycin-A5. *Curr Drug Metab* (2010) 11(7):595–602. doi:10.2174/138920010792927307
- 164. Damaraju VL, Damaraju S, Young JD, Baldwin SA, Mackey J, Sawyer MB, et al. Nucleoside anticancer drugs: the role of nucleoside transporters in resistance to cancer chemotherapy. *Oncogene* (2003) 22(47):7524–36. doi:10.1038/sj.onc.1206952
- Molina-Arcas M, Pastor-Anglada M. Role of nucleoside transporters in nucleoside-derived drug sensitivity. *Nucleosides Nucleotides Nucleic Acids* (2010) 29(4–6):335–46. doi:10.1080/15257771003729823
- 166. Pastor-Anglada M, Cano-Soldado P, Molina-Arcas M, Lostao MP, Larráyoz I, Martínez-Picado J, et al. Cell entry and export of nucleoside analogues. *Virus Res* (2005) 107(2):151–64. doi:10.1016/j.virusres.2004.11.005
- 167. Hubeek I, Stam RW, Peters GJ, Broekhuizen R, Meijerink JPP, van Wering ER, et al. The human equilibrative nucleoside transporter 1 mediates in vitro cytarabine sensitivity in childhood acute myeloid leukaemia. *Br J Cancer* (2005) 93(12):1388–94. doi:10.1038/sj.bjc.6602881
- Galmarini CM, Thomas X, Calvo F, Rousselot P, El Jafaari A, Cros E, et al. Potential mechanisms of resistance to cytarabine in AML patients. *Leuk Res* (2002) 26(7):621–9. doi:10.1016/S0145-2126(01)00184-9
- 169. Leslie EM, Deeley RG, Cole SPC. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicol Appl Pharmacol* (2005) 204(3):216–37. doi:10.1016/j.taap.2004.10.012
- Cole SPC. Multidrug resistance protein 1 (MRP1, ABCC1), a "multitasking" ATP-binding cassette (ABC) transporter. J Biol Chem (2014) 289(45):30880–8. doi:10.1074/jbc.R114.609248
- 171. Greenberg PL, Lee SJ, Advani R, Tallman MS, Sikic BI, Letendre L, et al. Mitoxantrone, etoposide, and cytarabine with or without valspodar in patients with relapsed or refractory acute myeloid leukemia and high-risk myelodysplastic syndrome: a phase III trial (E2995). *J Clin Oncol* (2004) 22(6):1078–86. doi:10.1200/JCO.2004.07.048
- 172. Feng D-D, Zhang H, Zhang P, Zheng Y-S, Zhang X-J, Han B-W, et al. Down-regulated miR-331-5p and miR-27a are associated with chemotherapy resistance and relapse in leukaemia. *J Cell Mol Med* (2011) 15(10):2164–75. doi:10.1111/j.1582-4934.2010.01213.x
- 173. Li Z, Hu S, Wang J, Cai J, Xiao L, Yu L, et al. miR-27a modulates MDR1/Pglycoprotein expression by targeting HIPK2 in human ovarian cancer cells. *Gynecol Oncol* (2010) 119(1):125–30. doi:10.1016/j.ygyno.2010.06.004
- 174. Zhu H, Wu H, Liu X, Evans BR, Medina DJ, Liu C-G, et al. Role of microRNA miR-27a and miR-451 in the regulation of MDR1/P-glycoprotein expression in human cancer cells. *Biochem Pharmacol* (2008) 76(5):582–8. doi:10.1016/j. bcp.2008.06.007
- 175. Shang Y, Feng B, Zhou L, Ren G, Zhang Z, Fan X, et al. The miR27b-CCNG1-P53-miR-508-5p axis regulates multidrug resistance of gastric cancer. Oncotarget (2016) 7(1):538–49. doi:10.18632/oncotarget.6374
- Lopes-Rodrigues V, Seca H, Sousa D, Sousa E, Lima RT, Vasconcelos MH. The network of P-glycoprotein and microRNAs interactions. *Int J Cancer* (2014) 135(2):253–63. doi:10.1002/ijc.28500

- 177. Ikemura K, Yamamoto M, Miyazaki S, Mizutani H, Iwamoto T, Okuda M. MicroRNA-145 post-transcriptionally regulates the expression and function of P-glycoprotein in intestinal epithelial cells. *Mol Pharmacol* (2013) 83(2):399–405. doi:10.1124/mol.112.081844
- 178. Bao L, Hazari S, Mehra S, Kaushal D, Moroz K, Dash S. Increased expression of P-glycoprotein and doxorubicin chemoresistance of metastatic breast cancer is regulated by miR-298. *Am J Pathol* (2012) 180(6):2490–503. doi:10.1016/j.ajpath.2012.02.024
- 179. Kovalchuk O, Filkowski J, Meservy J, Ilnytskyy Y, Tryndyak VP, Chekhun VF, et al. Involvement of microRNA-451 in resistance of the MCF-7 breast cancer cells to chemotherapeutic drug doxorubicin. *Mol Cancer Ther* (2008) 7(7):2152–9. doi:10.1158/1535-7163.MCT-08-0021
- 180. Munoz JL, Bliss SA, Greco SJ, Ramkissoon SH, Ligon KL, Rameshwar P. Delivery of functional anti-miR-9 by mesenchymal stem cell-derived exosomes to glioblastoma multiforme cells conferred chemosensitivity. *Mol Ther Nucleic Acids* (2013) 2:e126. doi:10.1038/mtna.2013.60
- 181. Pan Y-Z, Zhou A, Hu Z, Yu A-M. Small nucleolar RNA-derived microRNA hsa-miR-1291 modulates cellular drug disposition through direct targeting of ABC transporter ABCC1. *Drug Metab Dispos* (2013) 41(10):1744–51. doi:10.1124/dmd.113.052092
- Wu D, Li X-S, Meng X-N, Yan J, Zong Z-H. MicroRNA-873 mediates multidrug resistance in ovarian cancer cells by targeting ABCB1. *Tumour Biol* (2016) 37(8):10499–506. doi:10.1007/s13277-016-4944-y
- 183. Gullà A, Di Martino MT, Gallo Cantafio ME, Morelli E, Amodio N, Botta C, et al. A 13 mer LNA-i-miR-221 inhibitor restores drug sensitivity in melphalan-refractory multiple myeloma cells. *Clin Cancer Res* (2016) 22(5):1222–33. doi:10.1158/1078-0432.CCR-15-0489
- Liang Z, Wu H, Xia J, Li Y, Zhang Y, Huang K, et al. Involvement of miR-326 in chemotherapy resistance of breast cancer through modulating expression of multidrug resistance-associated protein 1. *Biochem Pharmacol* (2010) 79(6):817–24. doi:10.1016/j.bcp.2009.10.017
- 185. Haenisch S, Laechelt S, Bruckmueller H, Werk A, Noack A, Bruhn O, et al. Down-regulation of ATP-binding cassette C2 protein expression in HepG2 cells after rifampicin treatment is mediated by microRNA-379. *Mol Pharmacol* (2011) 80(2):314–20. doi:10.1124/mol.110.070714
- Pan Y-Z, Morris ME, Yu A-M. MicroRNA-328 negatively regulates the expression of breast cancer resistance protein (BCRP/ABCG2) in human cancer cells. *Mol Pharmacol* (2009) 75(6):1374–9. doi:10.1124/mol.108.054163
- 187. To KKW, Zhan Z, Litman T, Bates SE. Regulation of ABCG2 expression at the 3' untranslated region of its mRNA through modulation of transcript stability and protein translation by a putative microRNA in the S1 colon cancer cell line. *Mol Cell Biol* (2008) 28(17):5147–61. doi:10.1128/MCB.00331-08
- Liao R, Sun J, Zhang L, Lou G, Chen M, Zhou D, et al. MicroRNAs play a role in the development of human hematopoietic stem cells. *J Cell Biochem* (2008) 104(3):805–17. doi:10.1002/jcb.21668
- 189. Miyake K, Mickley L, Litman T, Zhan Z, Robey R, Cristensen B, et al. Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. *Cancer Res* (1999) 59(1):8–13.
- 190. Yeh C-H, Moles R, Nicot C. Clinical significance of microRNAs in chronic and acute human leukemia. *Mol Cancer* (2016) 15(1):37. doi:10.1186/ s12943-016-0518-2
- 191. Varma S, Pommier Y, Sunshine M, Weinstein JN, Reinhold WC. High resolution copy number variation data in the NCI-60 cancer cell lines from whole genome microarrays accessible through cell miner. *PLoS One* (2014) 9(3):e92047. doi:10.1371/journal.pone.0092047
- 192. de Necochea-Campion R, Shouse GP, Zhou Q, Mirshahidi S, Chen C-S. Aberrant splicing and drug resistance in AML. *J Hematol Oncol* (2016) 9(1):85. doi:10.1186/s13045-016-0315-9
- 193. Beg MS, Brenner AJ, Sachdev J, Borad M, Kang Y-K, Stoudemire J, et al. Phase I study of MRX34, a liposomal miR-34a mimic, administered twice weekly in patients with advanced solid tumors. *Invest New Drugs* (2017) 35(2):180–8. doi:10.1007/s10637-016-0407-y
- 194. Misso G, Di Martino MT, De Rosa G, Farooqi AA, Lombardi A, Campani V, et al. miR-34: a new weapon against cancer? *Mol Ther Nucleic Acids* (2014) 3:e194. doi:10.1038/mtna.2014.47
- 195. Navarro F, Lieberman J. miR-34 and p53: new insights into a complex functional relationship. *PLoS One* (2015) 10(7):e0132767. doi:10.1371/journal. pone.0132767

- 196. Takagi S, Nakajima M, Kida K, Yamaura Y, Fukami T, Yokoi T. MicroRNAs regulate human hepatocyte nuclear factor 4alpha, modulating the expression of metabolic enzymes and cell cycle. *J Biol Chem* (2010) 285(7):4415–22. doi:10.1074/jbc.M109.085431
- 197. Xu Y, Zalzala M, Xu J, Li Y, Yin L, Zhang Y. A metabolic stress-inducible miR-34a-HNF4α pathway regulates lipid and lipoprotein metabolism. *Nat Commun* (2015) 6:7466. doi:10.1038/ncomms8466
- 198. Reid G, Pel ME, Kirschner MB, Cheng YY, Mugridge N, Weiss J, et al. Restoring expression of miR-16: a novel approach to therapy for malignant pleural mesothelioma. *Ann Oncol* (2013) 24(12):3128–35. doi:10.1093/ annonc/mdt412
- 199. Johansson J, Berg T, Kurzejamska E, Pang MF, Tabor V, Jansson M, et al. miR-155-mediated loss of C/EBPβ shifts the TGF-β response from growth inhibition to epithelial-mesenchymal transition, invasion and metastasis in breast cancer. Oncogene (2013) 32(50):5614–24. doi:10.1038/onc.2013.322
- Burnett JC, Rossi JJ. RNA-based therapeutics: current progress and future prospects. *Chem Biol* (2012) 19(1):60–71. doi:10.1016/j.chembiol.2011.12.008

- 201. Mali S. Delivery systems for gene therapy. Indian J Hum Genet (2013) 19(1):3-8. doi:10.4103/0971-6866.112870
- 202. Markopoulos GS, Roupakia E, Tokamani M, Chavdoula E, Hatziapostolou M, Polytarchou C, et al. A step-by-step microRNA guide to cancer development and metastasis. *Cell Oncol (Dordr)* (2017) 40(4):303–39. doi:10.1007/ s13402-017-0341-9

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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New Challenges to Study Heterogeneity in Cancer Redox Metabolism

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Reactive oxygen species (ROS) are important pathophysiological molecules involved in vital cellular processes. They are extremely harmful at high concentrations because they promote the generation of radicals and the oxidation of lipids, proteins, and nucleic acids, which can result in apoptosis. An imbalance of ROS and a disturbance of redox homeostasis are now recognized as a hallmark of complex diseases. Considering that ROS levels are significantly increased in cancer cells due to mitochondrial dysfunction, ROS metabolism has been targeted for the development of efficient treatment strategies, and antioxidants are used as potential chemotherapeutic drugs. However, initial ROS-focused clinical trials in which antioxidants were supplemented to patients provided inconsistent results, i.e., improved treatment or increased malignancy. These different outcomes may result from the highly heterogeneous redox responses of tumors in different patients. Hence, population-based treatment strategies are unsuitable and patient-tailored therapeutic approaches are required for the effective treatment of patients. Moreover, due to the crosstalk between ROS, reducing equivalents [e.g., NAD(P)H] and central metabolism, which is heterogeneous in cancer, finding the best therapeutic target requires the consideration of system-wide approaches that are capable of capturing the complex alterations observed in all of the associated pathways. Systems biology and engineering approaches may be employed to overcome these challenges, together with tools developed in personalized medicine. However, ROS- and redox-based therapies have yet to be addressed by these methodologies in the context of disease treatment. Here, we review the role of ROS and their coupled redox partners in tumorigenesis. Specifically, we highlight some of the challenges in understanding the role of hydrogen peroxide (H₂O₂), one of the most important ROS in pathophysiology in the progression of cancer. We also discuss its interplay with antioxidant defenses, such as the coupled peroxiredoxin/thioredoxin and glutathione/glutathione peroxidase systems, and its reducing equivalent metabolism. Finally, we highlight the need for system-level and patient-tailored approaches to clarify the roles of these systems and identify therapeutic targets through the use of the tools developed in personalized medicine.

OPEN ACCESS

Edited by:

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Specialty section:

This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

> **Received:** 25 April 2017 **Accepted:** 26 June 2017 **Published:** 11 July 2017

Citation:

Benfeitas R, Uhlen M, Nielsen J and Mardinoglu A (2017) New Challenges to Study Heterogeneity in Cancer Redox Metabolism. Front. Cell Dev. Biol. 5:65. doi: 10.3389/fcell.2017.00065

Keywords: cancer heterogeneity, redox biology, reactive oxygen species, systems biology, personalized medicine

INTRODUCTION

Redox metabolism is closely intertwined with cell physiology, and reactive oxygen species (ROS) are central players in health and disease. For instance, these oxygen-derived species are involved in cancer (Reuter et al., 2010), neurodegenerative diseases (Sultana et al., 2006), aging (Höhn et al., 2013), and diabetes (Evans et al., 2002). They are produced intracellularly by several processes and dedicated enzymes, such as NADPH oxidases (Nauseef, 2008) and in multiple cellular compartments (Messner and Imlay, 2002; Chen et al., 2008; Murphy, 2009; Brown and Borutaite, 2012). Due to their high membrane permeability (Chance et al., 1979), extracellularly produced ROS (Hampton et al., 1998; Babior et al., 2002) may quickly enter cells, or they may diffuse across compartments (Bienert et al., 2007; Marchissio et al., 2012). In many diseases, imbalances in ROS metabolism lead to oxidative stress. As result, cells face toxic outcomes of protein, lipid, and nucleic acid oxidation (Garrison, 1987; Cooke et al., 2003; Smith and Murphy, 2008; Figure 1A). For instance, DNA oxidation by ROS promotes mutagenesis, cancer initiation, and progression (Shibutani et al., 1991; Cooke et al., 2003; Sabharwal and Schumacker, 2014), and at high concentrations, ROS may cause cell apoptosis (Gao et al., 2013).

In turn, low ROS concentrations have important physiological roles (D'Autréaux and Toledano, 2007). They regulate cell-cycle progression (Havens et al., 2006), proliferation (Choe et al., 2012), growth (Arnold et al., 2001), and important signaling processes (Finkel, 2011; Rigoulet et al., 2011). For instance, hydrogen peroxide (H₂O₂) regulates the activity of kinases (Gotoh and Cooper, 1998; Paulsen et al., 2012), which control proliferation, differentiation, and apoptosis. NFE2L2 (also known as NRF2) responds to oxidative stress and regulates GSH biosynthesis and reduction, the expression of several proteins involved in antioxidant defense (glutathione peroxidases, transferases, peroxiredoxins, thioredoxins, and thioredoxin reductases), and NADPH production (Gorrini et al., 2013). Many of these redoxregulated processes are not directly controlled by ROS but rather by their redox partners. For instance, redox signaling transduction is often mediated by peroxiredoxins, thioredoxins, and other thiol-reacting proteins (Saitoh et al., 1998; Giannoni et al., 2005; Morinaka et al., 2011). These proteins ensure the high specificity required for efficient signaling transduction (Nagy and Winterbourn, 2010; Winterbourn, 2013; Marinho et al., 2014; Netto and Antunes, 2016). Together with other cellular antioxidants, such as catalases and dismutases, these redox systems prevent toxic ROS accumulation while permitting special-temporal selectivity and the maintenance of important redox signaling functions.

In cancer, mitochondrial dysfunction and metabolic changes promote constant oxidative stress (Szatrowski and Nathan, 1991; Hileman et al., 2004); however, this does not result in apoptosis. Cancer cells promote the expression of antioxidant defenses or reducing equivalents that enable their activity (Janssen et al., 1999; Miranda et al., 2000; Hileman et al., 2004), thus avoiding ROS-induced apoptosis and enabling proliferation, despite high mutagenesis (Toyokuni et al., 1995; Kondo et al., 1999) and metastasis (Ishikawa et al., 2008; **Figure 1B**). ROS closely interact with iron (Galaris et al., 2008) and central (Robbins et al., 2012; Hart et al., 2015; Miar et al., 2015) metabolism, and they are controlled by several transcription factors and tumor suppressors (Gao et al., 2007; Frohlich et al., 2008; Gupta et al., 2012; Gorrini et al., 2013; Hornsveld and Dansen, 2016). Additionally, antioxidant enzymes may display high or low expression in cancer cells (Ray et al., 2000; Oltra et al., 2001; Skrzydlewska et al., 2005; Glorieux et al., 2015), suppress tumorigenesis or promote metastasization (Zhao et al., 2001; Liu et al., 2012; Robbins et al., 2012; Miar et al., 2015), and display synergistic responses (Harris et al., 2015). As result, systematic approaches may capture these complex responses and provide insights into the mechanisms underlying the diverse phenotypic responses in cancer.

Systems biology presents promising approaches for capturing and studying complex cellular responses (Mardinoglu et al., 2013b; Ghaffari et al., 2015b). The application of such frameworks to clinical challenges is referred to as systems or network medicine (Mardinoglu and Nielsen, 2012, 2016). The complexity of biological pathways in cells and tissues may be captured through reconstruction of biological networks, including genome-scale metabolic models (GEMs), transcriptional regulatory networks, protein-protein interaction networks, and signaling networks, in an integrated approach that aims to understand entire cell processes at the systems level (Mardinoglu and Nielsen, 2015; Zhang et al., 2016). These networks may also be integrated with each other for a holistic understanding of the relationships between cellular networks, function, and disease (Bjornson et al., 2016; Lee et al., 2016; Mardinoglu and Uhlén, 2016). Generation of omics data for major human tissues enabled the generation of comprehensive biological networks (Kampf et al., 2014a; Lindskog et al., 2015; Uhlén et al., 2015, 2016; Thul et al., 2017), which have been successfully employed in revealing the underlying mechanisms involved in the occurrence of obesity (Mardinoglu et al., 2013a, 2014b, 2015a), type 2 diabetes (Väremo et al., 2015), non-alcoholic fatty liver disease (Kampf et al., 2014b; Mardinoglu et al., 2014a, in press; Hyötyläinen et al., 2016), and cancer (Agren et al., 2012, 2014; Weinstein et al., 2013; Zack et al., 2013; Leiserson et al., 2014; Yizhak et al., 2014b; Aran et al., 2015; Bjornson et al., 2015; Peng et al., 2015; Elsemman et al., 2016). Personalized models have also been used in the identification of potential therapeutic targets and biomarkers (Faratian et al., 2009; Agren et al., 2014; Bjornson et al., 2015; Mardinoglu et al., 2017; Nielsen, 2017). To date, small scale redox networks have also been analyzed (Zhang et al., 2010; Zhou et al., 2011; Zhan et al., 2012). However, despite extensive evidence highlighting the importance of ROS, antioxidants and other redox players in cancer, systems approaches have yet to systematically examine the role of redox metabolism in this disease and uncover potential personalized treatment strategies.

Here, we highlight some recent findings about important biological processes that are crucial in tumorigenesis: ROS, their redox partners, and reducing equivalents. We start by overviewing some of the main biochemical properties of ROS and their effectors. We then discuss the role of antioxidants and their reactions in tumorigenesis, focusing on thiols and reducing equivalents due to their importance



their responses to oxidative stress. Under normal conditions, ROS production is low and antioxidant defenses are sufficient to prevent toxic damage. Under oxidative stress, the promoted production of ROS overcomes the cell's capacity for detoxification and results in increased toxic damage and pathway disruption, which may lead to mitochondrial dysfunction, mutagenesis and ultimately apoptosis. In turn, cancer cells are under constant oxidative stress, which through upregulation of antioxidant defenses, prevents apoptosis while maintaining ROS toxicity. Arrows indicate fluxes, increasing from dashed to continuous, in red. $O_2^{\bullet-}$, superoxide; H_2O_2 , hydrogen peroxide; HO•, hydroxyl radical; SOD, superoxide dismutase.

in ROS and redox homeostasis. Finally, given the high heterogeneity of redox responses and the intricate crosstalk between redox and central metabolism, we highlight how system-level and patient-tailored approaches may help to identify potential cancer targets and provide mechanistic insights into redox cancer responses. These discussions do not aim to be exhaustive descriptions of all biological processes and regulators of redox homeostasis, and the interested reader may find excellent reviews on these topics elsewhere (e.g., Gao et al., 2007; Frohlich et al., 2008; Gupta et al., 2012; Gorrini et al., 2013; Hornsveld and Dansen, 2016).

BIOCHEMISTRY OF ROS AND REDOX SYSTEMS

Molecular oxygen freely diffuses across cell membranes and promotes the formation of intracellular ROS through electron abstraction. ROS may be classified as radicals and non-radicals. Radicals have unpaired electrons and include superoxide $(O_2^{\bullet-})$ and the hydroxyl (HO•) radicals. Non-radical ROS do not have unpaired electrons, and they include H₂O₂. Here, we focus on these three ROS due to their patho-physiological importance. ROS metabolism yields many other less reactive, abundant, or stable ROS and is highly intertwined with other important reactive species (such as Reactive Nitrogen Species, Weidinger and Kozlov, 2015).

ROS are formed in several intracellular compartments. Most notably, they are produced in peroxisomes through fatty acid oxidation (Fransen et al., 2012), in mitochondria during oxidative phosphorylation and in the cellular and intracellular membranes by NADPH oxidases (EC 1.6.3.1; Nauseef, 2008; Kowaltowski et al., 2009; Murphy, 2009; Brown and Borutaite, 2012; Fransen et al., 2012). Some crosstalk exists between these systems. For instance, mitochondrial-produced ROS promote $O_2^{\bullet-}$ generation by NADPH oxidases, which may have important functions during phagocytosis (Dikalov, 2011). It is currently unclear which of the compartments above contributes the most to intracellular ROS production, although mitochondria are often cited as the main cellular ROS source (Brown and Borutaite, 2012).

The reactions involving ROS and their cellular targets lead to the interconversion of various types of ROS (**Figure 2**). For instance, mitochondrial- and cytoplasmic-produced $O_2^{\bullet-}$ is dismutated to H_2O_2 by superoxide dismutases (SOD, EC 1.15.1.1). $O_2^{\bullet-}$ is fairly unreactive to most electron-rich centers due to its anionic charge, but it reacts with nitric oxide to form peroxynitrite (Huie and Padmaja, 1993) and oxidizes ironsulfur clusters, thereby producing H_2O_2 and HO[•] (Rouault and Klausner, 1996). These clusters are found in multiple intracellular compartments (Tong et al., 2000), and their oxidation by $O_2^{\bullet-}$ (or H_2O_2) leads to iron release and the inactivation of metabolically important enzymes, such as those involved in amino acid biosynthesis (Wallace et al., 2004) or carbohydrate metabolism (Gardner et al., 1995). It is currently unclear whether most $O_2^{\bullet-}$ is used toward nitric oxide metabolism, if it reacts with metal clusters, or if it is dismutated to H₂O₂. Both dismutases and nitric oxide react with $O_2^{\bullet-}$ with near diffusion-limited rate constants ($k > 10^9 \text{ M}^{-1} \text{ s}^{-1}$, Bannister et al., 1973; Huie and Padmaja, 1993), and $O_2^{\bullet-}$ is very reactive with some iron-sulfur cluster-bearing enzymes ($k \approx 10^6 - 10^7 \text{ M}^{-1} \text{ s}^{-1}$, Flint et al., 1993), but it is unreactive with amino acid residues (Bielski and Shiue, 1979). The fate of $O_2^{\bullet-}$ depends on the local availability of the other reactants or enzymes and likely varies between cells and under different conditions, although it is generally assumed that most $O_2^{\bullet-}$ is dismutated to H₂O₂ (Forman et al., 2010).

In turn, H₂O₂ reacts slowly with most biological compounds, such as free glutathione ($k < 10 \text{ M}^{-1} \text{ s}^{-1}$, Winterbourn and Metodiewa, 1999) and phosphatases ($k \approx 10-200 \text{ M}^{-1} \text{ s}^{-1}$, LaButti et al., 2007; Marinho et al., 2014). However, it may display extremely high reactivities with selected protein thiols due to their neighboring chemical environment ($k \approx 10^5 - 10^8 \text{ M}^{-1} \text{s}^{-1}$, Peskin et al., 2007; Trujillo et al., 2007; Manta et al., 2009). It is decomposed into water and molecular oxygen as a result of dismutation by catalases (EC 1.11.1.6), or it is reduced to water by peroxidases and peroxiredoxins (EC 1.11.1.15). Often, several of these mechanisms are present in the same cells. Protection against H_2O_2 is accomplished through glutathione peroxidase, catalase, and peroxiredoxin 2 in human erythrocytes (Johnson et al., 2005; Low et al., 2007; Benfeitas et al., 2014). These defenses are unlikely to be redundant in their functions: while catalase is an efficient H₂O₂ scavenger, even under high oxidative loads, peroxiredoxin 2 has limited reduction under such conditions (Low et al., 2007), resulting in a lower contribution for H_2O_2 consumption. However, peroxiredoxin 2 and its coupled cycles





display desirable redox signaling properties (Benfeitas et al., 2014), which are further discussed below.

 $O_2^{\bullet-}$ and H_2O_2 are also involved in the production of HO[•] through iron-catalyzed Fenton reactions involving heme peroxidases or iron/sulfur clusters (Fenton, 1894; Chen and Schopfer, 1999; Koppenol, 2001). Iron accumulation and HO[•] production have been extensively associated with carcinogenesis, and iron chelators have been employed as therapeutic drugs in cancer (reviewed by Torti and Torti, 2013; Bystrom and Rivella, 2015). Due to its electrophilic nature, HO[•] preferably oxidizes electron-rich sites, reacting with nucleic acids, lipids, and proteins with diffusion-limited rate constants (Von Sonntag, 1987; Buxton et al., 1988; Stadtman and Levine, 2003; Sharma and Rokita, 2013). This promotes DNA strand breaks, lipid peroxidation and protein carbonylation, crosslinking, and cleavage. The products of these reactions are toxic, and they often promote radical propagation and damage to nearby molecules through subsequent chain reactions. Importantly, due to HO[•]'s very high and unselective reactions with biological compounds, no cellular antioxidants can feasibly scavenge this oxidant before it reacts with cellular contents. Instead, protection against the toxic outcomes of HO[•] comes from preventing its formation by shielding iron from ROS or by scavenging H_2O_2 and $O_2^{\bullet-}$ before they yield HO[•]. The role of H₂O₂ in HO[•] formation and consequential radical formation is also thought to be one of the main reasons behind H₂O₂'s toxicity (Winterbourn, 1995).

As result of their different reactivities with biological compounds, the above ROS present varying stabilities and cellular roles. For instance, the fast and indiscriminate reactions of HO[•] result in very small diffusion distances (\approx 80 Å, Roots

and Okada, 1975) that are approximately the size of a small peptide. For this reason, HO[•] is likely to oxidize molecules near its formation site (estimated half-life of 10⁻⁹ s, Pryor, 1986), and it is unfit to behave as a signaling molecule. $O_2^{\bullet-}$ is also regarded as a poor signaling effector because it does not permeate cell membranes and is quickly dismutated to H₂O₂, or it reacts with iron/sulfur clusters and nitric oxide. This results in low intracellular stability, hindering its diffusion across large distances. Therefore, while $O_2^{\bullet-}$ has some regulatory properties, these properties are possibly due to its role in nitric oxide and H₂O₂ metabolism (Brune, 2005; Thomas et al., 2006; D'Autréaux and Toledano, 2007; Kaewpila et al., 2008; Labunskyy and Gladyshev, 2013). In turn, H₂O₂ has emerged as the ROS that displays the best signaling properties. Its high stability and selective reactions with cellular compounds permit diffusion over distances of several micrometers (Winterbourn, 2008) and enable cell membrane crossing, which is also facilitated through specific channels (Bienert et al., 2007). H₂O₂ reacts with cellular thiols, including those contained in low molecular weight compounds, such as glutathione, and protein thiols, such as peroxiredoxins and thioredoxins (Box 1 and Figure 3). These reactions convert an oxidizing equivalent into a redox signal, which may be transduced from protein to protein via thiol disulfide exchange or between glutathione and proteins, forming mixed disulfides. Together with intracellular thiols, H₂O₂ regulates the redox state and activity of several target proteins and has pivotal importance in both physiological and pathological conditions (D'Autréaux and Toledano, 2007). Due to this intricate association, any discussion about the involvement of ROS in tumorigenesis also needs to consider the role of thiols and other antioxidant

BOX 1 | Thiols as important redox signaling sensors and effectors.

Cysteine's thiol side chains (R-SH) are often very reactive with H_2O_2 . They undergo a series of reversible or irreversible redox transitions, which are represented here through the catalytic cycles of a typical 2-Cys peroxiredoxin/thioredoxin (**Figure 3**). In this cycle, the reduced form is subsequently oxidized by H_2O_2 to sulfenic (R-SOH) and sulfinic (R-SO_2H) acids. The sulfinic form may be irreversibly oxidized to sulfonic (R-SO_3H) forms *in vitro*, but it is currently unclear whether this process occurs *in vivo*. Sulfinic acids may be reduced to sulfenic acids by specific proteins (e.g., sulfiredoxins, SRX) at the expense of ATP and the oxidation of TXN and GSH (Chang et al., 2004). Sulfenic acids may also conjugate to form intra- or inter-molecular disulfide bonds (R-SS-R'). Disulfides are then reduced at the expense of reducing equivalents, such as those found in NADPH (e.g., oxidized GSH or thioredoxin reduction by reductases), or by disulfide exchange with other proteins. Therefore, cysteine oxidation by H_2O_2 may be transduced to partner proteins or small molecular weight compounds.

Due to their chemical and kinetic properties, the systems above have potentially different signaling properties. Although they exhibit slow reactivities with H_2O_2 when isolated ($k \approx 2.9 \text{ M}^{-1} \text{ s}^{-1}$ for free Cys, Winterbourn and Metodiewa, 1999), some cysteine thiols display extremely high reactivities ($k \approx 10^5 - 10^8 \text{ M}^{-1} \text{ s}^{-1}$ for peroxiredoxins, Trujillo et al., 2007; Manta et al., 2009). GSH is very abundant, but it is relatively unreactive with H_2O_2 per se, so the kinetics of glutathione peroxidases should be considered when assessing glutathione's intracellular role in H_2O_2 detoxification and signaling. These differences in reactivity also manifest within the same pathway. Reduced and sulfenic forms quickly react with H_2O_2 , unlike sulfinic and sulfonic acids, which are relatively unreactive with H_2O_2 . The process of thiol oxidation to disulfide exchange may transduce oxidative equivalents to target proteins, as observed in the proteins above (Jarvis et al., 2012; Naticchia et al., 2013; Sobotta et al., 2015). For instance, thiol-disulfide exchange between peroxiredoxins and phosphatases/kinases is a mechanism for explaining H_2O_2 -induced signaling regulation despite the low reactivity of H_2O_2 with phosphatases/kinases (Ray et al., 2012; Marinho et al., 2014; Sobotta et al., 2015), Latimer and Veal, 2016). Importantly, peroxiredoxin-mediated disulfide exchange controls the activity of several proteins involved in cancer (Park et al., 2007; Jarvis et al., 2012; Sobotta et al., 2015), which reinforces the role of H_2O_2 and redox metabolism in this disease. Similar to disulfide exchange, the oxidation of glutathione function of glutathion dist regulation of proteins, which also regulates their activities (e.g., peroxiredoxin 2, Peskin et al., 2016). These and other properties possibly explain the involvement of PRDXs and TXNs in redox signaling, and they point toward these proteins as good redox sensors and signaling transducers (Benfeitas et al., 2014;

It should be noted that many of these redox systems depend on reducing equivalents to maintain their activity. Reducing equivalents are any molecules that act as electron donors in reactions, typically in reference to NADH and NADPH. These species are used by several enzymes, such as reductases, which couple their oxidation to the reduction of thioredoxin or glutathione. This provides another possible layer of selectivity in redox homeostasis: should NADPH utilization be prioritized toward one system over another, the physiological role of the former would also be prioritized over the latter. Interestingly, the link between energy metabolism and redox metabolism goes beyond NAD(P)H-enabling reductase activity. For instance, glyceraldehyde-3-phosphate dehydrogenase, an NADH-producing enzyme essential to glycolysis, is inactivated by glutathionylation and H_2O_2 -induced disulfide formation (Little and O'brien, 1969; Mohr et al., 1999). ROS and redox-coupled processes thus not only consume reducing equivalents but also regulate energy metabolism.


defenses. Although these systems have been widely studied in cancer, the role of ROS, antioxidant defenses, and redox signaling transducing partners in cancer is only now emerging beyond antioxidant activities. Further ROS-centered studies aimed at clarifying these properties and their involvement in cancer are still required.

ROS AS ONCOGENIC DRIVERS AND TARGETS OF THERAPEUTIC STRATEGIES

The role of ROS in cell physiology is highly dependent on their levels. Under physiological levels, ROS regulate a number of signaling processes by reacting with proteins, genes, and transcription factors. ROS control adaptation to hypoxia, regulation of differentiation, immunity, and longevity (Sena and Chandel, 2012). However, the accumulation of ROS beyond physiological levels promotes cell proliferation, angiogenesis, and even apoptosis (D'Autréaux and Toledano, 2007; Cairns et al., 2011; Figure 4), and ROS also control cell-cycle progression (Menon and Goswami, 2007). Oncogene-induced senescence promotes AMP-activated protein kinase activation, mitochondrial dysfunction and ROS production, which trigger senescence, thereby forming a positive feedback loop. Cancer cells display high ROS production (Szatrowski and Nathan, 1991; Ray et al., 2000), which is often also associated with antioxidant imbalances (Skrzydlewska et al., 2005). This results in damage to nuclear (Shibutani et al., 1991) and mitochondrial DNA (Ishikawa et al., 2008; Weinberg et al., 2010). Mutations in nucleic acids may be particularly toxic for the cell if they occur in tumor suppressors or oncogenes. DNA mutations (Higinbotham et al., 1992; Du et al., 1994), in turn, promote ROS generation, thereby resulting in a vicious cycle of ROS production and mutagenesis concomitant with high proliferation. Mitochondrial-generated ROS are also essential for tumor aggressiveness and metastasis (Ishikawa et al., 2008; Weinberg et al., 2010; Goh et al., 2011), and increased cytoplasmic and mitochondrial ROS levels are observed in metastatic nodules and circulating tumors when compared to subcutaneous tumors (Piskounova et al., 2015).



PIGDE 4 Targeting ROS nomeostasis as a strategy for changing cell fate. In proliferative conditions, such as cancer, targeting antioxidant systems could move the redox state of the cell to either promote normal redox homeostasis or apoptosis (strategies a and c, respectively). Unsuccessful tackling of antioxidant metabolism results in the cells maintaining a proliferative state, which potentially enhances malignancy. The three lines represent the high heterogeneity between individuals, tissues, and cancer types.

ROS also stabilize the factors that drive tumor initiation and progression (Gao et al., 2007), and promote protein oxidation and the formation of toxic protein carbonyls (Stadtman and Levine, 2003). Protein carbonylation is an irreversible process present in cancer cells (Thanan et al., 2012). Carbonyls may propagate to other proteins or lipids, which may result in the formation of toxic byproducts through chain reactions. ROS-induced lipid peroxidation products, such as 4-hydroxy-2nonenals, also have multiple physiological roles under low levels, but they become toxic upon accumulation. These species also accumulate in cancer cells (Skrzydlewska et al., 2005; Ayala et al., 2014; Zhong and Yin, 2015).

Due to the toxic effects of ROS accumulation in cancer cells and the fact that some ROS responses may be exclusive to cancer cells but not to their healthy counterparts (Hileman et al., 2004), antioxidants were envisioned as potentially important drug targets in cancer treatment. Initial ROS-focused clinical trials aimed to prevent ROS accumulation (Figure 4, strategy a). In the Linxian study (Blot et al., 1993) stomach cancer patients supplemented with selenium, vitamin E and β-carotene exhibited lower mortality. However, antioxidant-supplemented diets often failed to yield significant changes in cancer development, and in some cases, these diets even promoted tumorigenesis and metastasis (Omenn et al., 1996; Goodman et al., 2011; Klein et al., 2011; Sayin et al., 2014). This is because cancer cells often cope with increased ROS production by increasing the levels of antioxidant defenses or reducing equivalents that maintain their activity (Weinberg et al., 2010; DeNicola et al., 2011). This effect is also observed in metastases, where metastatic melanoma nodules are exposed to additional oxidative stress that is not observed in established subcutaneous tumors, and the nodules cope with this stress by promoting the expression of multiple NADPH-producing pathways (Piskounova et al., 2015). High antioxidant activities enable fast ROS-driven proliferation and metastasization, but the increased oxidative stress is insufficient to lead to apoptosis (Figure 4, strategy b). Higher antioxidant expression is also associated with the radioresistance observed

in certain cancer stem cell populations (Diehn et al., 2009). In turn, recent antioxidant-targeted therapeutic strategies have shifted their focus in the opposite direction, exploiting ROS toxicity as a means leading to cancer cell apoptosis (**Figure 4**, strategy c). These drugs often mimic ROS-generating enzymes (e.g., NADPH oxidases and superoxide dismutases), inhibit antioxidant enzymes (e.g., catalase), deplete thiol pools, such as GSH, or shift redox buffer ratios (e.g., GSSG/2 GSH), thereby promoting a more oxidizing intracellular state and cell apoptosis. These strategies have been discussed in great detail in recent reviews (Ushio-Fukai and Nakamura, 2008; Gupta et al., 2012; Gorrini et al., 2013; Tong et al., 2015).

The targets of many of these drugs are not completely understood, nor is it known whether the drugs are targeting the best redox effectors. Are the targeted compounds selective in treating cancer, but not healthy cells? Are they the best target in that pathway? Which combined drug treatments could improve treatment? These questions stem from an insufficient understanding of redox metabolism in tumorigenesis, which is greatly caused by the high variability in metabolic and redox responses. For instance, different antioxidant defenses that target the same ROS are up- and down-regulated in cancer cells (Skrzydlewska et al., 2005), complicating the interpretation of their role and that of their target ROS in cancer progression. This high variability is even observed between individuals with the same cancer type (Figure 5). Furthermore, genes involved in the same processes are differentially expressed. For instance, a high dispersion in gene expression levels is observed within peroxiredoxins (PRDX1-6), thioredoxins (TXN and TXN2), and thioredoxin reductases (TXNRD1-3), which are highly conserved protein systems involved in H_2O_2 scavenging and redox signaling transduction. As result of this variability, redox-focused therapeutic strategies (**Figure 4**) must consider patient-specific data to determine the best approach.

ROLE OF ANTIOXIDANT DEFENSES IN CANCER

Imbalances in antioxidant defenses are one of the hallmarks of cancer (Oberley and Oberley, 1997; Huang et al., 2000; Chungman Ho et al., 2001; Hu et al., 2005; Murawaki et al., 2008). For instance, increased expression of SOD has been observed in multiple cancers (Ray et al., 2000; Skrzydlewska et al., 2005; Holley et al., 2012; Miar et al., 2015) and metastatic tissues (Miar et al., 2015). Its overexpression promotes carcinogenesis (Lu et al., 1997) and aggressiveness (Hempel et al., 2011). In turn, SOD deficiency is associated with a higher cancer incidence and DNA damage in mice (Van Remmen et al., 2003; Elchuri et al., 2005), and it has also been observed in some cancers (Oltra et al., 2001). SOD levels fluctuate throughout the cell cycle and regulate growth factor cancer signaling (Nelson et al., 2003; Juarez et al., 2008), cell cycle progression, and the energetic changes of cells upon cancer transformation (Hempel et al., 2011; Sarsour et al., 2014). Catalases, often regarded as the main cellular defenses against H₂O₂ in human cells, may be up- or down-regulated in cancer cells (Ray et al., 2000; Oltra et al., 2001; Skrzydlewska et al., 2005; Glorieux et al., 2015). Catalase treatment of highly metastatic cancer cell lines decreases migration and invasion (Liu et al., 2012). Due to their antioxidant



downloaded from NCI's Genomic Data Commons, and fragments per kilobase transcript per million (FPKM) were computed. FPKM-values lower than one were considered to be non-expressed and were assigned a value of 0. The Log₂(FPKM + 1) were then computed. Bars are colored according to processes of the same pathway, as indicated on the left. Genes and respective proteins: CAT, catalase; GPX1-8, glutathione peroxidase; GSR, glutathione reductase; GCLM and GCLC, glutamate-cysteine ligase modifier and catalytic subunits, respectively; GSS, glutathione synthetase; PRDX1-6, peroxiredoxin; TXN and TXN2, thioredoxin; TXNRD1-3, thioredoxin reductase; GOD, superoxide dismutase; G6PD, glucose-6-phosphate dehydrogenase; TALDO1, transaldolase 1, TKT, transketolase, and PGD, 6-phosphogluconate dehydrogenase.

activities and correlation with decreased aggressiveness in certain tumors, catalases have been envisaged as potentially important therapeutic agents (Glorieux et al., 2011; de Oliveira et al., 2016). For instance, a recent study shows that catalase activity correlates well with the ability of pancreatic cancers to resist chemotherapeutic H₂O₂ treatment with ascorbate (Doskey et al., 2016). In turn, altered levels of glutathione peroxidases are amply reported in cancer (Lu et al., 1997; Skrzydlewska et al., 2005), and the reduction potential of glutathione is associated with the proliferative and apoptotic state of a cell (Buettner et al., 2013). For instance, several glutathione peroxidases are upregulated in hepatocellular cancer (Carlson et al., 2012), and GPX4-deficient mice die shortly after birth (Carlson et al., 2016). GPXs are responsible not only for reducing H₂O₂ but also for reducing other ROS, such as lipid peroxides (Thomas et al., 1990; Esworthy et al., 1993). Peroxiredoxins have also been associated with tumorigenesis. PRDX1-4 and 6 display significantly altered levels in some prostate cancers (Basu et al., 2011; Whitaker et al., 2013). PRDXs may act as tumor suppressors (Egler et al., 2005), and their increased gene expression is associated with metastasis and aggressiveness (Park et al., 2006; Chang et al., 2007; Stresing et al., 2012). For instance, PRDX2 is highly expressed in lung metastases, and its knockdown decreases the formation of lung metastasis (Stresing et al., 2012). PRDX2 is also highly expressed in breast carcinoma, which correlates with the formation of lung metastases. PRDXs are often upregulated in cancers, and they contribute to cancer survival and resistance to oxidative stress (Lu et al., 2014) and radiotherapy (Wang et al., 2005). Interestingly, knockouts of the PRDX genes do not always result in a favorable outcome. For instance, PRDX1 knockout mice show premature death, increased DNA oxidation and increased tumorigenesis and malignancy, and PRDX3 knockout results in increased protein carbonylation in adipose tissues (see Cao et al., 2009; Perkins et al., 2014 and references therein). These observations show the crucial role of antioxidant enzymes in cancerogenesis and survivability.

Alterations in antioxidant enzymes and ROS levels occur throughout cancer progression. Overexpression of mitochondrial SOD2 acts as a tumor suppressor in skin and breast cancers (Zhao et al., 2001; Robbins et al., 2012), suggesting that high SOD activity promotes tumor initiation. However, SOD levels also increase with tumor progression in these and other cancers (Ray et al., 2000; Chung-man Ho et al., 2001; Dhar et al., 2011; Miar et al., 2015), with similar changes occurring in other antioxidants, such as cytoplasmic SOD1 and catalase (Miar et al., 2015). Similarly, metastases show increased SOD2 protein levels when compared with matched tissues, less metastatic cell lines, or primary tumors (Liu et al., 2012; Miar et al., 2015). These changes are often accompanied by directly proportional changes in intracellular concentrations of H₂O₂, but they are not always followed by changes in other antioxidants. Changes in ratios of antioxidant proteins (e.g., SOD/catalase and SOD/GPX1) translate into differential intracellular concentrations of H₂O₂, and they vary with cancer stage and between metastatic and primary tumor cells (Miar et al., 2015). These observations show that while a comparison of the antioxidant expression levels between cancer and matched tissues is informative, such a comparison has to consider the developmental stage of the cancer.

Importantly, these antioxidant defenses are not found in all cell compartments, which should also differentially affect the responses of cancer cells to therapeutic targeting. For instance, mitochondria rely on superoxide dismutase 2, peroxiredoxin 3, thioredoxin 2, and thioredoxin reductase 2 for antioxidant defense and signaling transduction, but they do not rely on catalase (Rabilloud et al., 2001; Jones, 2006). Considering the crucial role that mitochondrial-generated ROS have in cancer initiation, progression, and apoptosis, the targeting of key antioxidant and redox signaling transduction systems in this compartment (e.g., PRDX3 Li and Yu, 2015) may have important consequences for global cell metabolism. However, it should be noted that targeting these antioxidants may lead to compensatory responses by the other antioxidants. For instance, knockdown of PRDX3 promotes the upregulation of other peroxiredoxins, including those located in the cytoplasm (PRDX1-2 and PRDX6; Li et al., 2008, 2013; Goncalves et al., 2012). This is particularly important because these proteins may catalyze similar reactions in H₂O₂ scavenging and disulfide exchange, albeit with different mechanisms and specificities (Perkins et al., 2014).

In addition, cancer cells cope with increased intracellular ROS by promoting the synthesis of compounds that enable their activity (e.g., glutathione for GPXs, Buettner et al., 2013 and TXNs for PRDXs, Arnér and Holmgren, 2006; Kaimul et al., 2007). GSSG/GSH ratios are higher in many cancers (Oltra et al., 2001; Skrzydlewska et al., 2005), in metastatic nodules and in circulating tumor cells (Piskounova et al., 2015), and the enzymes involved in GSH recycling or synthesis are often upregulated (Carlson et al., 2012; Harris et al., 2015; Lien et al., 2016) as response to oxidative stress. Mutations in PI(3)K/Akt, which are common in some cancer types, such as breast cancer, stabilize and activate NFE2L2, thereby promoting the upregulation of enzymes involved in synthesizing or reducing GSH (glutathione synthetase GSS, and glutathione reductase GSR; Lien et al., 2016). Upregulation of GSS and GSR is also associated with the increased resistance to oxidative stress observed in breast cancer. The inhibition of GSH biosynthesis sensitizes cancer cells to H₂O₂ and is potentiated by the utilization of other antioxidant inhibitors (Lien et al., 2016). High intracellular GSH concentrations also block drug-induced cytotoxicity in myeloma cells (Starheim et al., 2016). Cumulative evidence thus points toward GSH biosynthesis and homeostasis as a therapeutic target. However, in some cancers, inhibition of the GSH pathway alone does not prevent tumor progression. In addition to GSS, GSH is also synthesized by glutamatecysteine ligase (GCL), an enzyme that consists of a heavy catalytic (GCLC) subunit and a light regulatory (modifier, GCLM) subunit. In mouse models of breast cancer, GCLMdeficiency or GLCM inhibition by Buthionine-[S,R]-sulfoximine (BSO) significantly prevented cancer initiation. However, this effect occurs only before tumor onset, and BSO treatment after onset does not alter tumor burden due to a compensatory role of TXN (Harris et al., 2015). GSH and TXN both serve as substrates for proteins with redox-important roles, including glutathione peroxidases, peroxiredoxins, glutathione and thioredoxin reductases, glutaredoxins, and sulfiredoxins (Björnstedt et al., 1994; Sun et al., 2001; Chang et al., 2004; Johansson et al., 2004; Peskin et al., 2016). Several cancers display increased expression of *TXN* and thioredoxin reductase 1 (*TXNRD1*) to compensate for GSH deficiency in *GCLM*^{-/-} cells (Mandal et al., 2010; Harris et al., 2015). The inverse is also observed, where *TXNRD1*-deficiency promotes the expression of *GSR* and *GCLC*, but not of *PRDX1*, *CAT*, or *SOD1*, in liver cancer cells (Carlson et al., 2012). Given that GSH and thioredoxins are relatively unreactive with ROS *per se* (Chae et al., 1994; Winterbourn and Metodiewa, 1999), the promotion of GSH or TXN biosynthesis is possibly promoting the peroxidase activities of GPX and PRDX.

GLCM-deficient cells also present increased expression of the cystine transporters and stabilizers XCT and CD44 (Lu et al., 2015). This increased import is used toward promoting cysteine biosynthesis, which, in turn, is used toward TXN biosynthesis. Other observations show that chemotherapy treatment promotes XCT and GCLM expression with a concomitant increase in GSH biosynthesis in a HIF1-dependent mechanism related to therapeutic resistance (Lu et al., 2015). Targeting XCT, GCLM, and other pluripotency-involved transcription factors impaired malignant transformation. It is currently unclear whether other cysteine-based antioxidants, such as peroxiredoxins, also benefit from increased cystine import. Importantly, it was also observed that TXNs and thioredoxin reductases are upregulated and co-localize in several cancers, particularly in more aggressive cancers (Soini et al., 2001; Lincoln et al., 2003). These enzymes are associated with tumor initiation (Shen et al., 2016), and they correlated with worse prognosis (Cadenas et al., 2010). TXNRD1 knockdown significantly slowed tumor progression and metastasis in lung carcinomas (Yoo et al., 2006), but it promoted cancer incidence in liver (Carlson et al., 2012).

Due to their chemical similarities and synergistic properties, recent approaches have simultaneously targeted both TXN and GSH metabolism, and they are significantly more effective at reducing tumor volumes than when they are applied individually (Harris et al., 2015). Multiple other studies have shown this efficacy in combination with common cancer drugs or radiotherapy for multiple cancers (e.g., Lu et al., 2007; Sobhakumari et al., 2012; Rodman et al., 2016; Roh et al., 2016; Tanaka et al., 2016). Together, the intracellular redox states expressed in terms of reducing equivalents and thiol compounds (Box 1) not only influence several signaling processes but also control the reactivities of ROS and their redox partners by regulating ROS homeostasis. It is currently unclear whether the involvement of some of these antioxidant defenses in cancer is related to their detoxification role, redox signaling properties or both. For instance, PRDX2 has increased levels in some cancer cells, which correlates with lower cytoplasmic H₂O₂ concentrations and cellular resistance to oxidative stress (Stresing et al., 2012). PRDX2 displays peroxidase activity and is also involved in redox signaling transduction (Neumann and Fang, 2007), such as the positive regulation of JNK-dependent DNA repair (Lee et al., 2011). Since these H_2O_2 -scavenging and redox signaling properties stem from the kinetic properties of PRDX2/TXN/TXNRD1, GPX1/GSH/GSR, and catalase systems (Benfeitas et al., 2014; Tomalin et al., 2016), further studies

are required to understand whether the culprit of PRDX2's tumorigenic association is its role as a peroxidase, chaperone, or redox signaling transducer. It is unclear whether the alterations in GSH/TXN biosynthesis and PRDX/GPX levels are more important toward controlling redox signaling, detoxification, or both. For instance, some observations indicate that sulfiredoxins and peroxiredoxins promote tumor growth and metastasis by modulating phosphokinase signaling cascades (Wei et al., 2011). Do these proteins directly interact with their targets? PTEN binds to PRDX1, but not to PRDX2, and this promotes Akt-mediated proliferation (Cao et al., 2009). The PRDX1-PTEN complex dissociates upon H₂O₂-mediated oxidation. A localized accumulation of H2O2, such as that occurring near cell membranes or near ROS sources, would thereby alter PTEN-mediated signaling transduction and proliferation by direct PRDX1 oxidation (Woo et al., 2010) or by relaying a redox signal from another more abundant, H₂O₂-reactive PRDX. PRDX2 is a good candidate as a H₂O₂ sensor due to its high reactivity with H₂O₂ ($k \approx 10^7 - 10^8$ M⁻¹ s⁻¹), and it was recently observed to transmit oxidative equivalents to the transcription factor STAT3 (Sobotta et al., 2015), thereby controlling tumor proliferation and survival (Yu et al., 2014). While disulfide exchange between PRDX1 and 2 remains to be shown, the above observations indicate that the direct reactions of peroxiredoxins with transcription factors are important proliferative processes controlled by H₂O₂. It also remains to be seen whether the promotion of TXN biosynthesis (Harris et al., 2015), which is often linked to added ROS protection, is instead enabling secondary signaling transduction reactions, and whether multiple PRDX isoforms act synergistically in this process. Studying cancer ROS metabolism should therefore consider the toxicity of these oxidants and signaling disruption.

Overall, the observations above highlight important features that should be considered in cancer studies. First, targeting one antioxidant defense may elicit compensatory behaviors by other antioxidant defenses. Second, the close relationship between antioxidant proteins (e.g., GPX and PRDX) and their redox partners (e.g., GSH and TXN) requires that the choice of suitable therapeutic targets considers possible synergisms between them. Third, the high variability in responses, even for the same tumor, requires that cancer treatment is designed in a case- and stage-specific manner, rather than a cancertype approach. Finally, all of these considerations need to be considered to identify antioxidant pathways that are differentially regulated by cancer, but not by normal cells. The targeting of antioxidant defenses as an approach for cancer treatment should therefore require tissue- and subject-specific phenotypic characterization.

ENERGETIC CHANGES ARE COUPLED WITH MAINTENANCE OF THE ANTIOXIDANT ACTIVITY IN CANCER

Cancer cells display increased glycolytic activity and lower mitochondrial oxidative phosphorylation. Glucose uptake by breast, liver, colorectal, lung, and pancreatic cancers may reach 8–15 times the fluxes observed by surrounding normal

tissues (see Boros et al., 1998 and references therein). This metabolic shift, characterized by increased ATP production from glycolytic pathways rather than respiratory pathways, even under aerobiosis, is one of the most well-known metabolic hallmarks of cancer cells, and it is generally referred to as the Warburg effect (Warburg, 1956). This process is crucial to maintaining the high energetic demand of fast proliferative cells. However, the energetic changes extend beyond the Warburg effect and are intimately related to the redox responses of cancer cells. This is the case of the metabolic changes that alter NADPH production. For instance, the MYC-controlled expression of pyruvate kinase type M2 (PKM2) is higher in cancer cells and promotes the diversion of carbohydrate metabolism from glycolytic pathways to other pathways (Vander Heiden et al., 2009), including the pentose phosphate pathway (PPP). Carbohydrates are thus diverted from ATP production to generate reducing equivalents and building blocks, such as phosphopentoses and ribonucleotides, supporting the fast proliferation of cancer cells (Boros et al., 1997; Raïs et al., 1999). Because ROS also regulate carbohydrate metabolism (Robbins et al., 2012; Hart et al., 2015; Miar et al., 2015) and some enzymes couple redox metabolism and ATP phosphorylation (Chang et al., 2004), the crosstalk between energetic and redox metabolism extends beyond enabling NADPH-driven peroxidase antioxidant activities.

Many of the glycolytic and PPP enzymes that are involved in NADPH production are elevated in cancer cells. Glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD), which are both enzymes of the oxidative branch of PPP, catalyze the production of NADPH from hexoses entering the PPP from either glycolysis or the non-oxidative PPP. G6PD's activity is promoted by multiple oncogenic pathways upregulated in cancer (Stanton et al., 1991; Tian et al., 1994; Au et al., 2000; Wang et al., 2012; Zhang et al., 2014), and multiple studies have proposed that G6PD has pro-oncogenic activities (Wang et al., 2012; Patra and Hay, 2014; Zhang et al., 2014). G6PD overexpression leads to higher levels of intracellular NADPH, GSH, and nucleotide precursors, increased health span, and lower nucleotide oxidation (Nóbrega-pereira et al., 2016). These observations provide a clear link between PPP-mediated NADPH production and oxidative stress. In turn, G6PD deficiency severely limits cell resistance to oxidative stress (Pandolfi et al., 1995) and promotes oxidative damage to DNA (Jeng et al., 2013). These observations raise the hypothesis that a targeted inhibition of G6PD may be conducive to oxidative imbalance and ROS-mediated cell death. Considering that G6PD catalyzes the first and rate-limiting step of PPP and that it has a role in controlling the intracellular redox environment, this enzyme has been envisaged as one of the potentially most important therapeutic cancer redox targets (Wang et al., 2012; Patra and Hay, 2014; Zhang et al., 2014; Nóbrega-pereira et al., 2016), and it has been included in pre-clinical trials (Budihardjo et al., 1998; De Preter et al., 2015). 6PGD is also upregulated in many cancers, including thyroid (Giusti et al., 2008), lung (Sukhatme and Chan, 2012), and cervical (Jonas et al., 1992) tumors. This enzyme is important for proliferation and tumor growth (Sukhatme and Chan, 2012; Shan et al., 2014), and its inhibition promotes senescence in lung cancer (Sukhatme and Chan, 2012). This phenotype results from altered glucose levels, but not altered NADPH levels (Sukhatme and Chan, 2012; Lin et al., 2015), suggesting that NADPH metabolism, and ultimately glucose metabolism, may adapt in such a way that compensates for the selective targeting of the PPP's enzymes. Interestingly, 6PGD suppression limits lipid biosynthesis and elevates intracellular ROS levels, and this effect translates into decreased tumor growth (Lin et al., 2015). However, conflicting observations regarding its importance in cancerogenesis (Sukhatme and Chan, 2012; Lin et al., 2015) also suggest that its role may vary depending on tissue and oncogenic background (Lin et al., 2015).

Other enzymes also promote downstream NADPH production. For instance, transketolase (TKT) and transaldolase (TALDO) are both enzymes of the non-oxidative PPP. While neither of them catalyze NADPH production, they are both important in directing the phosphorylated pentoses generated in the PPP back to glycolysis. Both enzymes are upregulated in cancer (Heinrich et al., 1976; Liu et al., 2010). TKT is required for cancer growth and controls resistance to oxidative stress by modulating NADPH levels. Its inhibition leads to higher intracellular ROS and decreased NADPH/NADP+ ratios (Xu et al., 2016), and it sensitizes cells to drug treatment. Importantly, TKT knockdown increases oxidative PPP fluxes, but it also leads to lower NADPH levels, which is a striking observation considering that NADPH is produced through the oxidative PPP; this point remains to be clarified. TALDO's expression is linked to metastasis in hepatocellular carcinoma (Wang et al., 2011). TALDO deficiency also elicits hepatocellular carcinoma and promotes the formation of malignant tumors (Hanczko et al., 2009). These outcomes are associated with redox imbalances (lower NADPH and GSH levels) due to the insufficient recycling of PPP metabolites to support NADPH production, and they are reverted with dietary supplementation of antioxidants. This insufficient recycling exposes the liver to added oxidative stress and decreases lifespan. Other NADPH-producing enzymes are also upregulated in cancer cells, and some of the isoforms are exclusive to proliferating cells (Mazurek et al., 2005), suggesting that these enzymes may be selective therapeutic targets. Altered glycolytic and PPP metabolism has been proposed for potential therapeutic targeting in cancer (Wang et al., 2012; Patra and Hay, 2014; Zhang et al., 2014; Wen et al., 2015; Hay, 2016). Importantly, the crucial role of the PPP in cancer development seems to be associated with its redox homeostasis properties rather than its production of ribonucleotide precursors, as observed in hepatocellular carcinoma (Xu et al., 2016). Further studies are required to understand whether similar observations are present in other cancers.

While the oxidative PPP represents the main source of cytoplasmic NADPH in proliferating cells (Fan et al., 2014), other sources contribute significantly. Serine (Mehrmohamadi et al., 2014), folate (Tedeschi et al., 2013; Fan et al., 2014; Piskounova et al., 2015), and malate (Jiang et al., 2013) pathways also produce and regenerate NADPH and have crucial roles in maintaining the redox status and buffering oxidative stress in cancer cells. A system-wide comparison the contributions of these pathways to NADPH production and ROS metabolism in cancer is beginning

to emerge (e.g., Tedeschi et al., 2013; Mehrmohamadi et al., 2014). In the context of antioxidant defense, most of these studies have focused on GSH-mediated ROS protection due to the close relationship between the PPP, serine/glycine metabolism, and de novo GSH biosynthesis. However, an analysis addressing the role of these pathways in supporting the activities of other important antioxidant defenses and redox signaling processes is currently missing. This becomes an important issue because defenses have different reducing equivalent requirements for activity, which also reflect their different antioxidant capacities and redox signaling roles. For instance, while antioxidant systems like PRDX/TXN/TXNRD and GPX/GSH/GSR stoichiometrically couple ROS detoxification to NADPH consumption, others, such as catalase, scavenge ROS while oxidizing virtually no NADPH. In normal cells, where energetic metabolism is limited, NADPH must be utilized toward lipid and cholesterol biosynthesis, and ROS decomposition by NADPH-consuming systems is thus an energetically expensive process. In cancer cells, where NADPH-producing fluxes are promoted, NADPH may be sufficiently abundant for cells to afford utilizing PRDX- or GPX-mediated detoxification. Importantly, the differences in the energetic requirements and kinetics of the PRDX/TXN/TXNRD, GPX/GSH/GSR, and catalase systems become particularly important if cancer treatments are targeting ROS metabolism by inhibiting NADPH production. ROS defenses may then be maintained by catalase or other energetically inexpensive processes, which is similar to what is observed in non-cancerous cells (Johnson et al., 2005; Benfeitas et al., 2014). Nevertheless, the lower cancer progression and increased ROS levels induced by the inhibition of NADPH-producing pathways indicate that this is a viable cancer therapy. A further understanding of the energetic requirements of antioxidant defense (e.g., PRDX/TXN vs. GSH/GPX pathways) may permit an efficient combination of anti-oxidant- and energetic-focused drug utilization for effective cancer treatment. Importantly, the heterogeneous gene expression and synergistic responses that may occur between alternative metabolic pathways at different cancer stages require an assessment of possible targets that considers specific oncogenic backgrounds.

SYSTEMS BIOLOGY AND PERSONALIZED MEDICINE APPROACHES ARE FUNDAMENTAL TO REVEALING REDOX RESPONSE IN CANCER

The observations above highlight the extensive crosstalk within and between ROS detoxification, redox signaling transduction, energy metabolism, and central metabolism. As such, the therapeutic targeting of cancer is more effectively strategized by addressing multi-pathway dysregulation (Pawson and Linding, 2008). Thus, while targeting the activity of specific enzymes may yield promising results *in vitro* and to a certain extent, *in vivo*, methods that encompass global metabolism are required to devise viable, cancer-specific treatment targets. Furthermore, these redox responses are highly heterogeneous, as has been observed by the different redox responses displayed by different cancer types, between individuals with the same cancer type, and between different cancer stages. Finding the best targets (**Figure 4**) and elucidating the mechanisms behind cancer phenotypes hence requires integrative analysis of a large number of biological networks, together with tissue- and patient-tailored data.

Systems biology aims at analyzing assorted biological data (e.g., genomics, proteomics, metabolomics, fluxomics), and it has consistently assisted in understanding the complex underlying mechanisms in health and disease (Mardinoglu and Nielsen, 2012; Agren et al., 2014; Benfeitas et al., 2014; Ghaffari et al., 2015b; Mardinoglu et al., 2017). Using systems biology approaches, others successfully clarified the role of small molecular decision circuits (e.g., Faratian et al., 2009; Gaglio et al., 2011; Tyson et al., 2011) and found commonalities across different cancers through pan-cancer analyses (e.g., Weinstein et al., 2013; Zack et al., 2013; Leiserson et al., 2014; Aran et al., 2015; Peng et al., 2015). However, few studies have addressed ROS metabolism using systems approaches (Zhang et al., 2010; Zhou et al., 2011; Zhan et al., 2012). For instance, modeling of the NFE2L2 pathway suggests that the high NFE2L2 expression that typically occurs in cancer cells promotes chemoresistance (Zhan et al., 2012) and suggests apparently opposite roles in antioxidant and ROS-mediated cancer signaling (Zhang et al., 2010). However, certain antioxidants, such as peroxiredoxins, display both scavenging and redox sensor and signaling transducer properties (Box 1). Mathematical modeling of H₂O₂ metabolism indicates that cancer-related transcription factors are unlikely to be activated by direct reaction with H₂O₂, and it points toward protein thiols as the likely signaling sensors and transducers. Others have observed that the glutathione and NADPH synthesis pathways are simultaneously up- or down-regulated in breast, ovary, colon and lung cancers, establishing important interactions with de novo nucleotide synthesis (Mehrmohamadi et al., 2014), which suggests that cancers utilize redox homeostasis and biosynthesis pathways in parallel. ROS and redox responses of cancer cells have yet to be more extensively examined.

With the advent of big data, recent approaches aimed at understanding cell metabolism now incorporate large reaction networks derived from omics technologies (genomics, transcriptomics, proteomics, and others). By encompassing whole-cell reaction networks, GEMs have helped identify important redox alterations in metabolic diseases and physiological processes. For instance, disturbed H₂O₂ metabolism is observed (Mardinoglu et al., 2017) in nonalcoholic fatty liver disease, specifically due to deficient GSH biosynthesis (by GCLC/GCLM, GSR) and NADPH production (NNT). A lower abundance of plasma glycine, a substrate for de novo GSH biosynthesis, is also found in subjects with high hepatic steatosis. Observations in mice (Mardinoglu et al., 2015b) indicate that commensal gut microbes decrease glycine availability in the gastro-intestinal tract of the host, which results in decreased de novo GSH synthesis and promotes NNT and GSR expression, possibly to compensate for the decreased GSH pool. In the context of cancer, publicly deposited genomic data permit the stratification of cancer patients based on network-specific

mutations (Hofree et al., 2013), and these data have been used to find biomarkers and potential cancer therapeutic targets (Jerby and Ruppin, 2012; Agren et al., 2014). Others have combined experimental and interactome data with stochastic modeling to find that ROS and DNA damage are necessary and sufficient for senescent growth arrest (Passos et al., 2010). Chronic, non-toxic ROS supplementation reverses drug resistance in carcinoma cells (Maiti, 2010), which, through pathway analysis, identified several genes (e.g., TP53, Rac/Cdc42 guanine nucleotide exchange factor 6 ARHGEF6, and a DNA-activated protein kinase PRKDC) that mediate ROS-related apoptosis. Reconstruction of a generic human whole-cell GEMs encompassing >9,000 reactions and >3,000 metabolites and genes (Mardinoglu et al., 2014a) enabled context-specific integration and interpretation of cancer omics data. Comparisons between hepatocellular carcinoma and healthy liver samples using these models indicate that tumors display an increased abundance of NADPH-producing

enzymes (e.g., ME1, G6PD, TALDO1, and TKT) and that H₂O₂ may be used as reporter metabolite in some of the patients (Bjornson et al., 2015). Personalized medicine approaches would greatly benefit from clarifying redox cancer responses. GEMs were previously used to identify novel anticancer drugs by reconstructing patient- and tissue-specific GEMs (Agren et al., 2014) and to identify anti-growth factors in human cancer cell lines (Ghaffari et al., 2015a). Cell-specific GEMs have also been used to identify malonyl-CoA decarboxylase, an important enzyme in fatty acid metabolism, as a selective and effective cancer therapeutic target (Yizhak et al., 2014a). Its inhibition drains reducing equivalents, decreases GSH/GSSG ratios, and promotes oxidative stress, which may help in chemotherapeutic approaches. Patient-specific breast and lung cancer GEMs were also able to predict patient survivability, and they predicted that knockout of GSR significantly affects cancer growth.



cancer. Tumor profiling of patients may help in identifying up- and down-regulated pathways (continuous and dashed arrows, respectively) that are suitable for therapeutic targeting. Drug targeting of specific processes (red arrows), either to promote or inhibit the processes, will permit alterations in the consequences of redox processes in cancer and other diseases.

Interestingly, despite an enzyme- or process-specific focus in redox cancer metabolism in recent years, system-wide studies of ROS and redox metabolism and their interactions with central metabolism in multiple cancers are lacking. Antioxidant profiling of tumor cells and their surrounding cells may be used in conjunction with patient-specific GEMs to identify the best therapeutic targets in this disease (Figure 6). Studies using reaction network information with tissue- and patientspecific models will be useful in (1) Redox profiling of patientspecific cancer tissues; (2) Understanding mechanistic properties of redox responses; (3) Devising effective, selective and patientspecific therapeutic strategies to regulate redox responses; (4) Establishing redox-based therapies to synergize with existent drugs (Kasiappan and Safe, 2016) and identifying and averting drug-resistance mechanisms; and (5) Shortening the gap between pre-clinical and clinical trials, potentially overcoming issues faced by previous trials, such as those that assessed the impact of dietary antioxidants on cancerogenesis (Omenn et al., 1996; Goodman et al., 2011; Klein et al., 2011; Sayin et al., 2014). The adopted strategies (Figure 6) will benefit from patientspecific tumor profiling to identify single or multiple targetable processes within the same pathway or to identify processes that serve as metabolic central hubs. The combination of these approaches with drugs that target other metabolic processes may promote desirable synergisms. Redox systems medicine is thus an interesting emerging field with potentially important implications for disease treatment.

CONCLUSION

Redox, energetic and central metabolism are closely intertwined, and the view that ROS are simple secondary products of cell metabolism is long gone. Together with their redox partners, ROS and antioxidant defenses are now regarded as crucial processes in tumorigenic initiation, progression and

REFERENCES

- Agren, R., Bordel, S., Mardinoglu, A., Pornputtapong, N., Nookaew, I., and Nielsen, J. (2012). Reconstruction of genome-scale active metabolic networks for 69 human cell types and 16 cancer types using INIT. *PLoS Comput. Biol.* 8:e1002518. doi: 10.1371/journal.pcbi.1002518
- Agren, R., Mardinoglu, A., Asplund, A., Kampf, C., Uhlen, M., and Nielsen, J. (2014). Identification of anticancer drugs for hepatocellular carcinoma through personalized genome-scale metabolic modeling. *Mol. Syst. Biol.* 10, 1–13. doi: 10.1002/msb.145122
- Aran, D., Sirota, M., and Butte, A. J. (2015). Systematic pan-cancer analysis of tumour purity. *Nat. Commun.* 6:8971. doi: 10.1038/ncomms9971
- Arnér, E. S. J., and Holmgren, A. (2006). The thioredoxin system in cancer. Semin. Cancer Biol. 16, 420–426. doi: 10.1016/j.semcancer.2006. 10.009
- Arnold, R. S., Shi, J., Murad, E., Whalen, A. M., Sun, C. Q., Polavarapu, R., et al. (2001). Hydrogen peroxide mediates the cell growth and transformation caused by the mitogenic oxidase Nox1. *Proc. Natl. Acad. Sci. U.S.A.* 98, 5550–5555. doi: 10.1073/pnas.101505898
- Au, S. W. N., Gover, S., Lam, V. M. S., and Adams, M. J. (2000). Human glucose-6phosphate dehydrogenase: the crystal structure reveals a structural NADP(+) molecule and provides insights into enzyme deficiency. *Struct. Fold. Des.* 8, 293–303. doi: 10.1016/S0969-2126(00)00104-0

aggressiveness. However, the redox cancer responses are highly heterogeneous, manifesting not only between different cancer types but also between patients who suffer from the same cancer, and they are altered throughout cancer progression. The influence of ROS on different biochemical levels makes it necessary to seek an integrative analysis of these systems at the genomic, proteomic, and metabolomic levels. Approaches that are able to encompass these levels and integrate the crosstalk between antioxidant, redox, energetic, and central metabolism are able to capture and understand these complex responses. Systems biology approaches may be used to analyze omics data and understand the roles of each redox system in cancer. These approaches may be tissue- and patient-tailored, which enables the identification of the best therapeutic targets, while taking in account patient-specific oncogenic backgrounds. This is the aim of personalized systems medicine (Mardinoglu and Nielsen, 2015; Schork, 2015), an emerging field that presents a high potential to overcome some of the problems in therapeutic treatments, including the low (<25%) drug efficacies caused by population-rather than patient-wise data (Schork, 2015). Together with existing drugs, novel or existing redox-targeting drugs may be identified to produce synergistic responses for the treatment or prevention of cancer. Personalized medicine may thus enable an understanding of the role of redox systems in cancer and other diseases and may assist in drug discovery.

AUTHOR CONTRIBUTIONS

RB has written the manuscript. All authors actively contributed in writing and editing of the manuscript.

FUNDING

This work was financially supported by the Knut and Alice Wallenberg Foundation.

- Ayala, A., Muñoz, M. F., and Argüelles, S. (2014). Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2nonenal. Oxid. Med. Cell. Longev. 2014:360438. doi: 10.1155/2014/360438
- Babior, B. M., Lambeth, J. D., and Nauseef, W. (2002). The neutrophil NADPH oxidase. Arch. Biochem. Biophys. 397, 342–344. doi: 10.1006/abbi.2001.2642
- Bannister, J. V., Bannister, W. H., Bray, R. C., Fielden, E. M., Roberts, P. B., and Rotilio, G. (1973). The superoxide dismutase activity of human erythrocuprein. *FEBS Lett.* 32, 303–306. doi: 10.1016/0014-5793(73)80859-2
- Basu, A., Banerjee, H., Rojas, H., Martinez, S. R., Roy, S., Jia, Z., et al. (2011). Differential expression of peroxiredoxins in prostate cancer: consistent upregulation of PRDX3 and PRDX4. *Prostate* 71, 755–765. doi:10.1002/pros.21292
- Benfeitas, R., Selvaggio, G., Antunes, F., Coelho, P. M., and Salvador, A. (2014). Hydrogen peroxide metabolism and sensing in human erythrocytes: a validated kinetic model and reappraisal of the role of peroxiredoxin II. *Free Radic. Biol. Med.* 74, 35–49. doi: 10.1016/j.freeradbiomed.2014.06.007
- Bielski, B. H., and Shiue, G. G. (1979). "Reaction rates of superoxide radicals with the essential aminoacids," in *Oxygen Free Radicals and Tissue Damage*, ed I. Fridovich (Amsterdam: Excerpta Medica), 43–56.
- Bienert, G. P., Møller, A. L. B., Kristiansen, K. A., Schulz, A., Møller, I. M., Schjoerring, J. K., et al. (2007). Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. J. Biol. Chem. 282, 1183–1192. doi: 10.1074/jbc.M603761200

- Bjornson, E., Boren, J., and Mardinoglu, A. (2016). Personalized cardiovascular disease prediction and treatment—a review of existing strategies and novel systems medicine tools. *Front. Physiol.* 7:2. doi: 10.3389/fphys.2016.00002
- Bjornson, E., Mukhopadhyay, B., Asplund, A., Pristovsek, N., Cinar, R., Romeo, S., et al. (2015). Stratification of hepatocellular carcinoma patients based on acetate utilization. *Cell Rep.* 13, 2014–2026. doi: 10.1016/j.celrep.2015.10.045
- Björnstedt, M., Xue, J., Huang, W., Åkesson, B., and Holmgren, A. (1994). The thioredoxin and glutaredoxin systems are efficient electron donors to human plasma glutathione peroxidase. J. Biol. Chem. 269, 29382–29384.
- Blot, W. J., Li, J.-Y. Y., Taylor, P. R., Guo, W., Dawsey, S., Wang, G.-Q., et al. (1993). Nutrition intervention trials in linxian, china: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population. J. Natl. Cancer Inst. 85, 1483–1492. doi: 10.1093/jnci/85.18.1483
- Boros, L. G., Lee, P. W., Brandes, J. L., Cascante, M., Muscarella, P., Schirmer, W. J., et al. (1998). Nonoxidative pentose phosphate pathways and their direct role in ribose synthesis in tumors: is cancer a disease of cellular glucose metabolism? *Med. Hypotheses* 50, 55–59. doi: 10.1016/S0306-9877(98)90178-5
- Boros, L. G., Puigjaner, J., Cascante, M., Lee, W. N. P., Brandes, J. L., Bassilian, S., et al. (1997). Oxythiamine and dehydroepiandrosterone inhibit the nonoxidative synthesis of ribose and tumor cell proliferation. *Cancer Res.* 2, 4242–4248.
- Brown, G. C., and Borutaite, V. (2012). There is no evidence that mitochondria are the main source of reactive oxygen species in mammalian cells. *Mitochondrion* 12, 1–4. doi: 10.1016/j.mito.2011.02.001
- Brune, B. (2005). The intimate relation between nitric oxide and superoxide in apoptosis and cell survival. *Antioxid. Redox Signal.* 7, 497–507. doi: 10.1089/ars.2005.7.497
- Budihardjo, I. I., Walker, D. L., Svingen, P. A., Buckwalter, C. A., Desnoyers, S., Eckdahl, S., et al. (1998). 6-Aminonicotinamide sensitizes human tumor cell lines to cisplatin. *Clin. Cancer Res.* 4, 117–130.
- Buettner, G. R., Wagner, B. A., and Rodgers, V. G. J. (2013). Quantitative redox biology: an approach to understand the role of reactive species in defining the cellular redox environment. *Cell Biochem. Biophys.* 67, 477–483. doi: 10.1007/s12013-011-9320-3
- Buxton, G. V., Greenstock, C. L., Helman, W. P., and Ross, A. B. (1988). Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals (· OH/· O–) in aqueous solution. J. Phys. Chem. Ref. Data 17, 513–886. doi: 10.1063/1.555805
- Bystrom, L. M., and Rivella, S. (2015). Cancer cells with irons in the fire. *Free Radic. Biol. Med.* 79, 337–342. doi: 10.1016/j.freeradbiomed.2014.04.035
- Cadenas, C., Franckenstein, D., Schmidt, M., Gehrmann, M., Hermes, M., Geppert, B., et al. (2010). Role of thioredoxin reductase 1 and thioredoxin interacting protein in prognosis of breast cancer. *Breast Cancer Res.* 12, R44. doi: 10.1186/bcr2599
- Cairns, R. A., Harris, I. S., and Mak, T. W. (2011). Regulation of cancer cell metabolism. *Nat. Rev. Cancer* 11, 85–95. doi: 10.1038/nrc2981
- Cao, J., Schulte, J., Knight, A., Leslie, N. R., Zagozdzon, A., Bronson, R., et al. (2009). Prdx1 inhibits tumorigenesis via regulating PTEN/AKT activity. *EMBO J.* 28, 1505–1517. doi: 10.1038/emboj.2009.101
- Carlson, B. A., Tobe, R., Yefremova, E., Tsuji, P. A., Hoffmann, V. J., Schweizer, U., et al. (2016). Glutathione peroxidase 4 and vitamin E cooperatively prevent hepatocellular degeneration. *Redox Biol.* 9, 22–31. doi: 10.1016/j.redox.2016.05.003
- Carlson, B. A., Yoo, M. H., Tobe, R., Mueller, C., Naranjo-Suarez, S., Hoffmann, V. J., et al. (2012). Thioredoxin reductase 1 protects against chemically induced hepatocarcinogenesis via control of cellular redox homeostasis. *Carcinogenesis* 33, 1806–1813. doi: 10.1093/carcin/bgs230
- Chae, H. Z., Chung, S. J., and Rhee, S. G. (1994). Thioredoxin-dependent peroxide reductase from yeast. J. Biol. Chem. 269, 27670–27678.
- Chance, B., Sies, H., and Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* 59, 527–605.
- Chang, T.-S., Jeong, W., Hyun, A. W., Sun, M. L., Park, S., Sue, G. R., et al. (2004). Characterization of mammalian sulfiredoxin and its reactivation of hyperoxidized peroxiredoxin through reduction of cysteine sulfinic acid in the active site to cysteine. J. Biol. Chem. 279, 50994–51001. doi: 10.1074/jbc.M409482200

- Chang, X. Z., Li, D. Q., Hou, Y. F., Wu, J., Lu, J. S., Di, G. H., et al. (2007). Identification of the functional role of peroxiredoxin 6 in the progression of breast cancer. *Breast Cancer Res.* 9, R76. doi: 10.1186/bcr1789
- Chen, K., Kirber, M. T., Xiao, H., Yang, Y., and Keaney, J. F. Jr. (2008). Regulation of ROS signal transduction by NADPH oxidase 4 localization. *J. Cell Biol.* 181, 1129–1139. doi: 10.1083/jcb.200709049
- Chen, S. X., and Schopfer, P. (1999). Hydroxyl-radical production in physiological reactions. A novel function of peroxidase. *Eur. J. Biochem.* 260, 726–735. doi: 10.1046/j.1432-1327.1999.00199.x
- Choe, Y., Yu, J., Son, Y., Park, S., Kim, J., Shi, X., et al. (2012). Continuously generated H₂O₂ stimulates the proliferation and osteoblastic differentiation of human periodontal ligament fibroblasts. *J. Cell. Biochem.* 113, 1426–1436. doi: 10.1002/jcb.24017
- Chung-man Ho, J., Zheng, S., Comhair, S. A., Farver, C., and Erzurum, S. C. (2001). Differential expression of manganese superoxide dismutase and catalase in lung cancer. *Cancer Res.* 61, 8578–8585.
- Cooke, M. S., Evans, M. D., Dizdaroglu, M., and Lunec, J. (2003). Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J.* 17, 1195–1214. doi: 10.1096/fj.02-0752rev
- D'Autréaux, B., and Toledano, M. B. (2007). ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat. Rev. Mol. Cell Biol.* 8, 813–824. doi: 10.1038/nrm2256
- DeNicola, G. M., Karreth, F. A., Humpton, T. J., Gopinathan, A., Wei, C., Frese, K., et al. (2011). Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature* 475, 106–109. doi: 10.1038/nature10189
- de Oliveira, V. A., da Motta, L. L., de Bastiani, M. A., Lopes, F. M., Müller, C. B., Gabiatti, B. P., et al. (2016). *In vitro* evaluation of antitumoral efficacy of catalase in combination with traditional chemotherapeutic drugs against human lung adenocarcinoma cells. *Tumor Biol.* 37, 10775–10784. doi: 10.1007/s13277-016-4973-6
- De Preter, G., Neveu, M.-A., Danhier, P., Brisson, L., and Payen, V. L., Porporato, P. E., et al. (2015). Inhibition of the pentose phosphate pathway by dichloroacetate unravels a missing link between aerobic glycolysis and cancer cell proliferation. *Oncotarget* 7, 2910–2920. doi: 10.18632/oncotarget.6272
- Dhar, S. K., Tangpong, J., Chaiswing, L., Oberley, T. D., and Clair, D. K. S. (2011). Manganese superoxide dismutase is a p53-regulated gene that switches cancers between early and advanced stages. *Cancer Res.* 71, 6684–6695. doi: 10.1158/0008-5472.CAN-11-1233
- Diehn, M., Cho, R. W., Lobo, N. A., Kalisky, T., Dorie, M. J., Kulp, A. N., et al. (2009). Association of reactive oxygen species levels and radioresistance in cancer stem cells. *Nature* 458, 780–783. doi: 10.1038/nature07733
- Dikalov, S. (2011). Cross talk between mitochondria and NADPH oxidases. Free Radic. Biol. Med. 51, 1289–1301. doi: 10.1016/j.freeradbiomed.2011.06.033
- Doskey, C. M., Buranasudja, V., Wagner, B. A., Wilkes, J. G., Du, J., Cullen, J. J., et al. (2016). Tumor cells have decreased ability to metabolize H₂O₂: implications for pharmacological ascorbate in cancer therapy. *Redox Biol.* 10, 274–284. doi: 10.1016/j.redox.2016.10.010
- Du, M. Q., Carmichael, P. L., and Phillips, D. H. (1994). Induction of activating mutations in the human c-Ha-ras-1 proto-oncogene by oxygen free radicals. *Mol. Carcinog.* 11, 170–175. doi: 10.1002/mc.2940110308
- Egler, R. A., Fernandes, E., Rothermund, K., Sereika, S., de Souza-Pinto, N., Jaruga, P., et al. (2005). Regulation of reactive oxygen species, DNA damage, and c-Myc function by peroxiredoxin 1. *Oncogene* 24, 8038–8050. doi: 10.1038/sj.onc.1208821
- Elchuri, S., Oberley, T. D., Qi, W. B., Eisenstein, R. S., Roberts, L. J., Van Remmen, H., et al. (2005). CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life. *Oncogene* 24, 367–380. doi: 10.1038/sj.onc.1208207
- Elsemman, I. E., Mardinoglu, A., Shoaie, S., Soliman, T. H., and Nielsen, J. (2016). Systems biology analysis of hepatitis C virus infection reveals the role of copy number increases in regions of chromosome 1q in hepatocellular carcinoma metabolism. *Mol. Biosyst.* 12, 1496–1506. doi: 10.1039/C5MB00827A
- Esworthy, R. S., Chu, F. F., Geiger, P., Girotti, A. W., and Doroshow, J. H. (1993). Reactivity of plasma glutathione peroxidase with hydroperoxide substrates and glutathione. *Arch. Biochem. Biophys.* 307, 29–34. doi: 10.1006/abbi.19 93.1555

- Evans, J. L., Goldfine, I. D., Maddux, B. A., and Grodsky, G. M. (2002). Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. *Endocr. Rev.* 23, 599–622. doi: 10.1210/er.2001-0039
- Fan, J., Ye, J., Kamphorst, J. J., Shlomi, T., Thompson, C. B., and Rabinowitz, J. D. (2014). Quantitative flux analysis reveals folate-dependent NADPH production. *Nature* 510, 298–302. doi: 10.1038/nature13236
- Faratian, D., Goltsov, A., Lebedeva, G., Sorokin, A., Moodie, S., Mullen, P., et al. (2009). Systems biology reveals new strategies for personalizing cancer medicine and confirms the role of PTEN in resistance to trastuzumab. *Cancer Res.* 69, 6713–6720. doi: 10.1158/0008-5472.CAN-09-0777
- Fenton, H. J. H. (1894). LXXIII.—Oxidation of tartaric acid in presence of iron. J. Chem. Soc. Trans. 65, 899–910. doi: 10.1039/CT8946500899
- Finkel, T. (2011). Signal transduction by reactive oxygen species. J. Cell Biol. 194, 7–15. doi: 10.1083/jcb.201102095
- Flint, D. H., Tuminello, J. F., and Emptage, M. H. (1993). The inactivation of Fe-S cluster containing hydro-lyases by superoxide. J. Biol. Chem. 268, 22369–22376.
- Forman, H. J., Maiorino, M., and Ursini, F. (2010). Signaling Functions of Reactive Oxygen Species. *Biochemistry* 49, 835–842. doi: 10.1021/bi9020378
- Fransen, M., Nordgren, M., Wang, B., and Apanasets, O. (2012). Role of peroxisomes in ROS/RNS-metabolism: implications for human disease. *Biochim. Biophys. Acta Mol. Basis Dis.* 1822, 1363–1373. doi: 10.1016/j.bbadis.2011.12.001
- Frohlich, D. A., McCabe, M. T., Arnold, R. S., and Day, M. L. (2008). The role of Nrf2 in increased reactive oxygen species and DNA damage in prostate tumorigenesis. *Oncogene* 27, 4353–4362. doi: 10.1038/onc.2008.79
- Gaglio, D., Metallo, C. M., Gameiro, P. A., Hiller, K., Danna, L. S., Balestrieri, C., et al. (2011). Oncogenic K-Ras decouples glucose and glutamine metabolism to support cancer cell growth. *Mol. Syst. Biol.* 7, 523. doi: 10.1038/msb.2011.56
- Galaris, D., Skiada, V., and Barbouti, A. (2008). Redox signaling and cancer: the role of "labile" iron. *Cancer Lett.* 266, 21–29. doi: 10.1016/j.canlet.2008.02.038
- Gao, P., Zhang, H., Dinavahi, R., Li, F., Xiang, Y., Raman, V., et al. (2007). HIF-dependent antitumorigenic effect of antioxidants *in vivo. Cancer Cell* 12, 230–238. doi: 10.1016/j.ccr.2007.08.004
- Gao, X., Liu, Y., Deeb, D., Liu, P., Liu, A., Arbab, A. S., et al. (2013). ROS mediate proapoptotic and antisurvival activity of oleanane triterpenoid CDDO-Me in ovarian cancer cells. *Anticancer Res.* 33, 215–221.
- Gardner, P. R., Raineri, I., Epstein, L. B., and White, C. W. (1995). Superoxide radical and iron modulate aconitase activity in mammalian cells. J. Biol. Chem. 270, 13399–13405. doi: 10.1074/jbc.270.22.13399
- Garrison, W. (1987). Reaction mechanisms in the radiolysis of peptides, polypeptides, and proteins. *Chem. Rev.* 87, 381–398. doi: 10.1021/cr00078a006
- Ghaffari, P., Mardinoglu, A., Asplund, A., Shoaie, S., Kampf, C., Uhlen, M., et al. (2015a). Identifying anti-growth factors for human cancer cell lines through genome-scale metabolic modeling. *Sci. Rep.* 5:8183. doi: 10.1038/srep08183
- Ghaffari, P., Mardinoglu, A., and Nielsen, J. (2015b). Cancer metabolism: a modeling perspective. *Front. Physiol.* 6:382. doi: 10.3389/fphys.2015.00382
- Giannoni, E., Buricchi, F., Raugei, G., Ramponi, G., and Chiarugi, P. (2005). Intracellular reactive oxygen species activate Src tyrosine kinase during cell adhesion and anchorage-dependent cell growth. *Mol. Cell. Biol.* 25, 6391–6403. doi: 10.1128/MCB.25.15.6391-6403.2005
- Giusti, L., Iacconi, P., Ciregia, F., Giannaccini, G., Donatini, G. L., Basolo, F., et al. (2008). Fine-needle aspiration of thyroid nodules: proteomic analysis to identify cancer biomarkers. J. Proteome Res. 7, 4079–4088. doi: 10.1021/pr8000404
- Glorieux, C., Dejeans, N., Sid, B., Beck, R., Calderon, P. B., and Verrax, J. (2011). Catalase overexpression in mammary cancer cells leads to a less aggressive phenotype and an altered response to chemotherapy. *Biochem. Pharmacol.* 82, 1384–1390. doi: 10.1016/j.bcp.2011.06.007
- Glorieux, C., Zamocky, M., Sandoval, J. M., Verrax, J., and Calderon, P. B. (2015). Regulation of catalase expression in healthy and cancerous cells. *Free Radic. Biol. Med.* 87, 84–97. doi: 10.1016/j.freeradbiomed.2015.06.017
- Goh, J., Enns, L., Fatemie, S., Hopkins, H., Morton, J., Pettan-Brewer, C., et al. (2011). Mitochondrial targeted catalase suppresses invasive breast cancer in mice. *BMC Cancer* 11:191. doi: 10.1186/1471-2407-11-191
- Goncalves, K., Sullivan, K., and Phelan, S. (2012). Differential expression and function of peroxiredoxin 1 and peroxiredoxin 6 in cancerous MCF-7 and noncancerous MCF-10A breast epithelial cells. *Cancer Invest.* 30, 38–47. doi: 10.3109/07357907.2011.629382

- Goodman, M., Bostick, R. M., Kucuk, O., and Jones, D. P. (2011). Clinical trials of antioxidants as cancer prevention agents: past, present, and future. *Free Radic. Biol. Med.* 51, 1068–1084. doi: 10.1016/j.freeradbiomed.2011.05.018
- Gorrini, C., Harris, I. S., and Mak, T. W. (2013). Modulation of oxidative stress as an anticancer strategy. *Nat. Rev. Drug Discov.* 12, 931–947. doi: 10.1038/nrd4002
- Gotoh, Y., and Cooper, J. A. (1998). Reactive oxygen species-and dimerizationinduced activation of apoptosis signal-regulating kinase 1 in tumor necrosis factor-α signal transduction. J. Biol. Chem. 273, 17477–17482. doi: 10.1074/jbc.273.28.17477
- Gupta, S. C., Hevia, D., Patchva, S., Park, B., Koh, W., and Aggarwal, B. B. (2012). Upsides and downsides of reactive oxygen species for cancer: the roles of reactive oxygen species in tumorigenesis, prevention, and therapy. *Antioxid. Redox Signal.* 16, 1295–1322. doi: 10.1089/ars.2011.4414
- Hampton, M. B., Kettle, A. J., and Winterbourn, C. C. (1998). Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* 92, 3007–3017.
- Hanczko, R., Fernandez, D. R., Doherty, E., Qian, Y., Vas, G., Niland, B., et al. (2009). Prevention of hepatocarcinogenesis and increased susceptibility to acetaminophen-induced liver failure in transaldolase-deficient mice by Nacetylcysteine. J. Clin. Invest. 119, 1546–1557. doi: 10.1172/JCI35722
- Harris, I. S., Treloar, A. E., Inoue, S., Sasaki, M., Gorrini, C., Lee, K., et al. (2015). Glutathione and thioredoxin antioxidant pathways synergize to drive cancer initiation and progression. *Cancer Cell* 27, 211–222. doi:10.1016/j.ccell.2014.11.019
- Hart, P. C., Mao, M., de Abreu, A. L. P., Ansenberger-Fricano, K., Ekoue, D. N., Ganini, D., et al. (2015). MnSOD upregulation sustains the Warburg effect via mitochondrial ROS and AMPK-dependent signalling in cancer. *Nat. Commun.* 6:6053. doi: 10.1038/ncomms7053
- Havens, C. G., Ho, A., Yoshioka, N., and Dowdy, S. F. (2006). Regulation of late G1/S phase transition and APCCdh1 by reactive oxygen species. *Mol. Cell. Biol.* 26, 4701–4711. doi: 10.1128/MCB.00303-06
- Hay, N. (2016). Reprogramming glucose metabolism in cancer: can it be exploited for cancer therapy? *Nat. Rev. Cancer* 16, 635–649. doi: 10.1038/nrc.2016.77
- Heinrich, P. C., Morris, H. P., and Weber, G. (1976). Behavior of transaldolase (EC 2.2.1.2) and transketolase (EC 2.2.1.1) activities in normal, neoplastic, differentiating, and regenerating liver. *Cancer Res.* 36, 3189–3197.
- Hempel, N., Carrico, P. M., and Melendez, J. A. (2011). Manganese superoxide dismutase (Sod2) and redox-control of signaling events that drive metastasis. *Anticancer. Agents Med. Chem.* 11, 191–201. doi: 10.2174/1871520117952 55911
- Higinbotham, K. G., Rice, J. M., Kasprzak, K. S., Reed, C. D., and Perantoni, A. O. (1992). GGT to GTT transversions in codon 12 of the K-ras oncogene in rat renal sarcomas induced with nickel subsulfide or nickel subsulfide/iron are consistent with oxidative damage to DNA. *Cancer Res.* 52, 4747–4751.
- Hileman, E. O., Liu, J., Albitar, M., Keating, M. J., and Huang, P. (2004). Intrinsic oxidative stress in cancer cells: a biochemical basis for therapeutic selectivity. *Cancer Chemother. Pharmacol.* 53, 209–219. doi: 10.1007/s00280-003-0726-5
- Hofree, M., Shen, J. P., Carter, H., Gross, A., and Ideker, T. (2013). Networkbased stratification of tumor mutations. *Nat. Methods* 10, 1108–1115. doi: 10.1038/nmeth.2651
- Höhn, A., König, J., and Grune, T. (2013). Protein oxidation in aging and the removal of oxidized proteins. J. Proteomics 92, 132–159. doi: 10.1016/j.jprot.2013.01.004
- Holley, A. K., Dhar, S. K., Xu, Y., and Clair, D. K. S. (2012). Manganese superoxide dismutase: beyond life and death. *Amino Acids* 42, 139–158. doi: 10.1007/s00726-010-0600-9
- Hornsveld, M., and Dansen, T. B. (2016). The hallmarks of cancer from a redox perspective. Antioxid. Redox Signal. 25, 300–325. doi: 10.1089/ars.2015.6580
- Hu, Y., Rosen, D. G., Zhou, Y., Feng, L., Yang, G., Liu, J., et al. (2005). Mitochondrial manganese-superoxide dismutase expression in ovarian cancer: role in cell proliferation and response to oxidative stress. J. Biol. Chem. 280, 39485–39492. doi: 10.1074/jbc.M503296200
- Huang, P., Feng, L., Oldham, E. A., Keating, M. J., and Plunkett, W. (2000). Superoxide dismutase as a target for the selective killing of cancer cells. *Nature* 407, 390–395. doi: 10.1038/35030140
- Huie, R. E., and Padmaja, S. (1993). The reaction of NO with superoxide. *Free Radic. Res.* 18, 195–199. doi: 10.3109/10715769309145868

- Hyötyläinen, T., Jerby, L., Petäjä, E. M., Mattila, I., Jäntti, S., Auvinen, P., et al. (2016). Genome-scale study reveals reduced metabolic adaptability in patients with non-alcoholic fatty liver disease. *Nat. Commun.* 7:8994. doi: 10.1038/ncomms9994
- Ishikawa, K., Takenaga, K., Akimoto, M., Koshikawa, N., Yamaguchi, A., Imanishi, H., et al. (2008). ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. *Science* 320, 661–664. doi: 10.1126/science.1156906
- Janssen, A. M. L., Bosman, C. B., Kruidenier, L., Griffioen, G., Lamers, C., Van Krieken, J., et al. (1999). Superoxide dismutases in the human colorectal cancer sequence. J. Cancer Res. Clin. Oncol. 125, 327–335. doi: 10.1007/s004320050282
- Jarvis, R. M., Hughes, S. M., and Ledgerwood, E. C. (2012). Peroxiredoxin 1 functions as a signal peroxidase to receive, transduce, and transmit peroxide signals in mammalian cells. *Free Radic. Biol. Med.* 53, 1522–1530. doi: 10.1016/j.freeradbiomed.2012.08.001
- Jeng, W., Loniewska, M. M., and Wells, P. G. (2013). Brain glucose-6phosphate dehydrogenase protects against endogenous oxidative DNA damage and neurodegeneration in aged mice. ACS Chem. Neurosci. 4, 1123–1132. doi: 10.1021/cn400079y
- Jerby, L., and Ruppin, E. (2012). Predicting drug targets and biomarkers of cancer via genome-scale metabolic modeling. *Clin. Cancer Res.* 18, 5572–5584. doi: 10.1158/1078-0432.CCR-12-1856
- Jiang, P., Du, W., Mancuso, A., Wellen, K. E., and Yang, X. (2013). Reciprocal regulation of p53 and malic enzymes modulates metabolism and senescence. *Nature* 493, 689–693. doi: 10.1038/nature11776
- Johansson, C., Lillig, C. H., and Holmgren, A. (2004). Human mitochondrial glutaredoxin reduces S-glutathionylated proteins with high affinity accepting electrons from either glutathione or thioredoxin reductase. J. Biol. Chem. 279, 7537–7543. doi: 10.1074/jbc.M312719200
- Johnson, R. M., Goyette, G., Ravindranath, Y., and Ho, Y.-S. S. (2005). Hemoglobin autoxidation and regulation of endogenous H₂O₂ levels in erythrocytes. *Free Radic. Biol. Med.* 39, 1407–1417. doi: 10.1016/j.freeradbiomed.2005.07.002
- Jonas, S. K., Benedetto, C., Flatman, A., Hammond, R. H., Micheletti, L., Riley, C., et al. (1992). Increased activity of 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase in purified cell suspensions and single cells from the uterine cervix in cervical intraepithelial neoplasia. *Br. J. Cancer* 66, 185–191. doi: 10.1038/bjc.1992.240
- Jones, D. P. (2006). Disruption of mitochondrial redox circuitry in oxidative stress. *Chem. Biol. Interact.* 163, 38–53. doi: 10.1016/j.cbi.2006.07.008
- Juarez, J. C., Manuia, M., Burnett, M., Betancourt, O., Boivin, B., Shaw, D. E., et al. (2008). Superoxide dismutase 1 (SOD1) is essential for H₂O₂-mediated oxidation and inactivation of phosphatases in growth factor signaling. *Proc. Natl. Acad. Sci. U.S.A.* 105, 7147–7152. doi: 10.1073/pnas.0709451105
- Kaewpila, S., Venkataraman, S., Buettner, G. R., and Oberley, L. W. (2008). Manganese superoxide dismutase modulates hypoxia-inducible factor-1 alpha induction via superoxide. *Cancer Res.* 68, 2781–2788. doi: 10.1158/0008-5472.CAN-07-2635
- Kaimul, A. M., Nakamura, H., Masutani, H., and Yodoi, J. (2007). Thioredoxin and thioredoxin-binding protein-2 in cancer and metabolic syndrome. *Free Radic. Biol. Med.* 43, 861–868. doi: 10.1016/j.freeradbiomed.2007.05.032
- Kampf, C., Mardinoglu, A., Fagerberg, L., Hallström, B. M., Danielsson, A., Nielsen, J., et al. (2014a). Defining the human gallbladder proteome by transcriptomics and affinity proteomics. *Proteomics* 14, 2498–2507. doi: 10.1002/pmic.201400201
- Kampf, C., Mardinoglu, A., Fagerberg, L., Hallström, B. M., Edlund, K., Lundberg, E., et al. (2014b). The human liver-specific proteome defined by transcriptomics and antibody-based profiling. *FASEB J.* 28, 1–14. doi: 10.1096/fj.14-250555
- Kasiappan, R., and Safe, S. (2016). ROS-inducing agents for cancer chemotherapy. *React. Oxyg. Species* 1, 22–37. doi: 10.20455/ros.2016.805
- Klein, E. A., Thompson, I. M. Jr., Tangen, C. M., John, J., Lucia, M. S., Goodman, P. J., et al. (2011). Vitamin E and the risk of prostate cancer: updated results of the selenium and vitamin E cancer prevention trial (SELECT). J. Am. Med. Assoc. 306, 1549–1556. doi: 10.1001/jama.2011.1437
- Kondo, S., Toyokuni, S., Iwasa, Y., Tanaka, T., Onodera, H., Hiai, H., et al. (1999). Persistent oxidative stress in human colorectal carcinoma, but not in adenoma. *Free Radic. Biol. Med.* 27, 401–410. doi: 10.1016/S0891-5849(99)00087-8
- Koppenol, W. H. (2001). The Haber-Weiss cycle 70 years later. *Redox Rep.* 6, 229–234. doi: 10.1179/135100001101536373

- Kowaltowski, A. J., de Souza-Pinto, N. C., Castilho, R. F., and Vercesi, A. E. (2009). Mitochondria and reactive oxygen species. *Free Radic. Biol. Med.* 47, 333–343. doi: 10.1016/j.freeradbiomed.2009.05.004
- Labunskyy, V. M., and Gladyshev, V. N. (2013). Role of reactive oxygen species-mediated signaling in aging. *Antioxid. Redox Signal.* 19, 1362–1372. doi: 10.1089/ars.2012.4891
- LaButti, J. N., Chowdhury, G., Reilly, T. J., and Gates, K. S. (2007). Redox regulation of protein tyrosine phosphatase 1B by peroxymonophosphate (O3POOH). J. Am. Chem. Soc. 129, 5320–5321. doi: 10.1021/ja070194j
- Latimer, H. R., and Veal, E. A. (2016). Peroxiredoxins in regulation of MAPK signalling pathways; sensors and barriers to signal transduction. *Mol. Cells* 39, 40–45. doi: 10.14348/molcells.2016.2327
- Lee, K. W., Lee, D. J., Lee, J. Y., Kang, D. H., Kwon, J., and Kang, S. W. (2011). Peroxiredoxin II restrains DNA damage-induced death in cancer cells by positively regulating JNK-dependent DNA repair. *J. Biol. Chem.* 286, 8394–8404. doi: 10.1074/jbc.M110.179416
- Lee, S., Zhang, C., Kilicarslan, M., Piening, B. D. D., Bjornson, E., Hallström, B. M. M., et al. (2016). integrated network analysis reveals an association between plasma mannose levels and insulin resistance. *Cell Metab.* 24, 1–13. doi: 10.1016/j.cmet.2016.05.026
- Leiserson, M. D. M., Vandin, F., Wu, H.-T., Dobson, J. R., Eldridge, J. V., Thomas, J. L., et al. (2014). Pan-cancer network analysis identifies combinations of rare somatic mutations across pathways and protein complexes. *Nat. Genet.* 47, 106–114. doi: 10.1038/ng.3168
- Li, L., Shoji, W., Oshima, H., Obinata, M., Fukumoto, M., and Kanno, N. (2008). Crucial role of peroxiredoxin III in placental antioxidant defense of mice. *FEBS Lett.* 582, 2431–2434. doi: 10.1016/j.febslet.2008.05.050
- Li, L., and Yu, A. Q. (2015). The functional role of peroxiredoxin 3 in reactive oxygen species, apoptosis, and chemoresistance of cancer cells. J. Cancer Res. Clin. Oncol. 141, 2071–2077. doi: 10.1007/s00432-015-1916-3
- Li, L., Zhang, Y. G., and Chen, C. L. (2013). Anti-apoptotic role of peroxiredoxin III in cervical cancer cells. *FEBS Open Bio.* 3, 51–54. doi: 10.1016/j.fob.2012.12.002
- Lien, E. C., Lyssiotis, C. A., Juvekar, A., Hu, H., Asara, J. M., Cantley, L. C., et al. (2016). Glutathione biosynthesis is a metabolic vulnerability in PI(3)K/Aktdriven breast cancer. *Nat. Cell Biol.* 18, 572–578. doi: 10.1038/ncb3341
- Lin, R., Elf, S., Shan, C., Kang, H.-B., Ji, Q., Zhou, L., et al. (2015). 6-Phosphogluconate dehydrogenase links oxidative PPP, lipogenesis and tumour growth by inhibiting LKB1-AMPK signalling. *Nat. Cell Biol.* 17, 1484–1496. doi: 10.1038/ncb3255
- Lincoln, D. T., Ali Emadi, E. M., Tonissen, K. F., and Clarke, F. M. (2003). The thioredoxin-thioredoxin reductase system: over-expression in human cancer. *Anticancer Res.* 23, 2425–2433.
- Lindskog, C., Linné, J., Fagerberg, L., Hallström, B. M., Sundberg, C. J., Lindholm, M., et al. (2015). The human cardiac and skeletal muscle proteomes defined by transcriptomics and antibody-based profiling. *BMC Genomics* 16:475. doi: 10.1186/s12864-015-1686-y
- Little, C., and O'brien, P. J. (1969). Mechanism of peroxide-inactivation of the sulphydryl enzyme glyceraldehyde-3-phosphate dehydrogenase. *Eur. J. Biochem.* 10, 533–538.
- Liu, H., Huang, D., McArthur, D. L., Boros, L. G., Nissen, N., and Heaney, A. P. (2010). Fructose induces transketolase flux to promote pancreatic cancer growth. *Cancer Res.* 70, 6368–6376. doi: 10.1158/0008-5472.CAN-09-4615
- Liu, Z., Li, S., Cai, Y., Wang, A., He, Q., Zheng, C., et al. (2012). Manganese superoxide dismutase induces migration and invasion of tongue squamous cell carcinoma via H₂O₂-dependent Snail signaling. *Free Radic. Biol. Med.* 53, 44–50. doi: 10.1016/j.freeradbiomed.2012.04.031
- Low, F. M., Hampton, M. B., Peskin, A. V., and Winterbourn, C. C. (2007). Peroxiredoxin 2 functions as a noncatalytic scavenger of lowlevel hydrogen peroxide in the erythrocyte. *Blood* 109, 2611–2617. doi: 10.1182/blood-2006-09-048728
- Lu, H., Samanta, D., Xiang, L., Zhang, H., Hu, H., Chen, I., et al. (2015). Chemotherapy triggers HIF-1-dependent glutathione synthesis and copper chelation that induces the breast cancer stem cell phenotype. *Proc. Natl. Acad. Sci. U.S.A.* 112, E4600–E4609. doi: 10.1073/pnas.1513433112
- Lu, P., Vogel, C., Wang, R., Yao, X., and Marcotte, E. M. (2007). Absolute protein expression profiling estimates the relative contributions of transcriptional and translational regulation. *Nat. Biotechnol.* 25, 117–124. doi: 10.1038/nbt1270

- Lu, W., Fu, Z., Wang, H., Feng, J., Wei, J., and Guo, J. (2014). Peroxiredoxin 2 is upregulated in colorectal cancer and contributes to colorectal cancer cells' survival by protecting cells from oxidative stress. *Mol. Cell. Biochem.* 387, 261–270. doi: 10.1007/s11010-013-1891-4
- Lu, Y. P., Lou, Y. R., Yen, P., Newmark, H. L., Mirochnitchenko, O. I., Inouye, M., et al. (1997). Enhanced skin carcinogenesis in transgenic mice with high expression of glutathione peroxidase or both glutathione peroxidase and superoxide dismutase. *Cancer Res.* 57, 1468–1474.
- Maiti, A. K. (2010). Gene network analysis of oxidative stress-mediated drug sensitivity in resistant ovarian carcinoma cells. *Pharmacogenomics J.* 10, 94–104. doi: 10.1038/tpj.2009.49
- Mandal, P. K., Seiler, A., Perisic, T., Kölle, P., Canak, A. B., Förster, H., et al. (2010). System xc- and thioredoxin reductase 1 cooperatively rescue glutathione deficiency. J. Biol. Chem. 285, 22244–22253. doi: 10.1074/jbc.M110.121327
- Manta, B., Hugo, M., Ortiz, C., Ferrer-Sueta, G., Trujillo, M., and Denicola, A. (2009). The peroxidase and peroxynitrite reductase activity of human erythrocyte peroxiredoxin 2. Arch. Biochem. Biophys. 484, 146–154. doi: 10.1016/j.abb.2008.11.017
- Marchissio, M. J., Frances, D. E., Carnovale, C. E., and Marinelli, R. A. (2012). Mitochondrial aquaporin-8 knockdown in human hepatoma HepG2 cells causes ROS-induced mitochondrial depolarization and loss of viability. *Toxicol. Appl. Pharmacol.* 264, 246–254. doi: 10.1016/j.taap.2012.08.005
- Mardinoglu, A., Agren, R., Kampf, C., Asplund, A., Nookaew, I., Jacobson, P., et al. (2013a). Integration of clinical data with a genome-scale metabolic model of the human adipocyte. *Mol. Syst. Biol.* 9:649. doi: 10.1038/msb.2013.5
- Mardinoglu, A., Agren, R., Kampf, C., Asplund, A., Uhlen, M., and Nielsen, J. (2014a). Genome-scale metabolic modelling of hepatocytes reveals serine deficiency in patients with non-alcoholic fatty liver disease. *Nat. Commun.* 5:3083. doi: 10.1038/ncomms4083
- Mardinoglu, A., Bjornson, E., Zhang, C., Klevstig, M., Söderlund, S., Ståhlman, M., et al. (2017). Personal model-assisted identification of NAD+ and glutathione metabolism as intervention target in NAFLD. *Mol. Syst. Biol.* 13, 1–17. doi: 10.15252/msb.20167422
- Mardinoglu, A., Boren, J., Ulf, S., Uhlén, M., and Nielsen, J. (in press). The employment of systems biology in gastroenterology and hepatology. *Nat. Rev. Gastroenterol. Hepatol.*
- Mardinoglu, A., Gatto, F., and Nielsen, J. (2013b). Genome-scale modeling of human metabolism - a systems biology approach. *Biotechnol. J.* 8, 985–996. doi: 10.1002/biot.201200275
- Mardinoglu, A., Heiker, J. T., Gärtner, D., Björnson, E., Schön, M. R., Flehmig, G., et al. (2015a). Extensive weight loss reveals distinct gene expression changes in human subcutaneous and visceral adipose tissue. *Sci. Rep.* 5:14841. doi: 10.1038/srep14841
- Mardinoglu, A., Kampf, C., Asplund, A., Fagerberg, L., Hallström, B. M., Edlund, K., et al. (2014b). Defining the human adipose tissue proteome to reveal metabolic alterations in obesity. *J. Proteome Res.* 13, 5106–5119. doi: 10.1021/pr500586e
- Mardinoglu, A., and Nielsen, J. (2012). Systems medicine and metabolic modelling. J. Intern. Med. 271, 142–154. doi: 10.1111/j.1365-2796.2011.02493.x
- Mardinoglu, A., and Nielsen, J. (2015). New paradigms for metabolic modeling of human cells. *Curr. Opin. Biotechnol.* 34, 91–97. doi: 10.1016/j.copbio.2014.12.013
- Mardinoglu, A., and Nielsen, J. (2016). Editorial: the impact of systems medicine on human health and disease. *Front. Physiol.* 7:552. doi: 10.3389/fphys.2016.00552
- Mardinoglu, A., Shoaie, S., Bergentall, M., Ghaffari, P., Zhang, C., Larsson, E., et al. (2015b). The gut microbiota modulates host amino acid and glutathione metabolism in mice. *Mol. Syst. Biol.* 11:834. doi: 10.15252/msb.20156487
- Mardinoglu, A., and Uhlén, M. (2016). Liver: phenotypic and genetic variance: a systems approach to the liver. Nat. Rev. Gastroenterol. Hepatol. 13, 439–440. doi: 10.1038/nrgastro.2016.93
- Marinho, H. S., Real, C., Cyrne, L., Soares, H., and Antunes, F. (2014). Hydrogen peroxide sensing, signaling and regulation of transcription factors. *Redox Biol.* 2, 535–562. doi: 10.1016/j.redox.2014.02.006
- Mazurek, S., Boschek, C. B., Hugo, F., and Eigenbrodt, E. (2005). Pyruvate kinase type M2 and its role in tumor growth and spreading. *Semin. Cancer Biol.* 15, 300–308. doi: 10.1016/j.semcancer.2005.04.009

- Mehrmohamadi, M., Liu, X., Shestov, A. A., and Locasale, J. W. (2014). Characterization of the usage of the serine metabolic network in human cancer. *Cell Rep.* 9, 1507–1519. doi: 10.1016/j.celrep.2014.10.026
- Menon, S. G., and Goswami, P. C. (2007). A redox cycle within the cell cycle: ring in the old with the new. Oncogene 26, 1101–1109. doi: 10.1038/sj.onc.1209895
- Messner, K. R., and Imlay, J. A. (2002). Mechanism of superoxide and hydrogen peroxide formation by fumarate reductase, succinate dehydrogenase, and aspartate oxidase. J. Biol. Chem. 277, 42563–42571. doi: 10.1074/jbc.M204958200
- Miar, A., Hevia, D., Muñoz-Cimadevilla, H., Astudillo, A., Velasco, J., Sainz, R. M., et al. (2015). Manganese superoxide dismutase (SOD2/MnSOD)/catalase and SOD2/GPx1 ratios as biomarkers for tumor progression and metastasis in prostate, colon, and lung cancer. *Free Radic. Biol. Med.* 85, 45–55. doi: 10.1016/j.freeradbiomed.2015.04.001
- Miranda, A., Janssen, L., Bosman, C. B., van Duijn, W., Oostendorp-van de Ruit, M. M., Kubben, F. J., et al. (2000). Superoxide dismutases in gastric and esophageal cancer and the prognostic impact in gastric cancer. *Clin. Cancer Res.* 6, 3183–3192.
- Mohr, S., Hallak, H., de Boitte, A., Lapetina, E. G., and Brune, B. (1999). Nitric oxide-induced S-glutathionylation and inactivation of glyceraldehyde-3-phosphate dehydrogenase. J. Biol. Chem. 274, 9427–9430. doi: 10.1074/jbc.274.14.9427
- Morinaka, A., Funato, Y., Uesugi, K., and Miki, H. (2011). Oligomeric peroxiredoxin-I is an essential intermediate for p53 to activate MST1 kinase and apoptosis. *Oncogene* 30, 4208–4218. doi: 10.1038/onc.2011.139
- Murawaki, Y. Y., Tsuchiya, H., Kanbe, T., Harada, K., Yashima, K., Nozaka, K., et al. (2008). Aberrant expression of selenoproteins in the progression of colorectal cancer. *Cancer Lett.* 259, 218–230. doi: 10.1016/j.canlet.2007.10.019
- Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. *Biochem. J.* 417, 1–13. doi: 10.1042/BJ20081386
- Nagy, P., and Winterbourn, C. C. (2010). Redox chemistry of biological thiols. *Adv. Mol. Toxicol.* 4, 183–222. doi: 10.1016/S1872-0854(10)04006-3
- Naticchia, M. R., Brown, H. A., Garcia, F. J., Lamade, A. M., Justice, S. L., Herrin, R. P., et al. (2013). Bifunctional electrophiles cross-link thioredoxins with redox relay partners in cells. *Chem. Res. Toxicol.* 26, 490–497. doi: 10.1021/tx4000123
- Nauseef, W. M. (2008). Biological roles for the NOX family NADPH oxidases. J. Biol. Chem. 283, 16961–16965. doi: 10.1074/jbc.R700045200
- Nelson, K. K., Ranganathan, A. C., Mansouri, J., Rodriguez, A. M., Providence, K. M., Rutter, J. L., et al. (2003). Elevated Sod2 activity augments matrix metalloproteinase expression: evidence for the involvement of endogenous hydrogen peroxide in regulating metastasis. *Clin. Cancer Res.* 9, 424–432.
- Netto, L. E. S., and Antunes, F. (2016). The roles of peroxiredoxin and thioredoxin in hydrogen peroxide sensing and in signal transduction. *Mol. Cells* 39, 65–71. doi: 10.14348/molcells.2016.2349
- Neumann, C. A., and Fang, Q. (2007). Are peroxiredoxins tumor suppressors? *Curr. Opin. Pharmacol.* 7, 375–380. doi: 10.1016/j.coph.2007.04.007
- Nielsen, J. (2017). Systems biology of metabolism: a driver for developing personalized and precision medicine. *Cell Metab.* 25, 572–579. doi: 10.1016/j.cmet.2017.02.002
- Nóbrega-pereira, S., Fernandez-marcos, P. J., Brioche, T., Gomez-cabrera, M. C., Salvador, A., Flores, J. M., et al. (2016). G6PD protects from oxidative damage and improves healthspan in mice. *Nat. Commun.* 7, 1–9. doi: 10.1038/ncomms10894
- Oberley, T. D., and Oberley, L. W. (1997). Antioxidant enzyme levels in cancer. *Histol. Histopathol.* 12, 525–535.
- Oltra, A. M., Carbonell, F., Tormos, C., Iradi, A., and Sáez, G. T. (2001). Antioxidant enzyme activities and the production of MDA and 8-oxo-dG in chronic lymphocytic leukemia. *Free Radic. Biol. Med.* 30, 1286–1292. doi: 10.1016/S0891-5849(01)00521-4
- Omenn, G., Goodman, G., Thornquist, M., Balmes, J., Cullen, M., and Glass, A. (1996). Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *New Engl. J. Med.* 334, 1150–1155. doi: 10.1056/NEJM199605023341802
- Pandolfi, P. P., Sonati, F., Rivi, R., Mason, P., Grosveld, F., and Luzzatto, L. (1995). Targeted disruption of the housekeeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress. *EMBO J.* 14, 5209–5215.

- Park, J. H., Kim, Y. S., Lee, H. L., Shim, J. Y., Lee, K. S., Oh, Y. J., et al. (2006). Expression of peroxiredoxin and thioredoxin in human lung cancer and paired normal lung. *Respirology* 11, 269–275. doi: 10.1111/j.1440-1843.2006.00849.x
- Park, S. Y., Yu, X., Ip, C., Mohler, J. L., Bogner, P. N., and Park, Y. M. (2007). Peroxiredoxin 1 interacts with androgen receptor and enhances its transactivation. *Cancer Res.* 67, 9294–9303. doi: 10.1158/0008-5472.CAN-07-0651
- Passos, J. F., Nelson, G., Wang, C., Richter, T., Simillion, C., Proctor, C. J., et al. (2010). Feedback between p21 and reactive oxygen production is necessary for cell senescence. *Mol. Syst. Biol.* 6:347. doi: 10.1038/msb.2010.5
- Patra, K. C., and Hay, N. (2014). The pentose phosphate pathway and cancer. *Trends Biochem. Sci.* 39, 347–354. doi: 10.1016/j.tibs.2014.06.005
- Paulsen, C. E., Truong, T. H., Garcia, F. J., Homann, A., Gupta, V., Leonard, S. E., et al. (2012). Peroxide-dependent sulfenylation of the EGFR catalytic site enhances kinase activity. *Nat. Chem. Biol.* 8, 57–64. doi: 10.1038/nchembio.736
- Pawson, T., and Linding, R. (2008). Network medicine. FEBS Lett. 582, 1266–1270. doi: 10.1016/j.febslet.2008.02.011
- Peng, L., Bian, X. W., Li, D. K., Xu, C., Wang, G. M., Xia, Q. Y., et al. (2015). Large-scale RNA-Seq transcriptome analysis of 4043 cancers and 548 normal tissue controls across 12 TCGA cancer types. *Sci. Rep.* 5:13413. doi: 10.1038/srep13413
- Perkins, A., Poole, L. B., and Karplus, P. A. (2014). Tuning of peroxiredoxin catalysis for various physiological roles. *Biochemistry* 53, 7693–7705. doi: 10.1021/bi5013222
- Peskin, A. V., Low, F. M., Paton, L. N., Maghzal, G. J., Hampton, M. B., and Winterbourn, C. C. (2007). The high reactivity of peroxiredoxin 2 with H₂O₂ is not reflected in its reaction with other oxidants and thiol reagents. *J. Biol. Chem.* 282, 11885–11892. doi: 10.1074/jbc.M700339200
- Peskin, A. V., Pace, P. E., Behring, J. B., Paton, L. N., Soethoudt, M., Bachschmid, M. M., et al. (2016). Glutathionylation of the active site cysteines of peroxiredoxin 2 and recycling by glutaredoxin. *J. Biol. Chem.* 291, 3053–3062. doi: 10.1074/jbc.M115.692798
- Piskounova, E., Agathocleous, M., Murphy, M. M., Hu, Z., Huddlestun, S. E., Zhao, Z., et al. (2015). Oxidative stress inhibits distant metastasis by human melanoma cells. *Nature* 527, 186–191. doi: 10.1038/nature15726
- Pryor, W. A. (1986). Oxy-radicals and related species: their formation, lifetimes, and reactions. Annu. Rev. Physiol. 48, 657–667. doi: 10.1146/annurev.ph.48.030186.003301
- Rabilloud, T., Heller, M., Rigobello, M. P., Bindoli, A., Aebersold, R., and Lunardi, J. (2001). The mitochondrial antioxidant defence system and its response to oxidative stress. *Proteomics* 1, 1105–1110. doi: 10.1002/1615-9861(200109)1:9andlt;1105::AID-PROT1105andgt; 3.0.CO;2-M
- Raïs, B., Comin, B., Puigjaner, J., Brandes, J. L., Creppy, E., Saboureau, D., et al. (1999). Oxythiamine and dehydroepiandrosterone induce a G1 phase cycle arrest in Ehrlich's tumor cells through inhibition of the pentose cycle. *FEBS Lett.* 456, 113–118. doi: 10.1016/S0014-5793(99)00924-2
- Ray, G., Batra, S., Shukla, N. K., Deo, S., Raina, V., Ashok, S., et al. (2000). Lipid peroxidation, free radical production and antioxidant status in breast cancer. *Breast Cancer Res. Treat.* 59, 163–170. doi: 10.1023/A:1006357330486
- Ray, P. D., Huang, B. W., and Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell. Signal.* 24, 981–990. doi: 10.1016/j.cellsig.2012.01.008
- Reuter, S., Gupta, S. C., Chaturvedi, M. M., and Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic. Biol. Med.* 49, 1603–1616. doi: 10.1016/j.freeradbiomed.2010.09.006
- Rigoulet, M., Yoboue, E. D., and Devin, A. (2011). Mitochondrial ROS generation and its regulation: mechanisms involved in H₂O₂ signaling. *Antioxid. Redox Signal.* 14, 459–468. doi: 10.1089/ars.2010.3363
- Robbins, D., Wittwer, J. A., Codarin, S., Circu, M. L., Aw, T. Y., Huang, T. T., et al. (2012). Isocitrate dehydrogenase 1 is downregulated during early skin tumorigenesis which can be inhibited by overexpression of manganese superoxide dismutase. *Cancer Sci.* 103, 1429–1433. doi: 10.1111/j.1349-7006.2012.02317.x
- Rodman, S. N., Spence, J. M., Ronnfeldt, T. J., Zhu, Y., Solst, S. R., O'Neill, R. A., et al. (2016). Enhancement of radiation response in breast cancer stem cells by inhibition of thioredoxin-and glutathione-dependent metabolism. *Radiat. Res.* 186, 385–395. doi: 10.1667/RR14463.1

- Roh, J.-L., Jang, H. J., Kim, E. H., and Shin, D. (2016). Targeting of the glutathione, thioredoxin, and Nrf2 antioxidant systems in head and neck cancer. *Antioxid. Redox Signal.* 27, 106–114. doi: 10.1089/ars.2016.6841
- Roots, R., and Okada, S. (1975). Estimation of life times and diffusion distances of radicals involved in x-ray-induced DNA strand breaks of killing of mammalian cells. *Radiat. Res.* 64, 306–320. doi: 10.2307/3574267
- Rouault, T. A., and Klausner, R. D. (1996). Iron-sulfur clusters as biosensors of oxidants and iron. *Trends Biochem. Sci.* 21, 174–177. doi:10.1016/S0968-0004(96)10024-4
- Sabharwal, S. S., and Schumacker, P. T. (2014). Mitochondrial ROS in cancer: initiators, amplifiers or an Achilles' heel? *Nat. Rev. Cancer* 14, 709–721. doi: 10.1038/nrc3803
- Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., et al. (1998). Mammalian thioredoxin is a direct inhibitor of apoptosis signalregulating kinase (ASK) 1. EMBO J. 17, 2596–2606. doi: 10.1093/emboj/ 17.9.2596
- Sarsour, E. H., Kalen, A. L., and Goswami, P. C. (2014). Manganese superoxide dismutase regulates a redox cycle within the cell cycle. *Antioxid. Redox Signal.* 20, 1618–1627. doi: 10.1089/ars.2013.5303
- Sayin, V. I., Ibrahim, M. X., Larsson, E., Nilsson, J. A., Lindahl, P., and Bergo, M. O. (2014). Antioxidants accelerate lung cancer progression in mice. *Sci. Transl. Med.* 6:221ra15. doi: 10.1126/scitranslmed.3007653
- Schork, N. J. (2015). Personalized medicine: time for one-person trials. Nature 520, 609–611. doi: 10.1038/520609a
- Sena, L. A., and Chandel, N. S. (2012). Physiological roles of mitochondrial reactive oxygen species. *Mol. Cell* 48, 158–166. doi: 10.1016/j.molcel.2012.09.025
- Shan, C., Elf, S., Ji, Q., Kang, H. B., Zhou, L., Hitosugi, T., et al. (2014). Lysine acetylation activates 6-phosphogluconate dehydrogenase to promote tumor growth. *Mol. Cell* 55, 552–565. doi: 10.1016/j.molcel.2014.06.020
- Sharma, V. K., and Rokita, S. E. (2013). Oxidation of Amino Acids, Peptides, and Proteins: Kinetics and Mechanism. Hoboken, NJ: John Wiley and Sons, Inc.
- Shen, X., Burguillos, M. A., Osman, A. M., Frijhoff, J., Carrillo-Jiménez, A., Kanatani, S., et al. (2016). Glioma-induced inhibition of caspase-3 in microglia promotes a tumor-supportive phenotype. *Nat. Immunol.* 17, 1282–1290. doi: 10.1038/ni.3545
- Shibutani, S., Takeshita, M., and Grollman, A. P. (1991). Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. *Nature* 349, 431–434. doi: 10.1038/349431a0
- Skrzydlewska, E., Sulkowski, S., Koda, M., Zalewski, B., Kanczuga-Koda, L., and Sulkowska, M. (2005). Lipid peroxidation and antioxidant status in colorectal cancer. *World J. Gastroenterol.* 11, 403–406. doi: 10.3748/wjg.v11.i3.403
- Smith, W. L., and Murphy, R. C. (2008). Oxidized lipids formed nonenzymatically by reactive oxygen species. J. Biol. Chem. 283, 15513–15514. doi: 10.1074/jbc.R800006200
- Sobhakumari, A., Love-Homan, L., Fletcher, E. V. M., Martin, S. M., Parsons, A. D., Spitz, D. R., et al. (2012). Susceptibility of human head and neck cancer cells to combined inhibition of glutathione and thioredoxin metabolism. *PLoS ONE* 7:e48175. doi: 10.1371/journal.pone.0048175
- Sobotta, M. C., Liou, W., Stöcker, S., Talwar, D., Oehler, M., Ruppert, T., et al. (2015). Peroxiredoxin-2 and STAT3 form a redox relay for H₂O₂ signaling. *Nat. Chem. Biol.* 11, 64–70. doi: 10.1038/nchembio.1695
- Soini, Y., Kahlos, K., Näpänkangas, U., Kaarteenaho-Wiik, R., Säily, M., Koistinen, P., et al. (2001). Widespread expression of thioredoxin and thioredoxin reductase in non-small cell lung carcinoma. *Clin. Cancer Res.* 7, 1750–1757.
- Stadtman, E. R., and Levine, R. L. (2003). Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids* 25, 207–218. doi: 10.1007/s00726-003-0011-2
- Stanton, R. C., Seifter, J. L., Boxer, D. C., Zimmerman, E., and Cantley, L. C. (1991). Rapid release of bound glucose-6-phosphate dehydrogenase by growth factors correlation with increased enzymatic activity. *J. Biol. Chem.* 266, 12442–12448.
- Starheim, K. K., Holien, T., Misund, K., Johansson, I., Baranowska, K. A., Sponaas, A. M., et al. (2016). Intracellular glutathione determines bortezomib cytotoxicity in multiple myeloma cells. *Blood Cancer J.* 6, e446. doi: 10.1038/bcj.2016.56
- Stresing, V., Baltziskueta, E., Rubio, N., Blanco, J., Arriba, M., Valls, J., et al. (2012). Peroxiredoxin 2 specifically regulates the oxidative and metabolic

stress response of human metastatic breast cancer cells in lungs. *Oncogene* 32, 724–735. doi: 10.1038/onc.2012.93

- Sukhatme, V. P., and Chan, B. (2012). Glycolytic cancer cells lacking 6phosphogluconate dehydrogenase metabolize glucose to induce senescence. *FEBS Lett.* 586, 2389–2395. doi: 10.1016/j.febslet.2012.05.052
- Sultana, R., Perluigi, M., and Butterfield, D. A. (2006). Protein oxidation and lipid peroxidation in brain of subjects with Alzheimer's disease: insights into mechanism of neurodegeneration from redox proteomics. *Antioxid. Redox Signal.* 8, 2021–2037. doi: 10.1089/ars.2006.8.2021
- Sun, Q. A., Kirnarsky, L., Sherman, S., and Gladyshev, V. N. (2001). Selenoprotein oxidoreductase with specificity for thioredoxin and glutathione systems. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3673–3678. doi: 10.1073/pnas.051454398
- Szatrowski, T. P., and Nathan, C. F. (1991). Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res.* 51, 794–799.
- Tanaka, G., Inoue, K.-I., Shimizu, T., Akimoto, K., and Kubota, K. (2016). Dual pharmacological inhibition of glutathione and thioredoxin systems synergizes to kill colorectal carcinoma stem cells. *Cancer Med.* 5, 2544–2557. doi: 10.1002/cam4.844
- Tedeschi, P. M., Markert, E. K., Gounder, M., Lin, H., Dvorzhinski, D., Dolfi, S. C., et al. (2013). Contribution of serine, folate and glycine metabolism to the ATP, NADPH and purine requirements of cancer cells. *Cell Death Dis.* 4:e877. doi: 10.1038/cddis.2013.393
- Thanan, R., Oikawa, S., Yongvanit, P., Hiraku, Y., Ma, N., Pinlaor, S., et al. (2012). Inflammation-induced protein carbonylation contributes to poor prognosis for cholangiocarcinoma. *Free Radic. Biol. Med.* 52, 1465–1472. doi: 10.1016/j.freeradbiomed.2012.01.018
- Thomas, D. D., Ridnour, L. A., Espey, M. G., Donzelli, S., Ambs, S., Hussain, S. P., et al. (2006). Superoxide fluxes limit nitric oxide-induced signaling. *J. Biol. Chem.* 281, 25984–25993. doi: 10.1074/jbc.M602242200
- Thomas, J. P., Maiorino, M., Ursini, F., and Girotti, A. W. (1990). Protective action of phospholipid hydroperoxide glutathione peroxidase against membranedamaging lipid peroxidation: *in situ* reduction of phospholipid and cholesterol hydroperoxides. J. Biol. Chem. 265, 454–461.
- Thul, P. J., Åkesson, L., Wiking, M., Mahdessian, D., Geladaki, A., Ait Blal, H., et al. (2017). A subcellular map of the human proteome. *Science* 356:eaal3321. doi: 10.1126/science.aal3321
- Tian, W. N., Pignatare, J. N., and Stanton, R. C. (1994). Signal transduction proteins that associate with the platelet-derived growth factor (PDGF) receptor mediate the PDGF-induced release of glucose-6-phosphate dehydrogenase from permeabilized cells. J. Biol. Chem. 269, 14798–14805.
- Tomalin, L. E., Day, A. M., Underwood, Z. E., Smith, G. R., Dalle Pezze, P., Rallis, C., et al. (2016). Increasing extracellular H₂O₂ produces a bi-phasic response in intracellular H₂O₂, with peroxiredoxin hyperoxidation only triggered once the cellular H₂O₂-buffering capacity is overwhelmed. *Free Radic. Biol. Med.* 95, 333–348. doi: 10.1016/j.freeradbiomed.2016.02.035
- Tong, L., Chuang, C.-C., Wu, S., and Zuo, L. (2015). Reactive oxygen species in redox cancer therapy. *Cancer Lett.* 367, 18–25. doi: 10.1016/j.canlet.2015.07.008
- Tong, W.-H., Rouault, T., Agar, J., Zheng, L., Cash, V., Dean, D., et al. (2000). Distinct iron-sulfur cluster assembly complexes exist in the cytosol and mitochondria of human cells. *EMBO J.* 19, 5692–5700. doi: 10.1093/emboj/ 19.21.5692
- Torti, S. V., and Torti, F. M. (2013). Iron and cancer: more ore to be mined. *Nat. Rev. Cancer* 13, 342–355. doi: 10.1038/nrc3495
- Toyokuni, S., Okamoto, K., Yodoi, J., and Hiai, H. (1995). Persistent oxidative stress in cancer. *FEBS Lett.* 358, 1–3. doi: 10.1016/0014-5793(94)01368-B
- Trujillo, M., Clippe, A., Manta, B., Ferrer-Sueta, G., Smeets, A., Declercq, J. P., et al. (2007). Pre-steady state kinetic characterization of human peroxiredoxin 5: taking advantage of Trp84 fluorescence increase upon oxidation. *Arch. Biochem. Biophys.* 467, 95–106. doi: 10.1016/j.abb.2007.08.008
- Tyson, J. J., Baumann, W. T., Chen, C., Verdugo, A., Tavassoly, I., Wang, Y., et al. (2011). Dynamic modelling of oestrogen signalling and cell fate in breast cancer cells. *Nat. Rev. Cancer* 11, 523–532. doi: 10.1038/nrc3081
- Uhlén, M., Fagerberg, L., Hallström, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., et al. (2015). Tissue-based map of the human proteome. *Science* 347, 1260419–1260419. doi: 10.1126/science.1260419
- Uhlén, M., Hallstrom, B. M., Lindskog, C., Mardinoglu, A., Ponten, F., and Nielsen, J. (2016). Transcriptomics resources of human tissues and organs. *Mol. Syst. Biol.* 12, 862–862. doi: 10.15252/msb.20155865

- Ushio-Fukai, M., and Nakamura, Y. (2008). Reactive oxygen species and angiogenesis: NADPH oxidase as target for cancer therapy. *Cancer Lett.* 266, 37–52. doi: 10.1016/j.canlet.2008.02.044
- Vander Heiden, M. G., Cantley, L. C., and Thompson, C. B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 324, 1029–1034. doi: 10.1126/science.1160809
- Van Remmen, H., Ikeno, Y., Hamilton, M., Pahlavani, M., Wolf, N., Thorpe, S. R., et al. (2003). Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. *Physiol. Genomics* 16, 29–37. doi: 10.1152/physiolgenomics.00122.2003
- Väremo, L., Scheele, C., Broholm, C., Mardinoglu, A., Kampf, C., Asplund, A., et al. (2015). Proteome- and transcriptome-driven reconstruction of the human myocyte metabolic network and its use for identification of markers for diabetes. *Cell Rep.* 11, 921–933. doi: 10.1016/j.celrep.2015.04.010
- Von Sonntag, C. (1987). The Chemical Basis of Radiation Biology. London, UK: Taylor and Francis.
- Wallace, M. A., Liou, L. L., Martins, J., Clement, M. H. S., Bailey, S., Longo, V. D., et al. (2004). Superoxide inhibits 4Fe-4S cluster enzymes involved in amino acid biosynthesis. Cross-compartment protection by CuZn-superoxide dismutase. J. Biol. Chem. 279, 32055–32062. doi: 10.1074/jbc.M403590200
- Wang, C., Guo, K., Gao, D., Kang, X., Jiang, K., Li, Y., et al. (2011). Identification of transaldolase as a novel serum biomarker for hepatocellular carcinoma metastasis using xenografted mouse model and clinic samples. *Cancer Lett.* 313, 154–166. doi: 10.1016/j.canlet.2011.08.031
- Wang, J., Yuan, W., Chen, Z., Wu, S., Chen, J., Ge, J., et al. (2012). Overexpression of G6PD is associated with poor clinical outcome in gastric cancer. *Tumour Biol.* 33, 95–101. doi: 10.1007/s13277-011-0251-9
- Wang, T., Tamae, D., LeBon, T., Shively, J. E., Yen, Y., and Li, J. J. (2005). The role of peroxiredoxin II in radiation-resistant MCF-7 breast cancer cells. *Cancer Res.* 65, 10338–10346. doi: 10.1158/0008-5472.CAN-04-4614
- Warburg, O. (1956). On the origin of cancer cells. Sci. New Ser. 123, 309–314. doi: 10.1126/science.123.3191.309
- Wei, Q., Jiang, H., Xiao, Z., Baker, A., Young, M. R., Veenstra, T. D., et al. (2011). Sulfiredoxin-Peroxiredoxin IV axis promotes human lung cancer progression through modulation of specific phosphokinase signaling. *Proc. Natl. Acad. Sci.* U.S.A. 108, 7004–7009. doi: 10.1073/pnas.1013012108
- Weidinger, A., and Kozlov, A., V (2015). Biological Activities of reactive oxygen and nitrogen species: oxidative stress versus signal transduction. *Biomolecules* 5, 472–484. doi: 10.3390/biom5020472
- Weinberg, F., Hamanaka, R., Wheaton, W. W., Weinberg, S., Joseph, J., Lopez, M., et al. (2010). Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8788–8793. doi: 10.1073/pnas.1003428107
- Weinstein, J. N., Collisson, E. A., Mills, G. B., Shaw, K. R. M., Ozenberger, B. A., Ellrott, K., et al. (2013). The cancer genome atlas pan-cancer analysis project. *Nat. Genet.* 45, 1113–1120. doi: 10.1038/ng.2764
- Wen, D., Liu, D., Tang, J., Dong, L., Liu, Y., Tao, Z., et al. (2015). Malic enzyme 1 induces epithelial-mesenchymal transition and indicates poor prognosis in hepatocellular carcinoma. *Tumour Biol.* 36, 6211–6221. doi: 10.1007/s13277-015-3306-5
- Whitaker, H. C., Patel, D., Howat, W. J., Warren, A. Y., Kay, J. D., Sangan, T., et al. (2013). Peroxiredoxin-3 is overexpressed in prostate cancer and promotes cancer cell survival by protecting cells from oxidative stress. *Br. J. Cancer* 109, 983–993. doi: 10.1038/bjc.2013.396
- Winterbourn, C. C. (1995). Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicol. Lett.* 82–83, 969–974. doi: 10.1016/0378-4274(95) 03532-X
- Winterbourn, C. C. (2008). Reconciling the chemistry and biology of reactive oxygen species. Nat. Chem. Biol. 4, 278–286. doi: 10.1038/nchembio.85
- Winterbourn, C. C. (2013). The biological chemistry of hydrogen peroxide. *Meth. Enzymol.* 528, 3–25. doi: 10.1016/B978-0-12-405881-1.00001-X
- Winterbourn, C. C., and Metodiewa, D. (1999). Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. *Free Radic. Biol. Med.* 27, 322–328. doi: 10.1016/S0891-5849(99)00051-9
- Woo, H. A., Yim, S. H., Shin, D. H., Kang, D., Yu, D.-Y., and Rhee, S. G. (2010). Inactivation of peroxiredoxin I by phosphorylation allows localized H₂O₂ accumulation for cell signaling. *Cell* 140, 517–528. doi: 10.1016/j.cell.2010.01.009

- Xu, I. M.-J., Lai, R. K.-H., Lin, S.-H., Tse, A. P.-W., Chiu, D. K.-C., Koh, H.-Y., et al. (2016). Transketolase counteracts oxidative stress to drive cancer development. *Proc. Natl. Acad. Sci. USA.* 113, E725–E734. doi: 10.1073/pnas.15087 79113
- Yizhak, K., Gaude, E., Le Dévédec, S., Waldman, Y. Y., Stein, G. Y., van de Water, B., et al. (2014a). Phenotype-based cell-specific metabolic modeling reveals metabolic liabilities of cancer. *Elife* 3:e03641. doi: 10.7554/eLife. 03641
- Yizhak, K., Le Dévédec, S. E., Rogkoti, V. M., Baenke, F., de Boer, V. C., Frezza, C., et al. (2014b). A computational study of the Warburg effect identifies metabolic targets inhibiting cancer migration. *Mol. Syst. Biol.* 10:744. doi: 10.15252/msb.20134993
- Yoo, M. H., Xu, X. M., Carlson, B. A., Gladyshev, V. N., and Hatfield, D. L. (2006). Thioredoxin reductase 1 deficiency reverses tumor phenotype and tumorigenicity of lung carcinoma cells. *J. Biol. Chem.* 281, 13005–13008. doi: 10.1074/jbc.C600012200
- Yu, H., Lee, H., Herrmann, A., Buettner, R., and Jove, R. (2014). Revisiting STAT3 signalling in cancer: new and unexpected biological functions. *Nat. Rev. Cancer* 14, 736–746. doi: 10.1038/nrc3818
- Zack, T. I., Schumacher, S. E., Carter, S. L., Cherniack, A. D., Saksena, G., Tabak, B., et al. (2013). Pan-cancer patterns of somatic copy number alteration. *Nat. Genet.* 45, 1134–1140. doi: 10.1038/ng.2760
- Zhan, L., Zhang, H., Zhang, Q., Woods, C. G., Chen, Y., Xue, P., et al. (2012). Regulatory role of KEAP1 and NRF2 in PPARγ expression and chemoresistance in human non-small-cell lung carcinoma cells. *Free Radic. Biol. Med.* 53, 758–768. doi: 10.1016/j.freeradbiomed.2012.05.041
- Zhang, C., Lee, S., Mardinoglu, A., and Hua, Q. (2016). Investigating the combinatory effects of biological networks on gene co-expression. *Front. Physiol.* 7:160. doi: 10.3389/fphys.2016.00160

- Zhang, C., Zhang, Z., Zhu, Y., and Qin, S. (2014). Glucose-6phosphate dehydrogenase: a biomarker and potential therapeutic target for cancer. *Anticancer. Agents Med. Chem.* 14, 280–289. doi: 10.2174/18715206113136660337
- Zhang, Q., Pi, J., Woods, C. G., and Andersen, M. E. (2010). A systems biology perspective on Nrf2-mediated antioxidant response. *Toxicol. Appl. Pharmacol.* 244, 84–97. doi: 10.1016/j.taap.2009.08.018
- Zhao, Y., Xue, Y., Oberley, T. D., Hines, J., and Clair, D. S. (2001). Overexpression of manganese superoxide dismutase suppresses tumor formation by modulation of activator protein-1 signaling in a multistage skin carcinogenesis model. *Cancer Res.* 61, 6082–6088.
- Zhong, H., and Yin, H. (2015). Role of lipid peroxidation derived 4hydroxynonenal (4-HNE) in cancer: focusing on mitochondria. *Redox Biol.* 4, 193–199. doi: 10.1016/j.redox.2014.12.011
- Zhou, J. P., Chen, X., Feng, S., Luo, S. D., Pan, Y. L., Zhong, L., et al. (2011). Systems biology modeling reveals a possible mechanism of the tumor cell death upon oncogene inactivation in egfr addicted cancers. *PLoS ONE* 6:e28930. doi: 10.1371/journal.pone.0028930

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Metabolism and Transcription in Cancer: Merging Two Classic Tales

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Cellular plasticity, or the ability of a cancer cell to adapt to changes in the microenvironment, is a major determinant of cell survival and functionality that require the coordination of transcriptional programs with signaling and metabolic pathways. In this scenario, these pathways sense and integrate nutrient signals for the induction of coordinated gene expression programs in cancer. This minireview focuses on recent advances that shed light on the bidirectional relationship between metabolism and gene transcription, and their biological outcomes in cancer. Specifically, we will discuss how metabolic changes occurring in cancer cells impact on gene expression, both at the level of the epigenetic landscape and transcription factor regulation.

OPEN ACCESS

Edited by:

Angela Re, Fondazione Istituto Italiano di Technologia, Italy

Reviewed by:

Jun Yan, Nanjing University, China Varda Shoshan-Barmatz, Ben-Gurion University of the Negev, Israel

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Specialty section:

This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: 09 September 2017 Accepted: 18 December 2017 Published: 05 January 2018

Citation:

Martín-Martín N, Carracedo A and Torrano V (2018) Metabolism and Transcription in Cancer: Merging Two Classic Tales. Front. Cell Dev. Biol. 5:119. doi: 10.3389/fcell.2017.00119 Keywords: cancer metabolism, nutrient sensing networks, transcription factors, histone acetylation, DNA and histone methylation, gene expression regulation

INTRODUCTION

The advances toward curative treatments for cancer are nowadays based on three pillars of research: (i) early detection, (ii) molecular stratification of high-risk patients and (iii) the selection of the most appropriate therapeutic strategy. New insights in the molecular understanding of cancer has led to a paradigmatic change in the way we combat the disease, introducing the concept of precision medicine: patient's stratification and personalized therapy.

In the recent years there has been a renaissance in the study of the cross-interaction between two important "usual suspects" in cancer: gene expression and metabolism (Hanahan and Weinberg, 2011). Both research areas have inherited potential to be applied to precision medicine. On the one hand, the study of transcriptional regulators can potentially lead to the development of stratification tools. On the other, the stratification can define which cancer patients will benefit from a given metabolic-based therapeutic approach (**Figure 1A**).

Along the process of transformation, the acquisition of pro-survival abilities is a crucial determinant that enables cancer cells to adapt to the ever-changing environment (Hanahan and Weinberg, 2011). This master adaptation is based, in part, on the connection between nutrient sensing and gene expression programs. As a consequence, cancer cells rewire their metabolism to activate the fittest metabolic rate for cancer homeostasis. This type of response requires a circuit in which cellular metabolism and gene transcription must be bidirectionally connected and tightly coordinated (**Figure 1B**).

One of the most important cellular regulatory mechanism that determine which genes are activated is the packing of DNA and histones in chromatin or epigenetic remodeling. Post-translational modifications of histones and DNA—mainly acetylation and methylation—alter the structure of chromatin, helping or preventing the recruitment of transcription factors complexes that will ultimately regulate gene expression. At the same time, changes in gene expression in



response to environmental fluctuations are led by posttranslational modifications or activation of transcription factors. Metabolism is the process of energy transduction that encompasses a network of chemical reactions tightly regulated by environmental changes. The idea that epigenetics and gene transcription can be influenced by products of metabolic pathways was proposed many years ago (Shi and Shi, 2004), but the biological relevance of this concept in tumorigenic processes has remained largely unknown.

Systematic profiling of cancer specimens has determined the existence of epigenetic alterations across the genome that potentially regulate gene expression and are associated with tumor progression (Baylin and Jones, 2011). This expanding field is coming together with cancer metabolism. During transformation, the entire metabolic network is rewired to efficiently convert nutrients to biosynthetic precursors to sustain cancer cell growth (Hanahan and Weinberg, 2011).

Metabolic and epigenetic enzymes are frequently components of the same tumorigenic pathway. Thus, metabolic rewiring occurring in cancer can impact on the regulation of chromatin structure and, therefore, cancer-related gene expression. Conversely, nutrient availability, or extracellular signals within the tumor microenvironment can fine-tune the expression of metabolic genes through epigenetic modifications and transcriptional regulation (**Figure 2**).

IMPACT OF METABOLISM AND ITS PRODUCTS IN GENE EXPRESSION PROGRAMS

Most chromatin-modifying enzymes use co-factors and substrates that are critical metabolites of the intermediary metabolism. The availability of these metabolites can influence the capacity of the cell to write or erase chromatin marks, highlighting the intimate link between the metabolic state, epigenetic regulation and gene expression.

DNA and Histone Methylation

In human DNA, cytosines are typically methylated at CpG islands located in promoter regions and associated with

transcriptional regulation. Cancers frequently display global DNA hypomethylation but hypermethylation of CpG islands in genomic regions where tumor suppressor genes are located (Hansen et al., 2011). These histone methyl marks can either activate or repress gene expression (Kinnaird et al., 2016).

Methylation is linked to the intermediary metabolism through S-adenosyl methionine (SAM), the primary source of methyl groups that is generated in the folate and methionine cycles (Maddocks et al., 2016; Mentch and Locasale, 2016). The activities of both histone methyltransferases (HMT) and DNA methyltransferases (DNMT) depend on the levels of intracellular SAM which varies based on serine and methionine availability. The deprivation of these essential amino acids induce reversible and rapid changes in histone and DNA methylation, which in turn change the transcriptional landscape of cancer cells (Mentch et al., 2015; Maddocks et al., 2016). In the light of these data, the methionine cycle and the sensing of SAM availability provide a direct link between intermediary metabolism and chromatin state in cells.

Interestingly, system biology approaches have revealed methionine cycle and one-carbon metabolism gene networks as major determinants of DNA methylation status in human cancer and cancer survival predictors (Mehrmohamadi et al., 2016). Indeed, dysregulation of histone methylation in specific chromatin regions is a major selective force for tumor progression and metastatic potential (McDonald et al., 2017). Of note, the epigenetic changes associated with distant metastasis are strongly dependent on the oxidative branch of the pentose phosphate pathway (oxPPP). This dependency confers selective advantages to the disseminated cells enabling their metastatic spread. In distal metastasis sites, oxPPP is coupled to epigenetic programs that promote tumorigenesis (McDonald et al., 2017).

The demethylation reaction is also susceptible to metabolic fluctuations. The enzymatic removal of methyl groups is regulated by histone and DNA demethylases whose activities are modulated by the tricarboxylic acid (TCA) cycle intermediates alpha-ketoglutarate (α -KG), fumarate and succinate. When presented in sufficient concentration, α -KG acts as a positive co-factor of the demethylase activity, while fumarate and succinate are competitive inhibitors of multiple histone demethylases (Xiao et al., 2012). The activity of these enzymes can be dramatically



altered by mutations in key metabolic enzymes. Inactivating mutations affecting the mitochondrial succinate dehydrogenase (SDH) complex subunits and fumarate hydratase (FH) are driver mutations in a subset of cancers (Tomlinson et al., 2002; Janeway et al., 2011; Pantaleo et al., 2011; Castro-Vega et al., 2014; Clark et al., 2014). These mutations lead to the accumulation of succinate and fumarate and the subsequent inhibition of α -KG-dependent dioxygenases (Xiao et al., 2012). The direct contribution of fumarate accumulation and epigenetics to tumorigenesis has been elegantly shown in the context of FH loss. In this scenario, fumarate accumulation elicits epigenetic changes in a regulatory region of the antimetastatic miRNA cluster mir-200ba429. In turn, the suppression of miR-200 leads to the expression of epithelial-to-mesenchymal-transition (EMT)-related transcription factors and the enhancement of migratory properties (Sciacovelli et al., 2016). Deficiency of SDH is associated with global DNA methylation changes (Killian et al., 2013) and the downregulation of neuroendocrine differentiation genes linked to a migratory phenotype (Letouze et al., 2013).

Upstream of SDH in the TCA cycle, isocitrate dehydrogenase (IDH) catalyzes the oxidative decarboxylation of isocitrate, producing α -KG and CO₂. IDH genes are the most frequently mutated metabolic genes in cancers driving global epigenetic changes (Figueroa et al., 2010; Ward et al., 2010; Cairns et al.,

2012). Mutations in IDH1/2 have oncogenic properties and impede the synthesis of α -KG but favor the formation of the oncometabolite 2-hydroxyglutarate (2-HG) (Dang et al., 2009; Ye et al., 2013). In turn, 2-HG accumulation inhibits DNA demethylation (Losman et al., 2013) and primes cancer cells for transformation (Figueroa et al., 2010; Lu et al., 2012; Turcan et al., 2012). However, the production of 2-HG is not restricted to an IDH mutated background. For example, in hypoxia wild-type IDH2 produces 2-HG as a by-product (Wise et al., 2011). In ER-negative breast cancer patients, the accumulation of 2-HG define a subgroup of wild-type IDH2 patients with specific hypermethylation phenotype and poor clinical outcome (Terunuma et al., 2014). This work suggests that the metabolic-epigenetic axis could be reflected in tumor subtypes of clinical relevance.

Beyond cancer biology, but conceptually connected, 2-HG has been proposed to act as an immunometabolite that links the environmental context to immune fate and function through a metabolic-epigenetic axis (Tyrakis et al., 2016; Xu et al., 2017). Given the important role of the immune system in the maintenance of chronic inflammation during tumorigenic processes (Numasaki et al., 2003; Grivennikov et al., 2012), these results may have implications for tumor immunology.

In summary, the accumulation of succinate, fumarate, and 2-HG contribute to cancer progression and position the Krebs cycle as mitochondrial custodian of the methylome (**Figure 2**).

Histone Acetylation

Global levels of nuclear histone acetylation are sensitive to overall acetyl CoA levels. Acetyl CoA is a key intermediate of central metabolism, which not only fuels ATP production via the TCA cycle, but also functions as an essential building block for the synthesis of fatty acids and sterols, and importantly histone acetylation. Acetyl CoA is generated from catabolic pathways of intermediary metabolism and at the same time used by anabolic processes such as lipid synthesis. In mammalian cells, there are three major enzymes that generate acetyl CoA: acetate-dependent acetyl-CoA synthetase 2 (ACSS2), citratedependent ATP-citrate lyase (ACLY) and mitochondrial pyruvate dehydrogenase complex (PDC). The relative importance of ACSS2, ACLY and PDC for nuclear histone acetylation differs on the basis of the developmental state, disease, tissue type and even subcellular location.

ACLY is the primary enzyme responsible for the synthesis of acetyl CoA from glucose-derived citrate and connects oncogenic signals to histone acetylation (Wellen et al., 2009; Lee et al., 2014). In the absence of ACLY, under nutrient deprivation or stress conditions, cells upregulate ACSS2, enabling cancer cells to utilize acetate to sustain tumor growth (Comerford et al., 2014; Mashimo et al., 2014; Schug et al., 2015) by providing acetyl CoA for fatty acid and phospholipid synthesis and histone acetylation (Zhao et al., 2016). In addition, under hypoxic conditions, acetate mediates epigenetic changes that specifically activate a lipogenic program and promote cancer cell survival (Gao et al., 2016). Importantly, ACSS2 has been recently identified as a chromatin-bound factor that regulates and coordinates gene expression programs related to long-term spatial memory (Mews et al., 2017). This is the first evidence of the direct and causal contribution of ACSS2-derived acetyl CoA to epigenetic modulation and gene expression.

Lipid-derived carbons are also a bona fide physiological source of acetyl CoA for histone acetylation. The acetyl CoA produced via the activation of fatty acid oxidation (FAO) is selectively used by histone acetyl transferases located at gene locus where key lymphatic and lipid-specific genes reside (McDonnell et al., 2016; Wong et al., 2017). These studies expand the landscape of nutrient sensing and uncover how lipids and metabolism are integrated by epigenetic events that control gene expression. In a cancer scenario, the uptake of fatty acidsmediated by CD36-and their oxidation sustain cancer-initiating cells and promote metastasis. Interestingly, these metastasisinitiating cells with high expression of CD36 are defined by a lipid metabolism transcriptional signature (Pascual et al., 2017). Although no link with epigenetic changes have been associated with this phenotype, we could predict that lipid uptake, and presumably its oxidation could play and important role in cell survival and cancer progression by regulating the epigenetic and transcriptional landscapes.

Due to its biochemical properties, the biosynthesis of acetyl CoA is thought to occur in the subcellular compartment where it is required. Therefore, the localized production of acetyl CoA by spatial regulation of its enzymatic producers would confer a high degree of specificity to metabolic regulation of histone acetylation and gene expression.

In the mitochondria, acetyl CoA is the main product of FAO, branch chain amino acid catabolism and pyruvate oxidation through the activity of PDC. Although PDC has classically been localized to the mitochondria, under metabolic insults, functional PDC translocate to the nucleus. There, it generates a nuclear pool of acetyl CoA that increases the acetylation of core histones important for S phase entry (Sutendra et al., 2014). In line, spatial regulation of ACSS2 confers specificity to the metabolic regulation of histone acetylation and together with ACLY were found in the nucleus (Takahashi et al., 2006; Wellen et al., 2009). Importantly, the "on site" generation of ACSS2-derived acetyl CoA at specific chromatin domains favors histone acetylation of key genes involved in long-term spatial memory, autophagy, cell survival and tumorigenesis (Bulusu et al., 2017; Li et al., 2017a; Mews et al., 2017).

Interestingly, the modulation of the mitochondrial protein VDAC1 induced a coordinated cascade of changes in mitochondrial metabolites that elicited a global metabolic re-programming in glioblastoma cells. This metabolic rewiring led to the activation of neural cell differentiation transcriptional programs and reversal oncogenic properties of glioblastoma cells (Arif et al., 2017).

In summary, chromatin-associated enzymes sense intermediary metabolism products and process this information into dynamic chromatin modifications that will ultimately regulate adaptive transcriptional programs associated with oncogenic processes.

TRANSCRIPTIONAL REGULATION OF METABOLIC PROGRAMS

The metabolic switch in cancer encloses a plethora of discrete enzymatic activities that must be coordinately altered in order to ensure the adaptation of cancer cells to environmental alterations (Loo et al., 2015). In the recent years, numerous reports have provided evidences of the cues regulating one or few enzymes within a metabolic pathway in cancer. However, the means of coordinated regulation of complex metabolic networks is starting to be elucidated (Torrano et al., 2016; Valcarcel-Jimenez et al., 2017).

Nutrients perturbations can be sensed directly by master transcriptional regulators of metabolism that will ultimately elicit the coordinated expression of genes required for metabolic adaptation in cancer cells (**Figure 2**). These programs allow the rapid adaptation to new biological states or external insults, and their contribution to cancer pathogenesis and progression has begun to emerge (Mouchiroud et al., 2014). More than fifty years ago an association between lipid metabolism and tumor progression was reported (Weinhouse et al., 1951) and since that time, the involvement of lipid metabolism in tumorigenesis has been thoroughly investigated.

Peroxisome-proliferator-activated receptors (PPARs), PPAR- α , PPAR- δ (also known as PPAR- β) and PPAR- γ , are members

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of the nuclear receptor superfamily of transcription factors that control lipid sensing and the transcriptional regulation of metabolic pathways (Michalik et al., 2006). PPARs regulate gene expression programs that impact on proliferation, differentiation and survival, thus controlling carcinogenesis in various tissues including liver, breast, lung, colon and bone marrow. The role of these nuclear factors in transformation has been controversial during the past years, being described as either tumor suppressor or oncogenes (Carracedo et al., 2012; Ito et al., 2012; Peters et al., 2015; Lakshmi et al., 2017; Martin-Martin et al., 2017; Sun et al., 2017). The activity of PPARs is modulated upon ligand binding and by a number of coactivator and corepressor proteins, the presence of which can stimulate or inhibit the transcriptional function of the receptor (Feige and Auwerx, 2007; Martin-Martin et al., 2017). One of the most studied coregulators of PPARs function is PPAR gamma co-activator 1 alpha (PGC1α), a master transcriptional co-activator with broad functions in energy metabolism. Together, PPARs and PGC1a control mitochondrial function and FAO (Sugden et al., 2010) and have been implicated in the maintenance of hematopoietic stem cell pool, cancer survival and progression (Carracedo et al., 2012; Ito et al., 2012; Torrano et al., 2016; Valcarcel-Jimenez et al., 2017).

The classical nuclear receptors are known as the receptors for steroids such as estrogen, androgen, glucocorticoid, and progesterone, which are derivatives of cholesterol. Among these classical nuclear factors, the sterol regulatory element bindingproteins (SREBPs) are the master transcription factors that are highly sensitive to the intracellular levels of cholesterol. The cholesterol composition of cellular membranes is an essential metabolic requirement for cell division (Bengoechea-Alonso and Ericsson, 2016). Different cancer cell types adapt their metabolism to maintain high intracellular cholesterol levels through increased cholesterol uptake and the activation of lipogenic transcriptional programs dependent on SREBP-1 (Guo et al., 2011; Huang et al., 2012; Li et al., 2017b). These pathways converge into the accelerated endogenous production of cholesterol. It has been recently described the regulation of ACSS2 by SREBP in mammary epithelial cells, having this regulation an effect on fatty acid synthesis (Xu et al., 2018). Given the important role of ACSS2 as a central node between metabolism and epigenetic regulation in cancer, it is tempting to speculate that the cholesterol levels in cancer cells may have an impact on gene regulation through the modulation of ACSS2 enzymatic activity.

The transcriptional agonist properties of cholesterol are not limited to SREBPs. Cholesterol has been recently identified as a physiological and functional endogenous agonist of the estrogenrelated receptor alpha (ERR α). Upon cholesterol binding, ERR α recruits PGC1 α coactivators to DNA promoters and together serve as a critical metabolic sensors that regulate gene expression programs associated to osteogenesis, myogenesis and macrophage activation (Wei et al., 2016). This is the first evidence for cholesterol and the cholesterol biosynthetic pathway in the regulation of ERR α activity and biology.

Taken together, all these data position cholesterol as a master metabolite that control gene transcription programs via its interaction with nuclear factors.

ERRa and its transcriptional programs are implicated in metabolism and cancer progression. Increased ERRa activity is observed in melanoma, breast and ovarian cancer, colorectal carcinoma and osteosarcoma (Stein and McDonnell, 2006; Vazquez et al., 2013; Chen et al., 2014; Thewes et al., 2015). ERRs are nuclear receptors that exhibit ligand-dependent regulation, and their activity relies on the status of transcriptional coactivators and co-repressors (Feige and Auwerx, 2007). One such co-activators, PGC1a has been extensively studied in physiological conditions (Handschin, 2009). PGC1a controls transcriptional programs that increase the energetic yield (Scarpulla, 2011) and counteract oxidative stress (St-Pierre et al., 2006; Haq et al., 2013; Vazquez et al., 2013), which enables elevated oxidative mitochondrial activity (OXPHOS) coping with the accumulation of reactive oxidant species (ROS). PGC1a exerts paradoxical activities in different tumor types and biological conditions and recent studies highlight the importance of it in cancer metabolism (Vazquez et al., 2013; LeBleu et al., 2014; Sancho et al., 2015; Luo et al., 2016) and specifically through the regulation of ERRs (Haq et al., 2013; Vazquez et al., 2013; Torrano et al., 2016; Valcarcel-Jimenez et al., 2017).

The classical view of cancer metabolic wiring (Warburg effect) would predict that the PGC1a-ERRa axis and OXPHOS triggered are inherently tumor suppressive. However, recent studies uncover that factors such as mutational background, tissue or cell of origin and disease stage impose a pressure toward the best-adapted metabolic wiring during cancer progression. In melanoma and breast cancer, cells turn on PGC1a and their OXPHOS program, which impacts on cancer cell survival, proliferation and contribution to therapy resistance (Haq et al., 2013; Vazquez et al., 2013; LeBleu et al., 2014). Interestingly, during the process of metastasis, melanoma cells need to suppress PGC1a expression in order to regulate an adhesion and invasion transcriptional program (Luo et al., 2016). In line, OXPHOS PGC1a-induced metabolism represents a disadvantageous metabolic state in prostate cancer. Moreover, the decrease of PGC1a-ERRa transcriptional activity provides a selective advantage to metastasize and correlates with an increased disease recurrence (Torrano et al., 2016; Valcarcel-Jimenez et al., 2017).

These studies elegantly illustrate how the PGC1 α -ERR α transcriptional axis can exert opposing activities in cancer progression, highlighting the metabolic diversity leading to metabolic adaptations during cancer progression in different cancer types.

CONCLUDING REMARKS

Metabolic rewiring and gene deregulation are both hallmarks of cancer (Hanahan and Weinberg, 2011) and are addictive for tumor cells (Bradner et al., 2017; Vander Heiden and DeBerardinis, 2017). Thus, the crosstalk between gene expression and metabolism are fundamental aspects of cellular adaptation to nutritional changes during tumorigenesis. An attractive approach to understand cancer and identify therapeutic targets is to discover the key components on which deregulated transcriptional and metabolic programs depend in cancer cells. We have outlined recent advances that described how coordinated gene expression programs are tightly and dynamically regulated by the metabolome, either at the level of chromatin modifications and transcription factor activities. In this scenario, metabolic alterations during cellular transformation drive aberrant gene expression which in turn will be key contributors to tumor development and progression. However, much remains to be discovered, and the study of the bidirectional contribution of metabolism to gene expression regulation will bring a more integrated understanding of cellular adaptations during cancer progression and, possibly new therapeutic opportunities.

AUTHOR CONTRIBUTIONS

VT wrote the manuscript with essential contribution of AC and NM-M.

REFERENCES

- Arif, T., Krelin, Y., Nakdimon, I., Benharroch, D., Paul, A., Dadon-Klein, D., et al. (2017). VDAC1 is a molecular target in glioblastoma, with its depletion leading to reprogrammed metabolism and reversed oncogenic properties. *Neuro Oncol.* 19, 951–964. doi: 10.1093/neuonc/now297
- Baylin, S. B., and Jones, P. A. (2011). A decade of exploring the cancer epigenome - biological and translational implications. *Nat. Rev. Cancer* 11, 726–734. doi: 10.1038/nrc3130
- Bengoechea-Alonso, M. T., and Ericsson, J. (2016). The phosphorylationdependent regulation of nuclear SREBP1 during mitosis links lipid metabolism and cell growth. *Cell Cycle* 15, 2753–2765. doi: 10.1080/15384101.2016.1220456
- Bradner, J. E., Hnisz, D., and Young, R. A. (2017). Transcriptional addiction in cancer. *Cell* 168, 629–643. doi: 10.1016/j.cell.2016.12.013
- Bulusu, V., Tumanov, S., Michalopoulou, E., N. J., van den Broek, MacKay, G., Nixon, C., et al. (2017). Acetate recapturing by nuclear acetyl-CoA synthetase 2 prevents loss of histone acetylation during oxygen and serum limitation. *Cell Rep.* 18, 647–658. doi: 10.1016/j.celrep.2016.12.055
- Cairns, R. A., Iqbal, J., Lemonnier, F., Kucuk, C., de Leval, L., Jais, J. P., et al. (2012). IDH2 mutations are frequent in angioimmunoblastic T-cell lymphoma. *Blood* 119, 1901–1903. doi: 10.1182/blood-2011-11-391748
- Carracedo, A., Weiss, D., Leliaert, A. K., Bhasin, M., de Boer, V. C., Laurent, G., et al. (2012). A metabolic prosurvival role for PML in breast cancer. *J. Clin. Invest.* 122, 3088–3100. doi: 10.1172/JCI62129
- Castro-Vega, L. J., Buffet, A., De Cubas, A. A., Cascón, A., Menara, M., Khalifa, E., et al. (2014). Germline mutations in FH confer predisposition to malignant pheochromocytomas and paragangliomas. *Hum. Mol. Genet.* 23, 2440–2446. doi: 10.1093/hmg/ddt639
- Chen, P., Wang, H., Duan, Z., Zou, J. X., Chen, H., He, W., et al. (2014). Estrogen-related receptor alpha confers methotrexate resistance via attenuation of reactive oxygen species production and P53 mediated apoptosis in osteosarcoma cells. *Biomed. Res. Int.* 2014:616025. doi: 10.1155/2014/616025
- Clark, G. R., Sciacovelli, M., Gaude, E., Walsh, D. M., Kirby, G., Simpson, M. A., et al. (2014). Germline FH mutations presenting with pheochromocytoma. J. Clin. Endocrinol. Metab. 99, E2046–E2050. doi: 10.1210/jc.2014-1659
- Comerford, S. A., Huang, Z., Du, X., Wang, Y., Cai, L., Witkiewicz, A. K., et al. (2014). Acetate dependence of tumors. *Cell* 159, 1591–1602. doi: 10.1016/j.cell.2014.11.020
- Dang, L., White, D. W., Gross, S., Bennett, B. D., Bittinger, M. A., Driggers, E. M., et al. (2009). Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature* 462, 739–744. doi: 10.1038/nature08617
- Feige, J. N., and Auwerx, J. (2007). Transcriptional coregulators in the control of energy homeostasis. *Trends Cell Biol.* 17, 292–301. doi: 10.1016/j.tcb.2007.04.001
- Figueroa, M. E., Abdel-Wahab, O., Lu, C., Ward, P. S., Patel, J., Shih, A., et al. (2010). Leukemic IDH1 and IDH2 mutations result in a hypermethylation

ACKNOWLEDGMENTS

Apologies to those whose related publications were not cited due to space limitations. We would like to acknowledge the Carracedo lab for the continuous input and discussions. NM-M is funded by CIBERONC. VT is founded by Fundación Vasca de Innovación e Investigación Sanitarias, BIOEF (BIO15/CA/052), the AECC J.P. Bizkaia and the Basque Department of Health (2016111109). The work of AC is supported by the Ramón y Cajal award, the Basque Department of Industry, Tourism and Trade (Etortek) and the department of education (IKERTALDE IT1106-16), the BBVA foundation, the MINECO (SAF2016-79381-R (FEDER/EU); Severo Ochoa Excellence Accreditation SEV-2016-0644) and the European Research Council (Starting Grant 336343, PoC 754627). The participation of NM-M, AC, and VT as part of CIBERONC was co-funded with FEDER funds.

phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell* 18, 553–567. doi: 10.1016/j.ccr.2010.11.015

- Gao, X., Lin, S. H., Ren, F., Li, J. T., Chen, J. J., Yao, C. B., et al. (2016). Acetate functions as an epigenetic metabolite to promote lipid synthesis under hypoxia. *Nat. Commun.* 7:11960. doi: 10.1038/ncomms11960
- Grivennikov, S. I., Wang, K., Mucida, D., Stewart, C. A., Schnabl, B., Jauch, D., et al. (2012). Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature* 491, 254–258. doi: 10.1038/nature 11465
- Guo, D., Reinitz, F., Youssef, M., Hong, C., Nathanson, D., Akhavan, D., et al. (2011). An LXR agonist promotes glioblastoma cell death through inhibition of an EGFR/AKT/SREBP-1/LDLR-dependent pathway. *Cancer Discov.* 1, 442–456. doi: 10.1158/2159-8290.CD-11-0102
- Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646–674. doi: 10.1016/j.cell.2011.02.013
- Handschin, C. (2009). The biology of PGC-1α and its therapeutic potential. *Trends Pharmacol. Sci.* 30, 322–329. doi: 10.1016/j.tips.2009.03.006
- Hansen, K. D., Timp, W., Bravo, H. C., Sabunciyan, S., Langmead, B., McDonald, O. G., et al. (2011). Increased methylation variation in epigenetic domains across cancer types. *Nat. Genet.* 43, 768–775. doi: 10.1038/ng.865
- Haq, R., Shoag, J., Andreu-Perez, P., Yokoyama, S., Edelman, H., Rowe, G. C., et al. (2013). Oncogenic BRAF regulates oxidative metabolism via PGC1α and MITF. *Cancer Cell* 23, 302–315. doi: 10.1016/j.ccr.2013.02.003
- Huang, W. C., Li, X., Liu, J., Lin, J., and Chung, L. W. (2012). Activation of androgen receptor, lipogenesis, and oxidative stress converged by SREBP-1 is responsible for regulating growth and progression of prostate cancer cells. *Mol. Cancer Res.* 10, 133–142. doi: 10.1158/1541-7786.MCR-11-0206
- Ito, K., Carracedo, A., Weiss, D., Arai, F., Ala, U., Avigan, D. E., et al. (2012). A PML-PPAR-delta pathway for fatty acid oxidation regulates hematopoietic stem cell maintenance. *Nat. Med.* 18, 1350–1358. doi: 10.1038/nm.2882
- Janeway, K. A., Kim, S. Y., Lodish, M., Nosé, V., Rustin, P., Gaal, J., et al. (2011). Defects in succinate dehydrogenase in gastrointestinal stromal tumors lacking KIT and PDGFRA mutations. *Proc. Natl. Acad. Sci. U.S.A.* 108, 314–318. doi: 10.1073/pnas.1009199108
- Killian, J. K., Kim, S. Y., Miettinen, M., Smith, C., Merino, M., Tsokos, M., et al. (2013). Succinate dehydrogenase mutation underlies global epigenomic divergence in gastrointestinal stromal tumor. *Cancer Discov.* 3, 648–657. doi: 10.1158/2159-8290.CD-13-0092
- Kinnaird, A., Zhao, S., Wellen, K. E., and Michelakis, E. D. (2016). Metabolic control of epigenetics in cancer. Nat. Rev. Cancer 16, 694–707. doi: 10.1038/nrc.2016.82
- Lakshmi, S. P., Reddy, A. T., Banno, A., and Reddy, R. C. (2017). PPAR Agonists for the prevention and treatment of lung cancer. *PPAR Res.* 2017:8252796. doi: 10.1155/2017/8252796
- LeBleu, V. S., O'Connell, J. T., Gonzalez Herrera, K. N., Wikman, H., Pantel, K., Haigis, M. C., et al. (2014). PGC-1α mediates mitochondrial biogenesis and

oxidative phosphorylation in cancer cells to promote metastasis. *Nat. Cell Biol.* 16, 992–1003. doi: 10.1038/ncb3039

- Lee, J. V., Carrer, A., Shah, S., Snyder, N. W., Wei, S., Venneti, S., et al. (2014). Aktdependent metabolic reprogramming regulates tumor cell histone acetylation. *Cell Metab.* 20, 306–319. doi: 10.1016/j.cmet.2014.06.004
- Letouzé, E., Martinelli, C., Loriot, C., Burnichon, N., Abermil, N., Ottolenghi, C., et al. (2013). SDH mutations establish a hypermethylator phenotype in paraganglioma. *Cancer Cell* 23, 739–752. doi: 10.1016/j.ccr.2013.04.018
- Li, N., Zhou, Z. S., Shen, Y., Xu, J., Miao, H. H., Xiong, Y., et al. (2017b). Inhibition of the sterol regulatory element-binding protein pathway suppresses hepatocellular carcinoma by repressing inflammation in mice. *Hepatology* 65, 1936–1947. doi: 10.1002/hep.29018
- Li, X., Yu, W., Qian, X., Xia, Y., Zheng, Y., Lee, J. H., et al. (2017a). Nucleus-Translocated ACSS2 promotes gene transcription for lysosomal biogenesis and autophagy. *Mol. Cell* 66, 684–697.e9. doi: 10.1016/j.molcel.2017.04.026
- Loo, J. M., Scherl, A., Nguyen, A., Man, F. Y., Weinberg, E., Zeng, Z., et al. (2015). Extracellular metabolic energetics can promote cancer progression. *Cell* 160, 393–406. doi: 10.1016/j.cell.2014.12.018
- Losman, J. A., Looper, R. E., Koivunen, P., Lee, S., Schneider, R. K., McMahon, C., et al. (2013). (R)-2-hydroxyglutarate is sufficient to promote leukemogenesis and its effects are reversible. *Science* 339, 1621–1625. doi: 10.1126/science.1231677
- Lu, C., Ward, P. S., Kapoor, G. S., Rohle, D., Turcan, S., Abdel-Wahab, O., et al. (2012). IDH mutation impairs histone demethylation and results in a block to cell differentiation. *Nature* 483, 474–478. doi: 10.1038/nature10860
- Luo, C., Lim, J. H., Lee, Y., Granter, S. R., Thomas, A., Vazquez, F., et al. (2016). A PGC1α-mediated transcriptional axis suppresses melanoma metastasis. *Nature* 537, 422–426. doi: 10.1038/nature19347
- Maddocks, O. D., Labuschagne, C. F., Adams, P. D., and Vousden, K. H. (2016). Serine metabolism supports the methionine cycle and DNA/RNA methylation through *de novo* ATP synthesis in cancer cells. *Mol. Cell* 61, 210–221. doi: 10.1016/j.molcel.2015.12.014
- Martín-Martín, N., Zabala-Letona, A., Fernandez-Ruiz, S., Arreal, L., Camacho, L., Castillo-Martin, M., et al. (2017). PPAR& elicits ligand-independent repression of trefoil factor family to limit prostate cancer growth. *Cancer Res.* doi: 10.1158/0008-5472.CAN-17-0908. [Epub ahead of print].
- Mashimo, T., Pichumani, K., Vemireddy, V., Hatanpaa, K. J., Singh, D. K., Sirasanagandla, S., et al. (2014). Acetate is a bioenergetic substrate for human glioblastoma and brain metastases. *Cell* 159, 1603–1614. doi: 10.1016/j.cell.2014.11.025
- McDonald, O. G., Li, X., Saunders, T., Tryggvadottir, R., Mentch, S. J., Warmoes, M. O., et al. (2017). Epigenomic reprogramming during pancreatic cancer progression links anabolic glucose metabolism to distant metastasis. *Nat. Genet.* 49, 367–376. doi: 10.1038/ng.3753
- McDonnell, E., Crown, S. B., Fox, D. B., Kitir, B., Ilkayeva, O. R., Olsen, C. A., et al. (2016). Lipids reprogram metabolism to become a major carbon source for histone acetylation. *Cell Rep.* 17, 1463–1472. doi: 10.1016/j.celrep.2016.10.012
- Mehrmohamadi, M., Mentch, L. K., Clark, A. G., and Locasale, J. W. (2016). Integrative modelling of tumour DNA methylation quantifies the contribution of metabolism. *Nat. Commun.* 7:13666. doi: 10.1038/ncomms13666
- Mentch, S. J., and Locasale, J. W. (2016). One-carbon metabolism and epigenetics: understanding the specificity. Ann. N. Y. Acad. Sci. 1363, 91–98. doi: 10.1111/nyas.12956
- Mentch, S. J., Mehrmohamadi, M., Huang, L., Liu, X., Gupta, D., Mattocks, D., et al. (2015). Histone methylation dynamics and gene regulation occur through the sensing of one-carbon metabolism. *Cell Metab.* 22, 861–873. doi: 10.1016/j.cmet.2015.08.024
- Mews, P., Donahue, G., Drake, A. M., Luczak, V., Abel, T., and Berger, S. L. (2017). Acetyl-CoA synthetase regulates histone acetylation and hippocampal memory. *Nature* 546, 381–386. doi: 10.1038/nature22405
- Michalik, L., Auwerx, J., Berger, J. P., Chatterjee, V. K., Glass, C. K., Gonzalez, F. J., et al. (2006). International union of pharmacology. LXI. Peroxisome proliferator-activated receptors. *Pharmacol. Rev.* 58, 726–741. doi: 10.1124/pr.58.4.5
- Mouchiroud, L., Eichner, L. J., Shaw, R. J., and Auwerx, J. (2014). Transcriptional coregulators: fine-tuning metabolism. *Cell Metab.* 20, 26–40. doi: 10.1016/j.cmet.2014.03.027

- Numasaki, M., Fukushi, J., Ono, M., Narula, S. K., Zavodny, P. J., Kudo, T., et al. (2003). Interleukin-17 promotes angiogenesis and tumor growth. *Blood* 101, 2620–2627. doi: 10.1182/blood-2002-05-1461
- Pantaleo, M. A., Nannini, M., Astolfi, A., Biasco, G., and Bologna, S. G. (2011). A distinct pediatric-type gastrointestinal stromal tumor in adults: potential role of succinate dehydrogenase subunit A mutations. *Am. J. Surg. Pathol.* 35, 1750–1752. doi: 10.1097/PAS.0b013e318230a523
- Pascual, G., Avgustinova, A., Mejetta, S., Martín, M., Castellanos, A., Attolini, C. S., et al. (2017). Targeting metastasis-initiating cells through the fatty acid receptor CD36. *Nature* 541, 41–45. doi: 10.1038/nature20791
- Peters, J. M., Gonzalez, F. J., and Muller, R. (2015). Establishing the role of PPARbeta/delta in carcinogenesis. *Trends Endocrinol. Metab.* 26, 595–607. doi: 10.1016/j.tem.2015.09.004
- Sancho, P., Burgos-Ramos, E., Tavera, A., Bou Kheir, T., Jagust, P., Schoenhals, M., et al. (2015). MYC/PGC-1α balance determines the metabolic phenotype and plasticity of pancreatic cancer stem cells. *Cell Metab.* 22, 590–605. doi: 10.1016/j.cmet.2015.08.015
- Scarpulla, R. C. (2011). Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network. *Biochim. Biophys. Acta* 1813, 1269–1278. doi: 10.1016/j.bbamcr.2010.09.019
- Schug, Z. T., Peck, B., Jones, D. T., Zhang, Q., Grosskurth, S., Alam, I. S., et al. (2015). Acetyl-CoA synthetase 2 promotes acetate utilization and maintains cancer cell growth under metabolic stress. *Cancer Cell* 27, 57–71. doi: 10.1016/j.ccell.2014.12.002
- Sciacovelli, M., Gonçalves, E., Johnson, T. I., Zecchini, V. R., da Costa, A. S., Gaude, E., et al. (2016). Fumarate is an epigenetic modifier that elicits epithelialto-mesenchymal transition. *Nature* 537, 544–547. doi: 10.1038/nature 19353
- Shi, Y., and Shi, Y. (2004). Metabolic enzymes and coenzymes in transcription-a direct link between metabolism and transcription? *Trends Genet.* 20, 445–452. doi: 10.1016/j.tig.2004.07.004
- Stein, R. A., and McDonnell, D. P. (2006). Estrogen-related receptor alpha as a therapeutic target in cancer. *Endocr. Relat. Cancer* 13(Suppl. 1), S25–S32. doi: 10.1677/erc.1.01292
- St-Pierre, J., Drori, S., Uldry, M., Silvaggi, J. M., Rhee, J., Jäger, S., et al. (2006). Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell* 127, 397–408. doi: 10.1016/j.cell.2006.09.024
- Sugden, M. C., Caton, P. W., and Holness, M. J. (2010). PPAR control: it's SIRTainly as easy as PGC. J. Endocrinol. 204, 93–104. doi: 10.1677/JOE-09-0359
- Sun, L., Shi, Y., Wang, G., Wang, X., Zeng, S., Dunn, S. E., et al. (2017). PPAR-delta modulates membrane cholesterol and cytokine signaling in malignant B cells. *Leukemia* doi: 10.1038/leu.2017.162. [Epub ahead of print].
- Sutendra, G., Kinnaird, A., Dromparis, P., Paulin, R., Stenson, T. H., Haromy, A., et al. (2014). A nuclear pyruvate dehydrogenase complex is important for the generation of acetyl-CoA and histone acetylation. *Cell* 158, 84–97. doi: 10.1016/j.cell.2014.04.046
- Takahashi, H., McCaffery, J. M., Irizarry, R. A., and Boeke, J. D. (2006). Nucleocytosolic acetyl-coenzyme a synthetase is required for histone acetylation and global transcription. *Mol. Cell* 23, 207–217. doi:10.1016/j.molcel.2006.05.040
- Terunuma, A., Putluri, N., Mishra, P., Mathé, E. A., Dorsey, T. H., Yi, M., et al. (2014). MYC-driven accumulation of 2-hydroxyglutarate is associated with breast cancer prognosis. J. Clin. Invest. 124, 398–412. doi: 10.1172/JCI71180
- Thewes, V., Simon, R., Schroeter, P., Schlotter, M., Anzeneder, T., Büttner, R., et al. (2015). Reprogramming of the ERRα and ERα target gene landscape triggers tamoxifen resistance in breast cancer. *Cancer Res.* 75, 720–731. doi: 10.1158/0008-5472.CAN-14-0652
- Tomlinson, I. P., Alam, N. A., Rowan, A. J., Barclay, E., Jaeger, E. E., Kelsell, D., et al. (2002). Germline mutations in FH predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer. *Nat. Genet.* 30, 406–410. doi: 10.1038/ng849
- Torrano, V., Valcarcel-Jimenez, L., Cortazar, A. R., Liu, X., Urosevic, J., Castillo-Martin, M., et al. (2016). The metabolic co-regulator PGC1α suppresses prostate cancer metastasis. *Nat. Cell Biol.* 18, 645–656. doi: 10.1038/ncb3357
- Turcan, S., Rohle, D., Goenka, A., Walsh, L. A., Fang, F., Yilmaz, E., et al. (2012). IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. *Nature* 483, 479–483. doi: 10.1038/nature10866

- Tyrakis, P. A., Palazon, A., Macias, D., Lee, K. L., Phan, A. T., Velica, P., et al. (2016). S-2-hydroxyglutarate regulates CD8+ T-lymphocyte fate. *Nature* 540, 236–241. doi: 10.1038/nature20165
- Valcarcel-Jimenez, L., Torrano, V., and Carracedo, A. (2017). New insights on prostate cancer progression. *Cell Cycle* 16, 13–14. doi: 10.1080/15384101.2016.1235845
- Vander Heiden, M. G., and DeBerardinis, R. J. (2017). Understanding the Intersections between metabolism and cancer biology. *Cell* 168, 657–669. doi: 10.1016/j.cell.2016.12.039
- Vazquez, F., Lim, J. H., Chim, H., Bhalla, K., Girnun, G., Pierce, K., et al. (2013). PGC1α expression defines a subset of human melanoma tumors with increased mitochondrial capacity and resistance to oxidative stress. *Cancer Cell* 23, 287–301. doi: 10.1016/j.ccr.2012.11.020
- Ward, P. S., Patel, J., Wise, D. R., Abdel-Wahab, O., Bennett, B. D., Coller, H. A., et al. (2010). The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. *Cancer Cell* 17, 225–234. doi: 10.1016/j.ccr.2010.01.020
- Wei, W., Schwaid, A. G., Wang, X., Wang, X., Chen, S., Chu, Q., et al. (2016). Ligand activation of ERRα by cholesterol mediates statin and bisphosphonate effects. *Cell Metab.* 23, 479–491. doi: 10.1016/j.cmet.2015.12.010
- Weinhouse, S., Millington, R. H., and Wenner, C. E. (1951). Metabolism of neoplastic tissue. I. The oxidation of carbohydrate and fatty acids in transplanted tumors. *Cancer Res.* 11, 845–850.
- Wellen, K. E., Hatzivassiliou, G., Sachdeva, U. M., Bui, T. V., Cross, J. R., and Thompson, C. B. (2009). ATP-citrate lyase links cellular metabolism to histone acetylation. *Science* 324, 1076–1080. doi: 10.1126/science.1164097
- Wise, D. R., Ward, P. S., Shay, J. E., Cross, J. R., Gruber, J. J., Sachdeva, U. M., et al. (2011). Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of alpha-ketoglutarate to citrate to support cell growth and viability. *Proc. Natl. Acad. Sci. U.S.A.* 108, 19611–19616. doi: 10.1073/pnas.11177 73108

- Wong, B. W., Wang, X., Zecchin, A., Thienpont, B., Cornelissen, I., Kalucka, J., et al. (2017). The role of fatty acid beta-oxidation in lymphangiogenesis. *Nature* 542, 49–54. doi: 10.1038/nature21028
- Xiao, M., Yang, H., Xu, W., Ma, S., Lin, H., Zhu, H., et al. (2012). Inhibition of α -KG-dependent histone and DNA demethylases by fumarate and succinate that are accumulated in mutations of FH and SDH tumor suppressors. *Genes Dev.* 26, 1326–1338. doi: 10.1101/gad.191056.112
- Xu, H., Luo, J., Ma, G., Zhang, X., Yao, D., Li, M., et al. (2018). Acyl-CoA synthetase short-chain family member 2 (ACSS2) is regulated by SREBP-1 and plays a role in fatty acid synthesis in caprine mammary epithelial cells. *J. Cell Physiol.* 233, 1005–1016. doi: 10.1002/jcp.25954
- Xu, T., Stewart, K. M., Wang, X., Liu, K., Xie, M., Kyu Ryu, J., et al. (2017). Metabolic control of TH17 and induced Treg cell balance by an epigenetic mechanism. *Nature* 548, 228–233. doi: 10.1038/nature23475
- Ye, D., Ma, S., Xiong, Y., and Guan, K. L. (2013). R-2-hydroxyglutarate as the key effector of IDH mutations promoting oncogenesis. *Cancer Cell* 23, 274–276. doi: 10.1016/j.ccr.2013.03.005
- Zhao, S., Torres, A., Henry, R. A., Trefely, S., Wallace, M., Lee, J. V., et al. (2016). ATP-citrate lyase controls a glucose-to-acetate metabolic switch. *Cell Rep.* 17, 1037–1052. doi: 10.1016/j.celrep.2016.09.069

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Aberrations of DNA Repair Pathways in Prostate Cancer: Future Implications for Clinical Practice?

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Patients who are carriers of inherited mutations in essential component of DNA repair pathways have a significantly higher lifetime risk for developing cancer compared to the population of reference. Recent advances in DNA next-generation sequencing technology have allowed screening for carriers of those mutations, allowing development of promising risk-reduction strategies and providing the rationale to personalize the therapeutic approach for these patients. New intriguing scenarios are opening nowadays for the management of prostate cancer in patients with germline or somatic mutations in components of DNA repair pathways (e.g., *BRCA1* and *BRCA2* genes), such as specific screening policies and new therapeutic strategies involving PARP inhibitors or platinum-based chemotherapy.

OPEN ACCESS

Edited by:

Caterina Nardella, University of Trento, Italy

Reviewed by:

Fahd Al-Mulla, Genatak, Kuwait Gabriele Multhoff, Technische Universität München, Germany

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Specialty section:

This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

Received: 04 February 2018 Accepted: 18 June 2018 Published: 05 September 2018

Citation:

Caffo O, Veccia A, Kinspergher S, Rizzo M and Maines F (2018) Aberrations of DNA Repair Pathways in Prostate Cancer: Future Implications for Clinical Practice? Front. Cell Dev. Biol. 6:71. doi: 10.3389/fcell.2018.00071 Keywords: prostate cancer, PARP inhibitors, BRCA1, BRCA2, olaparib

INTRODUCTION

BRCA1 and *BRCA2* are tumor suppressor genes with essential functions in the maintenance of genome stability (Yoshida and Miki, 2004). Both genes are characterized by an autosomal dominant inheritance pattern with incomplete penetrance, and individuals with heterozygous germline mutations in BRCA1/2 genes are at risk of losing the functional allele as a consequence of a second damage induced by alkylating agents, ionizing radiation, reactive oxygen species or chemical mutagens (Evers et al., 2010). The functional loss of *BRCA1 or 2* leads to a defect in double-strand breaks repair through the homologous recombination process, which, in turn, drastically affects the ability of the cell to preserve genome fidelity and stability (Liu and West, 2002).

It is well established that women who are carriers of inherited harmful *BRCA 1* or 2 gene mutations have an increased risk to develop breast and/or ovarian cancer in lifetime compared to their wild-type counterparts (Easton et al., 2007). Importantly, these cancer susceptibility genes have been associated to an increased risk of developing several other types of tumor, such as fallopian tube and peritoneal cancer in women (Brose et al., 2002; Finch et al., 2006), breast and prostate cancer (PC) in men (Levy-Lahad and Friedman, 2007; Tai et al., 2007; Mersch et al., 2015) and pancreatic cancer in both sexes (Ferrone et al., 2009; Mersch et al., 2015) although not fully overlapping results were observed (van Asperen et al., 2005; Moran et al., 2012).

BRCA AND PROSTATE CANCER

The incidence of germline BRCA mutations in newly diagnosed PC, unselected for family predisposition, ranges approximately from 1.2 to 2% of the cases (Leongamornlert et al., 2012).

BRCA carriers have an increased risk to develop PC (at 1.8- to 4.5-fold for BRCA1 carriers and at 2.5- to 8.6-fold for BRCA2 carriers in patients aged <65 years), in particular at early onset (Kote-Jarai et al., 2011; Leongamornlert et al., 2012).

Management of high-risk men with germline mutations in DNA-repair genes is uncertain and controversial, without consensus on the screening for PC. In this population, the harms of overdiagnosis and overtreatment are mitigated by the increased incidence and risk of PC-specific mortality. The National Comprehensive Cancer Network guidelines recommend that men with BRCA mutations should perform breast self-examination starting at 35 years of age and annually thereafter. At 45 years of age BRCA2 carriers should begin PSA screening and BRCA1 carriers should be advised to consider it. Prostatic biopsy is recommended at PSA > 3.0 ng/mL in this population [15].

The IMPACT study (Identification of Men with a genetic predisposition to ProstAte Cancer: Targeted screening in BRCA1/2 mutation carriers and controls) evaluated a tailored PC screening in 1522 men with BRCA1/2 germline mutation, proposing annual PSA tests and a prostate biopsy if PSA >3 ng/mL (Bancroft et al., 2014). Despite the lack of statistically significant difference in PC detection rate between carriers and controls, the authors observed a higher incidence of PC in BRCA2 carriers compared to BRCA1 and controls (3.3 vs. 2.6% and <2%, respectively); in addition more than 2/3 of the PCs detected in the BRCA2 carriers and 61% in BRCA1 carriers were classified as intermediate or high risk. In light of these results, they suggested that mainly in BRCA2 carriers PSA testing should be proposed earlier, repeated at shorter screening intervals, and with lower PSA thresholds compared to the general population, in order to detect the tumorigenic transformation earlier. It is clear that according to the actual limitations of PSA-based screening, final results of the study are waiting to define the optimal screening program in this subset of patient, although the preliminary results seem to support PSA routine testing in BRCA carriers (Mitra et al., 2011).

An ongoing clinical trial in Toronto, Canada (NCT01990521) is evaluating the role of prostate MRI in male BRCA carriers, regardless of the PSA values.

BRCA2 germline mutations in men with PC have been associated with an aggressive tumor phenotype, a more advanced tumor stage at the diagnosis and poor survival outcomes at any disease stage, including the localized/locally advanced disease (Tryggvadottir et al., 2007; Castro et al., 2013). In particular, it has been recently observed that BRCA mutation carriers with localized PC have worse outcomes than those who are wild type regardless of the local treatment they have previously undergone (radical prostatectomy or radiation therapy; Castro et al., 2015). The 5-year metastasis-free survival was significantly higher in wild-type patients compared to mutation carriers (93 vs. 77%; p = 0.009). BRCA carriers had higher rate of lymph-nodes involvement, higher Gleason score, developed distant metastasis earlier and had a shorter survival. Overall, the independent prognostic value of this mutation at the multivariate analysis strongly suggests the need of a timely management in BRCA carriers (Castro et al., 2013, 2015). Furthermore, these patients developed more frequently castration-resistant PC (CRPC) upon occurrence of metastases (Castro et al., 2013).

Overall, several studies suggested that BRCA mutation is an independent negative prognostic factor for both overall survival and PC-specific survival (Modena et al., 2016).

Taken together, all these findings suggest that active surveillance may not be a valid treatment option for BRCA mutation carriers, even in the low-risk PC population, according to more aggressive behavior and poor disease outcomes observed in such subjects. For all these reasons screening for BRCA1/2 might be useful in early diagnosis and potentially have a beneficial impact on the management of these patients.

Aberrations in genes involved in DNA integrity seem to increase in the late-stages of PC disease with 20-30% men with metastatic CRPC (mCRPC) carrying genomic defects in DNArepair pathways (Mateo et al., 2017). At the moment it is not clear if the increased incidence of defects in DNA-repair in mCRPC is related to progression to a more aggressive disease phenotype or rather it is the result of a secondary pressure due to specific treatments.

IMPLICATIONS FOR THE TREATMENT

In the last decades the therapeutic landscape of mCRPC patients has dramatically changed due to availability of several agents able to significantly improve survival: chemotherapeutic agents, docetaxel (Tannock et al., 2004) and cabazitaxel (de Bono et al., 2010), new generation hormone agents, abiraterone (de Bono et al., 2011; Ryan et al., 2014), and enzalutamide (Scher et al., 2012; Beer et al., 2014), and one radiopharmacetical agent, radium 223 (Parker et al., 2013).

In addition, there is a growing interest for strategies based on the immunotherapy: after the studies on vaccines leading to the FDA approval of sipuleucel-T (Kantoff et al., 2010a) or to the development of PROSTVAC-VF (Kantoff et al., 2010b), immunecheckpoints inhibitors are currently being tested in mCRPC, such as pembrolizumab (NCT02787005) and atezolizumab in combination with enzalutamide (NCT03016312).

The use of targeted therapies such as poly-(ADP-ribose) polymerase (PARP) inhibitors in BRCA-associated breast and ovarian cancers (Audeh et al., 2010; Tutt et al., 2010; Ledermann et al., 2012) suggests a potential role of these drugs also in BRCA carriers affected by other solid tumors, including PC. PARP polymerase is a nuclear DNA-binding enzyme involved in the single-strand break DNA repair, through the base excision and repair (BER) pathway (Morales et al., 2014). Impairment of BER activity through PARP inhibition determines the so called *synthetic lethality* interaction in homologous recombination deficient BRCA-mutant cancer cells ("BRCA-ness"), an overwhelming genome instability condition which drives cancer cells to die (Farmer et al., 2005). BRCAness tumors seem to be highly sensitive to PARP inhibitors, independently of the site of origin of the tumor (Underhill et al., 2011).

Several PARP inhibitors (olaparib, rucaparib, niraparib, velaparib, and talazoparib) are currently being investigated in

several tumor types. In the case of BRCAness PCs, phase I clinical trials (Fong et al., 2009; Sandhu et al., 2013a), small mCRPC series (Sandhu et al., 2013b), and translational studies (Brenner et al., 2011) suggested a role for these agents also in PC patients. Orally administered Olaparib at 400 mg twice a day was tested in 298 patients with germline BRCA1/2 mutation and recurrent advanced cancers, including eight pre-treated PC patients (Kaufman et al., 2015). Among these PC patients, seven had a BRCA2 mutation and the remaining was a BRCA1 carrier. All these patients had previously received an average of two lines of treatment for the advanced disease. Although the very limited number of PC patients, the results were encouraging since 4 out of 8 patients showed tumor response while in 2 the disease remained stable. The median progression-free survival (PFS) was 7.2 months with two patients having a favorable response for longer than 1 year. It is noteworthy that only one out of four PC patients who had previously received platinum chemotherapy responded to the PARP inhibitor, suggesting a potential crossresistance between the mechanisms of action of these drugs (Kaufman et al., 2015).

On the basis of these promising results, the study TOPARP-A (a larger phase II clinical trial) investigated the activity of Olaparib in 50 mCRPC patients who had shown progression disease after one or two treatments, including docetaxel (Mateo et al., 2015). In this study genomic defects in DNA-repair genes were prospectively evaluated with next-generation sequencing analyses on fresh tumor-biopsy performed before the treatment. The primary endpoint of the study was composite: radiological response according to RECIST 1.1 and/or PSA declines >50% and/or conversion in circulating tumor cells (CTC). PFS and OS were secondary endpoints of the study. A response to Olaparib was observed in 16 patients (33%), who received the drug for 6 months in 12 cases and for 12 months in four cases. The median OS was 10.1 months (5.1-15.6). Molecular analyses identified aberrations in DNA-repair genes [BRCA 1/2, ataxia-telangiectasia mutated (ATM), Fanconi's anemia genes, CHEK2, PALB2, FANCA, HDCA2, and others] in 16/49 patients. Of these 16 biomarker-positive patients, 14/16 (88%) showed a response to Olaparib, of particular relevance, 7/7 patients with BRCA2 mutation and 4/5 patients with ATM aberrations. The PSA response in those who had a clinical benefit from PARP inhibitors (13/16, 81%) suggested that PSA monitoring during the treatment could be useful to rapidly identify the responders. Radiologic PFS and OS were significantly longer in the biomarker-positive compared to the biomarker-negative group (median 9.8 vs. 2.7 months, p < 0.0001; median 13.8 vs. 7.5 months, p = 0.05, respectively; Mateo et al., 2015).

The tolerability profile of the drug was manageable and mainly related to hematological toxicities (anemia, thrombocytopenia), fatigue and gastrointestinal side effects. The striking results in BRCA1/2 and ATM gene-mutated mCRPC patients led the US Food and Drug Administration (FDA) to approve this breakthrough therapy with Olaparib for this population, although efficacy and safety results of the phase II trial need to be confirmed in larger trials. Noteworthy, it could be reductive to restrict treatment with Olaparib to patients with BRCA or ATM mutations only since this drug showed to be effective in additional 25% of patients who are very likely carriers of unknown defects in homologous recombination.

The Part B of TOPARP study (NCT01682772) (TOPARP-B) aims to validate the role of Olaparib in BRCA2 or ATM carriers and to provide additional efficacy data in presence of less common mutations in other genes involved in DNA repair such as FANC, CDK12, RAD51, PALB2, ATR, CHEK1, CHEK2, DSS1, MRE11, XRCC2/3, and ETS gene fusions (TMPRSS2-ERG) which have been previously linked to PARP inhibitors sensitivity (McCabe et al., 2006; Yang et al., 2011; Hussain et al., 2014).

Additional studies are waiting to confirm that the frequency of DNA-repair defects in mCRPC patients is higher than that observed in other disease settings or in untreated patients. Preliminary results suggest that somatic BRCA mutations are more often observed in late stages of prostate cancer disease; for this reason, in the next future, genomic re-assessment of the disease with a new fresh biopsy or using isolated circulating cells or circulating DNA will become desirable to personalize the therapeutic approach.

Mateo J et al. retrospectively reviewed the clinical outcome of mPC patients with and without germline DNA damage repair gene mutation (gDDRm); medical records were reviewed for 390 mPC patients with known gDDRm status. Data suggested that mPC patients with inherited mutations in DDR genes, including those with BRCA2 mutations, can achive similar benefit from standard of care therapies in terms of both response rate and PFS compared to patients without mutations [38].

Additional clinical trials are testing efficacy and safety of PARP inhibitors in combination with chemotherapy, radiotherapy or biological agents in several disease setting, including the localized disease. Phase II clinical trials are evaluating the combination of olaparib with abiraterone vs. placebo in mCRCP (NCT01972217), the combination of veliparib with abiraterone vs. abiraterone (NCT01576172) or the association of niraparib with enzalutamide (NCT02500901).

Another study evaluated the combination of veliparib (ABT-888) plus temozolamide in 26 mCRPC patients pre-treated with docetaxel: the authors demonstrated a very modest efficacy of the combination therapy with 12% of the patients achieving a PSA response >30% within 3 months (Hussain et al., 2014). Median PFS and OS were 9 weeks and 39.6 weeks, respectively. Hematological toxicities were observed; in particular, grade III/IV thrombocytopenia was noted in 15% of the patients. Despite the promising preclinical activity, this combination demonstrated disappointing results. The authors suggest that the administration of a low, sub-optimal dose of veliparib in this trial could explain the limited activity.

As observed in BRCA carriers patients affected by breast or ovarian cancer (Ahn et al., 1997; Yang et al., 2011), also carriers of mutations in DNA repair pathways could benefit from platinum-based chemotherapy and recent observations seem to support this hypothesis.

Cheng and colleagues reported some cases of very good response (complete or partial response) to platinum chemotherapy in advanced prostate cancer (Cheng et al., 2016). Retrospective DNA sequencing of these patients demonstrated a biallelic inactivation of BRCA2.

Additionaly, the retrospective multicentre analysis from Pomerantz et al confirmed that mCRPC carriers of BRCA2 mutations have a higher likelihood of positive response to carboplatin-based chemotherapy than non-carriers (Pomerantz et al., 2017). These authors retrospectively assessed a cohort of 141 mCRPC patients treated with carboplatin and docetaxel and found that 75% of the 8 BRCA2 carriers showed a PSA decline >50% compared with 17% of the 133 noncarriers.

It is clear that the studies evaluating the efficacy of platinum-based therapies in selected subgroup of patients with defects in DNA-repair genes are necessary, but the detection of mutations in DNA-repair genes could represent a predictive biomarker able to drive the therapeutic strategy. Despite the number of agents efficacious in mCRPC patients, today no robust available biomarkers are able to predict the response to a specific class of agents. Growing retrospective data could suggest a reduced activity of new hormone agents compared to chemotherapeutic agents in presence of the splice variant of androgen receptor AR-V7 (Antonarakis et al., 2014, 2015; Scher et al., 2016). Unfortunately, to date, the expression of this biomarker has only a prognostic value, since only prospective randomized trials will be able to assess its predictive value.

REFERENCES

- Ahn, M. J., Oh, S. J., Lee, Y. Y., Jung, T. J., Kim, I. S., Choi, I. Y., et al. (1997). A case of prostate cancer in 34 year old man presenting with generalized lymphadenopathy mimicking malignant lymphoma. J. Korean Med. Sci. 12, 262–265.doi: 10.3346/jkms.1997.12.3.262
- Antonarakis, E. S., Lu, C., Luber, B., Wang, H., Chen, Y., Nakazawa, M., et al. (2015). Androgen receptor splice variant 7 and efficacy of taxane chemotherapy in patients with metastatic castration-resistant prostate cancer. *JAMA Oncol.* 1, 582–591. doi: 10.1001/jamaoncol.2015.1341
- Antonarakis, E. S., Lu, C., Wang, H., Luber, B., Nakazawa, M., Roeser, J. C., et al. (2014). AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. N. Engl. J. Med. 371, 1028–1038. doi: 10.1056/NEJMoa1315815
- Audeh, M. W., Carmichael, J., Penson, R. T., Friedlander, M., Powell, B., Bell-McGuinn, K. M., et al. (2010). Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and recurrent ovarian cancer: a proof-of-concept trial. *Lancet* 376, 245–251. doi: 10.1016/S0140-6736(10)60893-8
- Bancroft, E. K., Page, E. C., Castro, E., Lilja, H., Vickers, A., Sjoberg, D., et al. (2014). Targeted prostate cancer screening in BRCA1 and BRCA2 mutation carriers: results from the initial screening round of the IMPACT study. *Eur. Urol.* 66, 489–499. doi: 10.1016/j.eururo.2014.01.003
- Beer, T. M., Armstrong, A. J., Rathkopf, D. E., Loriot, Y., Sternberg, C. N., Higano, C. S., et al. (2014). Enzalutamide in metastatic prostate cancer before chemotherapy. *N. Engl. J. Med.* 371, 424–433. doi: 10.1056/NEJMoa1405095
- Brenner, J. C., Ateeq, B., Li, Y., Yocum, A. K., Cao, Q., Asangani, I. A., et al. (2011). Mechanistic rationale for inhibition of poly(ADP-ribose) polymerase in ETS gene fusion-positive prostate cancer. *Cancer Cell.* 19, 664–678. doi: 10.1016/j.ccr.2011.04.010
- Brose, M. S., Rebbeck, T. R., Calzone, K. A., Stopfer, J. E., Nathanson, K. L., and Weber, B. L. (2002). Cancer risk estimates for BRCA1 mutation carriers identified in a risk evaluation program. *J. Natl. Cancer Inst.* 94, 1365–1372.

CONCLUSIONS

Carcinogenesis is mediated by the accumulation of inherited or acquired genetic aberrations that promote the tumor growth advantage. DNA-repair defects can lead to an increase in genetic changes in cells resulting in an improved risk of developing cancer. The identification of the carriers of these genomic aberrations allows not only to identify people who have cancer susceptibility but also to define cancer subtypes with a different sensitivity to the treatments. It is likely that DNA sequencing will change the therapeutic approach to prostate cancer in the next years, improving molecular classification of this tumor and therefore the personalized therapeutic approach. Molecular characterization of prostate cancer seems to be promising to define also cancer prognosis.

In order to maximize the efficacy of cancer therapies avoiding unnecessary side effects, identification and prospective validation of predictive biomarkers are strongly advocated. In this context there is the need of carefully designed clinical trials which will be able to guide the tailored therapeutic approach and thus the clinical decision making process.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

- Castro, E., Goh, C., Leongamornlert, D., Saunders, E., Tymrakiewicz, M., Dadaev, T., et al. (2015). Effect of BRCA Mutations on metastatic relapse and causespecific survival after radical treatment for localised prostate cancer. *Eur. Urol.* 68, 186–193. doi: 10.1016/j.eururo.2014.10.022
- Castro, E., Goh, C., Olmos, D., Saunders, E., Leongamornlert, D., Tymrakiewicz, M., et al. (2013). Germline BRCA mutations are associated with higher risk of nodal involvement, distant metastasis, and poor survival outcomes in prostate cancer. J. Clin. Oncol. 31, 1748–1757. doi: 10.1200/JCO.2012.43.1882
- Cheng, H. H., Pritchard, C. C., Boyd, T., Nelson, P. S., and Montgomery, B. (2016). Biallelic Inactivation of BRCA2 in Platinum-sensitive Metastatic Castration-resistant Prostate Cancer. *Eur. Urol.* 69, 992–995. doi: 10.1016/j.eururo.2015.11.022
- de Bono, J. S., Logothetis, C. J., Molina, A., Fizazi, K., North, S., Chu, L., et al. (2011). Abiraterone and increased survival in metastatic prostate cancer. N. Engl. J. Med. 364, 1995–2005. doi: 10.1056/NEJMoa1014618
- de Bono, J. S., Oudard, S., Ozguroglu, M., Hansen, S., Machiels, J. P., Kocak, I., et al. (2010). Prednisone plus cabazitaxel or mitoxantrone for metastatic castrationresistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial. *Lancet.* 376, 1147–1154.
- Easton, D. F., Deffenbaugh, A. M., Pruss, D., Frye, C., Wenstrup, R. J., Allen-Brady, K., et al. (2007). A systematic genetic assessment of 1,433 sequence variants of unknown clinical significance in the BRCA1 and BRCA2 breast cancerpredisposition genes. Am. J. Hum. Genet. 81, 873–883. doi: 10.1086/521032
- Evers, B., Helleday, T., and Jonkers, J. (2010). Targeting homologous recombination repair defects in cancer. *Trends Pharmacol. Sci.* 31, 372–380. doi: 10.1016/j.tips.2010.06.001
- Farmer, H., McCabe, N., Lord, C. J., Tutt, A. N., Johnson, D. A., Richardson, T. B., et al. (2005). Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 434, 917–921. doi: 10.1038/nature03445
- Ferrone, C. R., Levine, D. A., Tang, L. H., Allen, P. J., Jarnagin, W., Brennan, M. F., et al. (2009). BRCA germline mutations in Jewish patients with pancreatic adenocarcinoma. J. Clin. Oncol. 27, 433–438. doi: 10.1200/JCO.2008.18.5546

- Finch, A., Beiner, M., Lubinski, J., Lynch, H. T., Moller, P., Rosen, B., et al. (2006). Salpingo-oophorectomy and the risk of ovarian, fallopian tube, and peritoneal cancers in women with a BRCA1 or BRCA2 Mutation. *JAMA* 296, 185–192. doi: 10.1001/jama.296.2.185
- Fong, P. C., Boss, D. S., Yap, T. A., Tutt, A., Wu, P., Mergui-Roelvink, M., et al. (2009). Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N. Engl. J. Med.*. 361, 123–134. doi: 10.1056/NEJMoa0900212
- Hussain, M., Carducci, M. A., Slovin, S., Cetnar, J., Qian, J., McKeegan, E. M., et al. (2014). Targeting DNA repair with combination veliparib (ABT-888) and temozolomide in patients with metastatic castration-resistant prostate cancer. *Invest. New Drugs.* 32, 904–912. doi: 10.1007/s10637-014-0099-0
- Kantoff, P. W., Higano, C. S., Shore, N. D., Berger, E. R., Small, E. J., Penson, D. F., et al. (2010a). Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N. Engl. J. Med. 363, 411–422. doi: 10.1056/NEJMoa1001294
- Kantoff, P. W., Schuetz, T. J., Blumenstein, B. A., Glode, L. M., Bilhartz, D. L., Wyand, M., et al. (2010b). Overall survival analysis of a phase II randomized controlled trial of a Poxviral-based PSA-targeted immunotherapy in metastatic castration-resistant prostate cancer. J. Clin. Oncol. 28, 1099–1105. doi: 10.1200/JCO.2009.25.0597
- Kaufman, B., Shapira-Frommer, R., Schmutzler, R. K., Audeh, M. W., Friedlander, M., Balmana, J., et al. (2015). Olaparib monotherapy in patients with advanced cancer and a germline BRCA1/2 mutation. *J. Clin. Oncol.* 33, 244–250. doi: 10.1200/JCO.2014.56.2728
- Kote-Jarai, Z., Leongamornlert, D., Saunders, E., Tymrakiewicz, M., Castro, E., Mahmud, N., et al. (2011). BRCA2 is a moderate penetrance gene contributing to young-onset prostate cancer: implications for genetic testing in prostate cancer patients. *Br. J. Cancer* 105, 1230–1234. doi: 10.1038/bjc.2011.383
- Ledermann, J., Harter, P., Gourley, C., Friedlander, M., Vergote, I., Rustin, G., et al. (2012). Olaparib maintenance therapy in platinum-sensitive relapsed ovarian cancer. N. Engl. J. Med. 366, 1382–1392. doi: 10.1056/NEJMoa1105535
- Leongamornlert, D., Mahmud, N., Tymrakiewicz, M., Saunders, E., Dadaev, T., Castro, E., et al. (2012). Germline BRCA1 mutations increase prostate cancer risk. *Br. J. Cancer* 106, 1697–1701. doi: 10.1038/bjc.2012.146
- Levy-Lahad, E., and Friedman, E. (2007). Cancer risks among BRCA1 and BRCA2 mutation carriers. *Br. J. Cancer.* 96, 11–15. doi: 10.1038/sj.bjc.660 3535
- Liu, Y., and West, S. C. (2002). Distinct functions of BRCA1 and BRCA2 in double-strand break repair. *Breast Cancer Res.* 4, 9–13. doi: 10.1186/bcr417
- Mateo, J., Boysen, G., Barbieri, C. E., Bryant, H. E., Castro, E., Nelson, P. S., et al. (2017). DNA Repair in prostate cancer: biology and clinical implications. *Eur.* Urol. 71, 417–425. doi: 10.1016/j.eururo.2016.08.037
- Mateo, J., Carreira, S., Sandhu, S., Miranda, S., Mossop, H., Perez-Lopez, R., et al. (2015). DNA-Repair Defects and Olaparib in Metastatic Prostate Cancer. N. Engl. J. Med.. 373, 1697–1708. doi: 10.1056/NEJMoa1506859
- McCabe, N., Turner, N. C., Lord, C. J., Kluzek, K., Bialkowska, A., Swift, S., et al. (2006). Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP-ribose) polymerase inhibition. *Cancer Res.* 66, 8109–8115. doi: 10.1158/0008-5472.CAN-06-0140
- Mersch, J., Jackson, M. A., Park, M., Nebgen, D., Peterson, S. K., Singletary, C., et al. (2015). Cancers associated with BRCA1 and BRCA2 mutations other than breast and ovarian. *Cancer* 121, 269–275. doi: 10.1002/cncr.29041
- Mitra, A. V., Bancroft, E. K., Barbachano, Y., Page, E. C., Foster, C. S., Jameson, C., et al. (2011). Targeted prostate cancer screening in men with mutations in BRCA1 and BRCA2 detects aggressive prostate cancer: preliminary analysis of the results of the IMPACT study. *BJU Int.* 107, 28–39. doi: 10.1111/j.1464-410X.2010.09648.x
- Modena, A., Iacovelli, R., Scarpa, A., Brunelli, M., Ciccarese, C., Fantinel, E., et al. (2016). Investigating BRCA mutations: a breakthrough in precision medicine of castration-resistant prostate cancer. *Target. Oncol.*11, 569–577.doi: 10.1007/s11523-016-0450-9
- Morales, J., Li, L., Fattah, F. J., Dong, Y., Bey, E. A., Patel, M., et al. (2014). Review of poly (ADP-ribose) polymerase (PARP) mechanisms of action and rationale for targeting in cancer and other diseases. *Crit. Rev. Eukaryot. Gene Expr.* 24, 15–28.
- Moran, A., O'Hara, C., Khan, S., Shack, L., Woodward, E., Maher, E. R., et al. (2012). Risk of cancer other than breast or ovarian in individuals with BRCA1 and BRCA2 mutations. *Fam. Cancer* 11, 235–242. doi: 10.1007/s10689-011-9506-2

- Parker, C., Nilsson, S., Heinrich, D., Helle, S. I., O'Sullivan, J. M., Fossa, S. D., et al. (2013). Alpha emitter radium-223 and survival in metastatic prostate cancer. *N. Engl. J. Med.* 369, 213–223. doi: 10.1056/NEJMoa1213755
- Pomerantz, M. M., Spisak, S., Jia, L., Cronin, A. M., Csabai, I., Ledet, E., et al. (2017). The association between germline BRCA2 variants and sensitivity to platinum-based chemotherapy among men with metastatic prostate cancer. *Cancer* 123, 3532–3539. doi: 10.1002/cncr.30808
- Ryan, C., Smith, M. R., Fizazi, K., Miller, K., Mulders, P. F., Sternberg, C., et al. (2014). Final overall survival analysis of COU-AA-302, a randomized phase 3 study of abiraterone acetate in metastatic castration-resistant prostate cancer patients without prior chemotherapy. *Ann. Oncol.* 25(Suppl 4):iv255. doi: 10.1093/annonc/mdu336.1
- Sandhu, S. K., Omlin, A., Hylands, L., Miranda, S., Barber, L. J., Riisnaes, R., et al. (2013a). Poly (ADP-ribose) polymerase (PARP) inhibitors for the treatment of advanced germline BRCA2 mutant prostate cancer. *Ann. Oncol.* 24, 1416–1418. doi: 10.1093/annonc/mdt074
- Sandhu, S. K., Schelman, W. R., Wilding, G., Moreno, V., Baird, R. D., Miranda, S., et al. (2013b). The poly(ADP-ribose) polymerase inhibitor niraparib (MK4827) in BRCA mutation carriers and patients with sporadic cancer: a phase 1 dose-escalation trial. *Lancet Oncol.* 14, 882–892. doi: 10.1016/S1470-2045(13)70240-7
- Scher, H. I., Fizazi, K., Saad, F., Taplin, M. E., Sternberg, C. N., Miller, K., et al. (2012). Increased survival with enzalutamide in prostate cancer after chemotherapy. N. Engl. J. Med. 367, 1187–1197.doi: 10.1056/NEJMoa1207506
- Scher, H. I., Lu, D., Schreiber, N. A., Louw, J., Graf, R. P., Vargas, H. A., et al. (2016). Association of AR-V7 on circulating tumor cells as a treatment-specific biomarker with outcomes and survival in castration-resistant prostate cancer. *JAMA Oncol.* 2, 1441–1449. doi: 10.1001/jamaoncol.2016.1828
- Tai, Y. C., Domchek, S., Parmigiani, G., and Chen, S. (2007). Breast cancer risk among male BRCA1 and BRCA2 mutation carriers. J. Natl. Cancer Inst. 99, 1811–1814. doi: 10.1093/jnci/djm203
- Tannock, I. F., de Wit, R., Berry, W. R., Horti, J., Pluzanska, A., Chi, K. N., et al. (2004). Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N. Engl. J. Med. 351, 1502–1512.
- Tryggvadottir, L., Vidarsdottir, L., Thorgeirsson, T., Jonasson, J. G., Olafsdottir, E. J., Olafsdottir, G. H., et al. (2007). Prostate cancer progression and survival in BRCA2 mutation carriers. *J. Natl. Cancer Inst.* 99, 929–935.doi: 10.1093/jnci/djm005
- Tutt, A., Robson, M., Garber, J. E., Domchek, S. M., Audeh, M. W., Weitzel, J. N., et al. (2010). Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-ofconcept trial. *Lancet* 376, 235–244. doi: 10.1016/S0140-6736(10)60892-6
- Underhill, C., Toulmonde, M., and Bonnefoi, H. (2011). A review of PARP inhibitors: from bench to bedside. *Ann. Oncol.*. 22, 268–279. doi:10.1093/annonc/mdq322
- van Asperen, C. J., Brohet, R. M., Meijers-Heijboer, E. J., Hoogerbrugge, N., Verhoef, S., Vasen, H. F., et al. (2005). Cancer risks in BRCA2 families: estimates for sites other than breast and ovary. J. Med. Genet. 42, 711–719. doi: 10.1136/jmg.2004.028829
- Yang, D., Khan, S., Sun, Y., Hess, K., Shmulevich, I., Sood, A. K., et al. (2011). Association of BRCA1 and BRCA2 mutations with survival, chemotherapy sensitivity, and gene mutator phenotype in patients with ovarian cancer. *JAMA* 306, 1557–1565. doi: 10.1001/jama.2011.1456
- Yoshida, K., and Miki, Y. (2004). Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage. *Cancer Sci.* 95, 866–871.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Synergistic Protective Activity of **Tumor-Specific Epitopes Engineered** in Bacterial Outer Membrane Vesicles

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Introduction: Bacterial outer membrane vesicles (OMVs) are naturally produced by all Gram-negative bacteria and, thanks to their plasticity and unique adjuvanticity, are emerging as an attractive vaccine platform. To test the applicability of OMVs in cancer immunotherapy, we decorated them with either one or two protective epitopes present in the B16F10EGFRvIII cell line and tested the protective activity of OMV immunization in C57BL/6 mice challenged with B16F10EGFRvIII.

OPEN ACCESS

Edited by:

Stephen G. Maher, Trinity College, Dublin, Ireland

Reviewed by:

Gabriele Multhoff, Technische Universität München. Germanv Sujuan Guo, Dana-Farber Cancer Institute, United States

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Specialty section:

This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: 14 July 2017 Accepted: 09 October 2017 Published: 07 November 2017

Citation:

Grandi A, Tomasi M, Zanella I, Ganfini L, Caproni E, Fantappiè L, Irene C, Frattini L, Isaac SJ, König E, Zerbini F, Tavarini S, Sammicheli C, Giusti F, Ferlenghi I, Parri M and Grandi G (2017) Synergistic Protective Activity of Tumor-Specific Epitopes Engineered in Bacterial Outer Membrane Vesicles. Front. Oncol. 7:253. doi: 10.3389/fonc.2017.00253

Materials and methods: The 14 amino acid B cell epitope of human epidermal growth factor receptor variant III (EGFRvIII) and the mutation-derived CD4+ T cell neo-epitope of kif18b gene (B16-M30) were used to decorate OMVs either alone or in combination. C57BL/6 were immunized with the OMVs and then challenged with B16F10EGFRvIII cells, Immunogenicity and protective activity was followed by measuring anti-EGFRyIII antibodies, M30-specific T cells, tumor-infiltrating cell population, and tumor growth.

Results: Immunization with engineered EGFRvIII-OMVs induced a strong inhibition of tumor growth after B16F10EGFRvIII challenge. Furthermore, mice immunized with engineered OMVs carrying both EGFRvIII and M30 epitopes were completely protected from tumor challenge. Immunization was accompanied by induction of high anti-EGFRvIII antibody titers, M30-specific T cells, and infiltration of CD4+ and CD8+ T cells at the tumor site.

Conclusion: OMVs can be decorated with tumor antigens and can elicit antigen-specific, protective antitumor responses in immunocompetent mice. The synergistic protective activity of multiple epitopes simultaneously administered with OMVs makes the OMV platform particularly attractive for cancer immunotherapy.

Keywords: bacterial outer membrane vesicles, cancer immunotherapy, EGRRvIII, cancer neoepitopes, BALB/ c-CT26 cancer mouse model, precision medicine

INTRODUCTION

All cancer therapies attempt to exploit the differences existing between tumor and normal cells. Since our immune system is built to target and destroy the "non-self," theoretically cancer vaccination is the safest, most natural, and effective therapeutic approach against cancer. Indeed, a large number of preclinical and clinical studies involving cancer vaccines have been described over the last two decades. Unfortunately, in the clinical settings, the results so far have been disappointing. Klebanoff et al. (1) reported a cumulative analysis of several vaccine trials run from 2004 to 2009 and included 936 patients with different types of solid tumors. Using response rate as a measure of positive outcome, the conclusion of the study was that only 3.6% of the patients had an objective benefit from vaccination. The authors concluded that for cancer vaccines to become effective the strategies so far used for their formulation need be substantially revisited.

An ideal cancer vaccine should include three elements: (1) a cocktail of tumor-specific and/or tumor-associated antigens (TSA/TAAs), (2) one or more potent immune-stimulatory molecules (adjuvants), and (3) a delivery system which allows the co-delivery of cancer antigens and adjuvant(s) to antigen presenting cells (APCs). The absence of just one of these elements can make the vaccine incapable of counteracting the corrupted tumor microenvironment (containing regulatory T cells and aberrantly matured myeloid cells), and the highly mutable tumor targets (driving antigen loss and immune evasion).

Enthusiasm for therapeutic cancer vaccines has been recently rejuvenated by two major discoveries. First, it has been shown that the large number of mutations occurring in most tumors (2) creates "neo-epitopes," which can become the targets of both CD4+ and CD8+ T cells. Neo-epitope-specific T cells have been found among tumor-infiltrating lymphocytes (TILs), and when amplified ex vivo from tumor biopsies and introduced back into patients, TILs can exert antitumor activities (3). Moreover, the impressive therapeutic effect of checkpoint inhibitor antibodies observed in a fraction of patients has been shown to correlate with the number of tumor-associated mutations (4-6). Consequently, vaccines formulated with neo-epitopes have recently been created and shown to be highly effective in preventing tumor growth in different preclinical settings (7). Second, Kranz and coworkers (8) have demonstrated that when administered intravenously (i.v.) in melanoma patients, negatively charged liposomes carrying TSA encoding synthetic RNAs were efficiently taken up by splenic DCs, resulting in a potent elicitation of TAA-specific CD4+ and CD8+ T cells. Overall, these data support the hypothesis that therapeutic cancer vaccines can drive protective antitumor immune responses as long as specific TSAs/TAAs are formulated with the appropriate combination of adjuvant(s) and delivery system.

In our laboratories, we have become interested in bacterial outer membrane vesicles (OMVs) both from a scientific and translation viewpoint. More than 40 years ago, researchers made the observation that all Gram-negative bacteria release OMVs, closed spheroid particles, 20–300 nm in diameter, generated through the "budding out" of the outer membrane (9, 10). Consistent with their origin, the majority of OMV components are represented by lipopolysaccharide (LPS), glycerophospholipids, and outer membrane and periplasmic proteins (11, 12). OMVs have a multitude of functions, including inter and intra species cell-to-cell cross-talk, biofilm formation, genetic transformation, defense against host immune responses, and toxin and virulence factor delivery to host cells (11). From a translational standpoint, OMVs can be an attractive vaccine platform for three main reasons. First, they carry many microbe-associated molecular patterns, including LPS, lipoproteins, peptidoglycan, and flagellin, which by binding to pathogen recognition receptors play a key role in stimulating innate immunity and promoting adaptive immune responses (13-15). Such stimulatory molecules can work synergistically, thus potentiating the built-in adjuvanticity of OMVs (16). Second, OMVs can be easily decorated with foreign antigens/epitopes by manipulating the OMV-producing strain through different Synthetic Biology approaches. This feature was demonstrated for the first time by Kesty and Kuehn who showed that Yersinia enterocolitica outer membrane protein Ail assembled on OMV surface when expressed in Escherichia coli, and that the Green Fluorescence Protein fused to the "twin arginine transport" signal sequence was incorporated in the OMV lumen (17). Following this observation, an increasing number of heterologous proteins have been successfully delivered to OMVs using a variety of strategies (16, 18). Recently, we showed that different bacterial antigens could be delivered to the lumen of E. coli vesicles by fusing their coding sequences to a leader peptide for secretion (19). Moreover, we showed that heterologous lipoproteins could be incorporated into the OMV membrane and that such proteins could serve as chaperones to transport heterologous polypeptides to the OMV surface (20). Third, OMVs can be rapidly and easily purified from bacterial culture supernatant. The original OMV production methods, currently in use at industrial scale for Neisseria meningitidis group B vaccines, involve the treatment of bacterial biomass with mild detergents (21). More recently, detergent-free methods for OMV production have been proposed which make use of mutant strains featuring a hyper-vesiculating phenotype (19, 22-25). Once the supernatant is separated from the biomass of these mutant strains, the purification of the vesicles can be easily carried out using tangential flow filtration with production yield higher than 100 mg of vesicles (protein content) per liter of culture (26).

In this work, we addressed two main questions. First, we were interested to know whether OMVs decorated with a well-known, B cell cancer-specific epitope could induce epitope-specific immune responses and whether such responses could protect immunocompetent mice from the challenge with a syngeneic cancer cell line expressing the epitope on its surface. Second, we wanted to investigate whether the addition of a second cancerspecific epitope also expressed in the same cell line could result in a synergistic effect, thus potentiating the overall efficacy of the OMV cancer vaccine. As a second epitope, we selected a protective CD4+ T cell epitope with the idea that the combination of humoral and cell-mediated immune responses could strengthen the overall anticancer effect of immunization. The data indicate that immunization with OMVs engineered with the B cell epitope strongly protected mice from tumor challenge and that 100% protection was achieved with OMVs decorated with both the B and the T cell epitopes.

RESULTS

Selection of Cancer Antigens and Mouse Model

Since our first objective was to test whether the OMV-based vaccine platform could induce protective immune responses

in a cancer model of immunocompetent mice, we focused our attention on C57BL/6-B16F10 model and we selected two peptide antigens, LEEKKGNYVVTDH (EGFRvIIIpep) and PSKPSFQEFVDWENVSPELNSTDQPFL (B16-M30pep), previously shown to be protective in the same model.

EGFRvIIIpep belongs to EGFRvIII, a mutated form of the human epidermal growth factor receptor (EGFR), expressed on several tumors and associated with the expression of epithelialmesenchymal transition and cancer stem cell genes. EGFRvIII contains an in-frame deletion in the extracellular domain of EGFR, creating a novel antigenic epitope which is exquisitely tumor-specific (27). Immunization with EGFRvIIIpep conjugated to limpet hemocyanin (KLH) was shown to protect mice from the challenge of syngeneic cell lines stably transfected with human EGFRvIII. In particular, Heimberger and coworkers showed that the conjugated peptide formulated with GM-CSF protected C57BL/6 mice from both extracerebral and intracerebral challenge with B16F10-EGFRvIII cells (28). Based on these data, a vaccine (Rindopepimut) for EGFRvIII-positive glioblastoma patients was proposed and tested in different trials (29). As far as the B16-M30pep is concerned, it was recently described by Kreiter and coworkers (7) as a CD4+ T cells epitope expressed in the B16F10 cell line as a consequence of a mutation occurred in the kif18b gene. Therefore, M30 is a B16F10-specific neoepitope not expressed in the syngeneic healthy C57BL/6 mouse tissues. Interestingly, the authors showed that immunization with liposome-formulated synthetic RNA coding for B16-M30 induced robust T cell-mediated protection in C57BL/6 mice when challenged with B16F10 cells.

Immunogenicity and Protective Activity of EGFRvIII-OMVs

We first tested whether OMVs decorated with the Nm-fHbpvIII fusion protein carrying three copies of EGFRvIIIpep at its C-terminus could induce anti-EGFRvIIIpep antibodies and whether such anti-EGFRvIIIpep immune response could protect mice from B16F10EGFRvIII challenge. The expression of EGFRvIIIpep in the OMVs from E. coli BL21*ΔompA* strain has been recently described (20). Briefly, a synthetic DNA encoding three copies of EGFRvIIIpep was fused to the 3' end of the Neisseria meningitidis fHbp gene, thus generating a chimera (Nm-fHbp-vIII) constituted of the full length fHbp protein and the EGFRvIII tri-peptide attached to its C-terminus (Figure 1A). The fusion protein was shown to be incorporated into the outer membrane of *E. coli* BL21*△ompA* and importantly to be exposed on the cell surface (Figures 1C,D). Furthermore, Nm-fHbpvIII accumulates in the vesicle compartment, as demonstrated by SDS-PAGE and Western Blot analyses (Figure 1B) and by immune gold transmission electron microscopy (TEM) analysis of OMVs (Figure 1E).

C57BL/6 mice (16 mice per group) were immunized with either "empty" OMVs (not carrying the fused antigen) from *E. coli* BL21 Δ ompA (control group) or with Nm-fHbp-vIII-OMVs. Vaccination was carried out at days 0, 14, and 28 (**Figure 2A**) and 1 week after the third immunization sera were collected and the induction of anti-EGFRvIII-antibodies was confirmed by ELISA

(Figure 2B). A good fraction of EGFRvIII-specific antibodies belonged to the IgG2a isotype, in line with our previous data showing that OMVs from *E. coli* BL21 Δ ompA elicit a Th1-skewed immune response (19). Next, at day 35, mice were challenged with a s.c. injection of 0.5 × 10⁵ B16F10EGFRvIII cells and tumor growth was followed both in control mice and in mice immunized with Nm-fHbp-vIII-OMVs. While all but one control mice developed large tumors 20 days after challenge (average tumor volume = 850 mm³, with three mice sacrificed having developed tumors >1,500 mm³), immunization with Nm-fHbp-vIII-OMVs markedly reduced tumor growth in a statistically significant manner. In particular, eight mice were completely protected while the remaining mice developed tumors with average volumes of approximately 400 mm³ (Figure 2C).

We also analyzed the tumor-infiltrating cell population in both control and Nm-fHbp-vIII-OMVs immunized mice. At the end of the challenge study, two tumors per group were randomly collected. Cells were mechanically and enzymatically isolated and the fraction of CD4+ T cells, CD8+ T cells, Treg, and MDSCs populations was determined by flow cytometry analysis after cell staining with specific antibodies. As shown in **Figure 2D** (Figure S1 in Supplementary Material), in line with the Th1 profile of the immune response, Nm-fHbp-vIII-OMVs immunization promoted a significant increase of CD4+ and CD8+ T cells at tumor site and a concomitant reduction of both CD4+ Treg and MDSC cells.

Synergistic Protective Activity of EGFRvIIIpep and M30

Having demonstrated that EGFRvIII-OMVs induced a robust protection in C57BL/6 mice challenged with B16F10EGFRvIII cell line, we investigated whether protection could be further potentiated by formulating Nm-fHbp-OMVs with B16-M30pep, a second antigen expressed in B16F10EGFRvIII and generated by one of the several B16F10-specific mutations (7). Therefore, we set up a second immunization/challenge experiment involving four groups of eight mice each. The first group received three doses of "empty" OMVs from E. coli BL21*ΔompA* (control group). The second group was injected with "empty" OMVs together with B16-M30 synthetic peptide (100 µg/dose) (peptide-"absorbed" M30-OMVs). Finally, the third and the fourth groups received three doses of Nm-fHbp-vIII-OMVs and three doses of Nm-fHbp-vIII-OMVs mixed with B16-M30pep, respectively (peptide-"absorbed" M30-Nm-fHbp-vIII-OMVs). One week after the last immunization, all mice were challenged with 0.5×10^5 B16F10EGFRvIII cells and tumor growth was followed over a period of 30 days. Figure 3 summarizes the result of this experiment. In line with the previous experiment, EGFRvIII-OMVs induced a strong protective immunity against B16F10EGFRvIII. Five out of eight mice were completely protected and the other three mice developed tumors with an average size of approximately 350 mm³. All but one control mice developed tumors \geq 1,500 mm³ and were euthanized. As far as M30-"absorbed" vesicles are concerned, M30-OMVs immunization resulted in a marginal, non-statistically significant protection, with only two out of eight mice protected. However, when B16-M30 peptide



with rabbit anti-EGFRvIIIpep polyclonal antibodies and mouse anti-LPS mAb, and subsequently with goat anti-rabbit IgG, Alexa Fluor 594 conjugated-antibodies (red), and goat anti-mouse IgG, Alexa Fluor 488 conjugated-antibodies (green). (E) Immuno Transmission Electron Microscopy (TEM) analysis of OMVs purified from BL2140mpA(pET-Nm-fHbpvIII) strain using primary anti-EGFRvIIIpep rabbit antibodies and 5-nm gold-labeled anti-rabbit secondary antibody (see Materials and Methods for details).

was "absorbed" to Nm-fHbp-vIII-OMVs, protection from tumor growth was complete, with only one mouse scored as having a "barely detectable tumor" (**Figure 3A**).

The conclusion from these experiments is that the M30 peptide "absorbed" to "Empty" OMVs induced an M30-specific immune response not sufficient to protect mice from the challenge with B16F10EGFRvIII cell line, but capable of synergizing with a second antigen (a B cell epitope) to the point that together the two antigens completely abrogated tumor growth. To evaluate the immunogenicity of the M30 peptide "absorbed" to OMVs, the presence of M30-specific, IFN- γ -positive T cells was analyzed in the spleens of mice sacrificed at the end of the challenge experiment. As shown in **Figure 3B**, mice immunized with both M30-OMVs and M30-EGFRvIII-OMVs had a higher number of M30-specific, CD4+ T cells with respect to spleens of control-group mice. By contrast, no appreciable amounts of M30-specific CD8+ T cells were measured (not shown), in line with the fact that M30 was described as a MHC II neo-epitope (7).



Analysis of tumor development in C57BL/6 mice immunized with "Empty" OMVs and with Nm-fHbpvIII-OMVs. The figure reports the tumor size in each mouse as measured at day 30 after challenge with 0.5×10^5 B16F10EGFRvIII cells. *** indicates a statistically significant difference of P < 0.001. (**D**) Analysis of tumor-infiltrating cell populations. At the end of the challenge experiment, two tumors/group were randomly selected and the percentage of infiltrating CD4+ T cells, CD8+ T cells, MDSCs, and Tregs was determined by flow cytometry, as described in Section "Materials and Methods" (*P < 0.05; **P < 0.01).

Finally, to further confirm the synergistic effect of EGFRvIIIpep and M30 in protecting mice from B16F10EGFRvIII cell line challenge, we created a second fusion protein in which

fHbp was fused to three copies of M30 peptide followed by three copies of EGFRvIII pep (**Figure 4A**). The construction details of plasmid pET-Nm-fHbp-M30-vIII encoding the fusion protein



are reported in the Section "Materials and Methods." The fusion protein accumulated in the OMV compartment and the engineered OMVs induced anti-EGFRvIII antibody titers similar to the titers induced by Nm-fHbp-vIII-OMVs (**Figure 4B**). Moreover, Nm-fHbp-M30-vIII-OMVs induced IFN- γ positive, M30-specific, CD4+ T cells to a level comparable to the induction observed upon immunization with M30—"absorbed" OMVs (100 µg M30pep + 20 µg OMVs) (**Figure 4C**). Finally, when mice immunized with Nm-fHbp-M30-vIII-OMVs were challenge with the B16F10EGFRvIII cell line, all animals were completely protected with no sign of tumor development at the site of injection (**Figure 4B**).

DISCUSSION

This work delivers a few relevant messages.

First of all, we have shown that the OMV vaccine platform can be potentially applicable in cancer immunotherapy. OMVs are being extensively and successfully utilized in the preclinical and clinical settings for prophylactic vaccination against infectious diseases [for a recent review, see Ref. (30)]. Their unique adjuvanticity, which directs the immune responses toward a marked Th1 profile, and the ease with which they can be manipulated and purified have attracted the attention of several academic and industrial groups and bacterial OMV-based vaccines are already available for human use. However, there is a paucity of information regarding the applicability of this platform technology in cancer vaccines. Our data demonstrate that OMVs are a promising alternative to other adjuvants/delivery systems. EGFRvIII-decorated vesicles are capable of inducing a potent anti-EGFRvIII antibody response which, in immune competent C57BL/6 mice, strongly reduced the growth of B16F10 tumor cells expressing human EGFRvIII. Moreover, the Th1 profile of the response favored the migration of IFN γ -producing CD4+ and CD8+ T cells at the tumor site, eventually contributing to the overall protective activity of vaccination. The level of protection obtained appears to be similar to the one described by Heimberger and coworkers using the same mouse model and a KLM-conjugated EGFRvIII peptide in the presence of GM-CSF (28).

A second important message from this work is that the decoration of OMVs with more than one antigen further potentiate the protective efficacy of the vaccine. In particular, we combined a B cell epitope to a CD4+ T cell epitope and we showed that, together, the two epitopes completely abrogate tumor growth. This is an interesting observation also in light of the fact that in glioblastoma patients, vaccination with EGFRvIII-conjugated peptide was shown to prolong overall survival but ultimately EGFRvIII-negative tumor cells escape vaccine-induced protection (29). This immunoediting mechanism can in part explain the disappointing results obtained with the EGFRvIII-conjugated vaccine in a large Phase III trial (31). Our data pointing to the synergistic effect of EGFRvIII-OMV in combination with other cancer-specific epitopes might rejuvenate the interest in EGFRvIII antigen in the near future.



pET-Nm-fHbp-M30-vIII plasmid. The DNA sequence refers to the 3' end of the gene fusion encoding three copies of both M30 and EGFRvIII epitoestination of pET-Nm-fHbp-M30-vIII plasmid. The DNA sequence refers to the 3' end of the gene fusion encoding three copies of both M30 and EGFRvIII epitopes. (B) Mice were immunized with (i) "Empty" OMVs, (ii) Nm-fHbpvIII OMVs, and (iii) Nm-fHbpvIII OMVs + M30pep, and (iv) Nm-fHbp-M30-vIII-OMVs and subsequently challenged with $0.5 \times 10^{\circ}$ B16F10EGFRvIII cells following the schedule indicated in **Figure 2A**. Tumor size in each mouse was measured at day 30 post challenge. Seven days after the last immunization, serum samples were also collected from mice immunized with (i) "Empty" OMVs, (ii) Nm-fHbpvIII OMVs, and (iii) Nm-fHbpvIII OMVs, and total anti-EGFRvIII lgGs were measured by ELISA (**P < 0.01; ***P < 0.01). (C) M30-specific CD4+ T cells induced in mice immunized with OMVs decorated with M30 peptide. Mice were immunized twice i.p. at days 0 and 7 with either 20 µg "Empty" OMVs + 100 mg M30pep or 20 µg Nm-fHbp-M30-vIII-OMVs. Five days after the second immunization, splenocytes were stimulated with either an irrelevant peptide or with M30 peptide and IFN_Y-positive CD4+ T cells were counted by FACS (**P < 0.01).

A third message from our work is the confirmation that the OMV platform can efficiently elicit not only humoral but also cell-mediated immunity against OMV-associated heterologous antigens. Even though the elicitation of protective T cell responses using pathogen-derived whole vesicles (32) or using OMVs decorated with heterologous antigens (33) was described, information on the general applicability of the OMV platform to induce antigen-specific cell-mediated immunity is still limited. Our work further provides evidence that OMVs combined or engineered with new T cell epitopes elicit epitope-specific T cell responses. We recently corroborated this conclusion by engineering OMVs with seven additional cancer CD4+/CD8+ T cell epitopes and by demonstrating the induction by all seven engineered OMVs of epitope-specific T cell responses (manuscript in preparation). Considering the ease with which

OMVs can be manipulated with foreign antigens, these results lead to the attractive possibility of exploiting the OMV platform in cancer precision medicine.

One last comment deserves the strength of T cell responses induced by OMVs. Kreiter and coworkers previously shown that the M30 CD4+ T cell epitope completely inhibited tumor growth using a mouse model similar to the one tested in this study. Furthermore, the same authors showed that M30 immunization could also reduce the formation of lung metastases in the same model (7). In our hands, protection mediated by M30-"absorbed" OMV immunization could only be appreciated in combination with EGFRvIII epitope. There are a number of arguments to explain the different results. First, differently from the data reported by Kreiter and coworkers (7), we used a "classical" prophylactic modality according to which three

immunizations were followed by the tumor challenge. While this schedule is indicated for eliciting antibody responses, it is not typically recommended for T cell responses, which usually require several administrations few days apart. Second, the i.v. route of immunization used by Kreiter and coworkers appears to be a key element to obtain the remarkable protection of M30 peptide. By delivering the vaccine intravenously, these authors showed that the vaccine could reach the spleen where it could be taken up by dendritic and phagocytic cells. Third, Kreiter and coworkers used as vaccine synthetic RNA coding for the M30 peptide. RNA vaccines have the property to drive the expression of the antigen directly into the cytoplasm of receiving cells and to act as potent adjuvant. While the armamentarium of adjuvants present in OMVs, which work through the elicitation of several TLR and NOD signaling pathways, should guarantee excellent Th1 immune responses, we are currently testing whether different immunization schedules and routes of immunization might improve the level of protection of M30-formulated OMVs. In this respect, we recently challenged BALB/c mice with CT26 cell line, and after challenge, mice were given seven immunizations 3 days apart with OMVs decorated with five protective CT26 neoeptopes described by Kreiter et al. (7). Following this therapeutic immunization modality tumor growth was remarkably reduced (manuscript in preparation). Finally, it has to be pointed out that when tested alone in our immunization/ challenge experiments M30 peptide was "absorbed" to OMVs. In reality, we do not know the interaction of the M30 peptide to the OMVs and in fact, considering the hydrophobic nature of several amino acids and the presence of a few negatively charged amino acids, the peptide might not stably interact with the vesicles at all. Should this be the case, since adjuvant/ antigen co-delivery to DCs is a pre-requisite to elicit good T cell responses, M30-engineered OMVs should outperform the M30-"absorbed" OMVs. We have not tested yet the protective activity of M30-engineered OMVs but it is interesting to note that Nm-fHbp-M30-vIII-OMVs fusion induced good levels of M30-specific, CD4+ T cells and fully protected mice from tumor challenge even if, on a molar basis, the amount of M30 peptide present in the engineered OMVs was approximately 1,000-folds lower than the 100 μ g theoretically "absorbed" to the OMVs. In fact, assuming that the fusion protein represents 2-5% of total OMV proteins (**Figure 4B**), each mouse received approximately 0.5-1 µg of fusion protein/vaccine dose, corresponding to no more than 50-100 ng of M30 peptide.

In conclusion, our work demonstrates that bacterial OMVs represent a promising platform for cancer immunotherapy. The main interesting aspects of the technology includes (i) the rapidity with which they can be decorated with foreign epitopes (we routinely engineer the OMV-producing strains with heterologous antigens in less than 2 weeks), (ii) the high yield of OMVs from bacterial fermentation (usually more than 100 mg of purified OMVs are obtained from a 1-l fermentation), and (iii) the simplicity of the OMV purification process, which only involves tangential flow ultrafiltration. Considering that OMVs are already part of specific human vaccines for which the safety and the quality control assays have already been developed, the platform is potentially ready to be tested in the clinics.

MATERIALS AND METHODS

Bacterial Strains, Cell Line, and Mice

Escherichia coli HK100 strain was used for cloning experiments using the PIPE method.

B16F10 melanoma cell line that stably expresses the EGFRvIII variant gene was kindly provided by Prof. Sampson (Department of Neurosurgery of the Duke University, Duhram, NC, USA). Cells were tested for mycoplasma before animal injection.

To verify the presence of the M30-associated mutation in B16F10 cell line, RNA from B16F10 cells was purified using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Subsequently, purified RNA was reverse-transcribed to cDNA using qScript cDNA synthesis kit (Quanta Bioscences). Finally, the region spanning the M30-associated mutation was PCR amplified from B16F10 cDNA with the forward (TCCTCCCGAGTCTGCCCAGCCACGGTCATT) and the reverse (ACAGCTGCGGCCTCGGGAGACTGAGGGCCT) primers. The amplification reaction product was purified from agarose gel using the PCR clean-up Kit (Macherey Nagel) and sequenced.

C57bl/6 female 4-week-old mice were purchased from Charles River Laboratories and kept and treated in accordance with the Italian policies on animal research at the Toscana Life Sciences animal facility (Siena, Italy).

Construction of Plasmids

The construction of pET21-Nm-fHbp and pET-Nm-fHbp-vIII plasmids expressing the Neisseria meningitidis fHbp and fHbp fused to three repeated copies of EGFRvIII peptide, respectively, was previously described (20). pET-Nm-fHbp-M30vIII plasmid carries the N. meningitidis fHbp gene fused to a synthetic DNA fragment encoding three copies of B16-M30 peptide and three copies of EGFRvIII peptide, each copy intercalated by a Glycine–Serine (GS) spacer (Figure 4A). To construct the plasmid, the PIPE method was applied. Briefly, pET-Nm-fHbp-vIII plasmid was linearized by PCR, using F-vIIIM30 (5'-ATCAGCCATTCCTGGGTTCCC TGGAAGAAAAGAAGGGT-3') primer, which anneals upstream of the vIII coding sequence, and R-fHbpM30(5'-TGCCTAGT CGGTAAGGACTTATTGCTTGGCGGCAAGGC-3') primer. In parallel, the synthetic DNA encoding three copies of M30 peptide (Thermo Fisher, 1 ng/µl in MilliQ water) was amplified by PCR with the forward primer 5'-CTTGCCGCCAAGCAAC CGAGCAAACCGAGCT-3', complementary to the 5' end of the N. meningitidis fHbp gene, and the reverse primer 5'-TCT TCCAGGGAACCCAGGAATGGCTGATCCGTTGA-3' complementary to the vIII sequence and encoding a GS spacer. The PCR products were mixed together and the mixture was used to transform E. coli HK100 strain. After confirmation of the correctness of the gene fusion by sequence analysis, E. coli BL21DE3*dompA* strain was transformed with pET-Nm-fHbp-M30-vIII plasmid and the derived recombinant strain was used for the production of engineered M30-vIII-OMVs.

Synthetic Peptides and Antibodies

The EGFRvIII peptide LEEKKGNYVVTDH unconjugated or conjugated to KLH protein was purchased from GeneScript in
lyophilic form and solubilized in PBS at the final concentration of 1 mg/ml. Polyclonal antibodies against EGFRvIII peptide were obtained from GenScript by immunizing rabbits with KLHconjugated LEEKKGNYVVTDH peptide.

The 27 amino acid M30 peptide PSKPSFQEFVDWEN VSPELNSTDQPFL was purchased from GeneScript in lyophilic form and solubilized in milliQ water at final concentration of 5 mg/ml.

Bacterial Total Lysate and OMV Preparation

Plasmids containing the genes of interest were used to transform *E. coli* BL21DE3 $\Delta ompA$ strain. Recombinant clones were grown in 200 ml LB medium (starting OD₆₀₀ = 0.05) and, when the cultures reached an OD₆₀₀ value of 0.5, protein expression was induced by addition of 1 mM IPTG. After 2 h, OMVs were collected from culture supernatants by filtration through a 0.22-µm pore size filter (Millipore) followed by high-speed centrifugation (200,000 g for 2 h). Pellets containing OMVs were finally re-suspended in PBS. Total bacterial lysates were prepared by suspending bacterial cells from 1 ml cultures (centrifuged at 13,000 g for 5 min) in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Laemli buffer and heated at 100°C for 5 min. Proteins were separated by 4–12% or 10% SDS-PAGE (Invitrogen), run in MES buffer (Invitrogen), and finally stained with Coomassie Blue.

Western Blot Analysis

Total lysates were prepared from bacteria grown in LB. Liquid cultures were pelleted in a bench-top centrifuge and suspended in SDS-PAGE loading buffer in an appropriate volume to normalize cell density to a final $OD_{600} = 10$. Each sample (10 µl) was then separated on a 4-12% SDS-polyacrylamide gel (Invitrogen). Proteins separated by SDS-PAGE were then transferred onto nitrocellulose membrane by standard methods. The membranes were blocked either 1 h at room temperature (RT) or overnight at 4°C by agitation in blocking solution (10% skimmed dry milk and 0.05% Tween 20 dissolved in PBS). Primary antibodies or sera were diluted in 1% skimmed dry milk plus 0.05% Tween 20 dissolved in PBS and incubated 1 h at RT. After three washing steps in 0.05% Tween 20 dissolved in PBS, the membranes were incubated in a 1:2,000 dilution of peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin (Dako) in 1% skimmed dry milk and 0.05% Tween 20 dissolved in PBS for 1 h, and after three washing steps, antibody binding was detected by using the SuperSignal West Pico chemiluminescent substrate (Pierce).

Flow Cytometry Analysis

20 ml of LB medium supplemented with 100 μ g/ml Ampicillin were inoculated at OD₆₀₀ = 0.05 with an overnight culture of BL21 Δ ompA(pET-Nm-fHbp-vIII). The culture was then grown and IPTG-induced as described above. BL21 Δ ompA(pET21b+) strain was used as negative control. Bacterial cells from 1 ml were harvested by centrifugation at 10,000 g for 5 min at 4°C and re-suspended with 1% BSA in

PBS to obtain a cell density of 2×10^7 CFUs/ml. 50 µl were then dispensed in a round bottom 96 well plate. Anti-EGFRvIIII peptide rabbit antibodies were added at a concentration of 5 µg/ml and incubated 1 h on ice. After three washes with 1% BSA in PBS, 20 µl of FITC-labeled anti-rabbit secondary antibodies (1:200 dilution) (Life Technologies) were added and incubated 30 min on ice. Each well was then washed twice with 200 µl 1% BSA in PBS, and plates were centrifuged at 4,000 g for 5 min. Samples were then re-suspended in 2% formaldehyde solution, incubated 15 min at 4°C and centrifuged again at 4,000 g for 5 min. Finally, samples were re-suspended in 200 µl of PBS, and data were acquired by using BD FACS Canto II cell analyzer (BD).

Confocal Microscopy Analysis

To verify fHbp-EGFRvIII localization on the cell surface, 20 ml of LB medium were inoculated at $OD_{600} = 0.05$ with an overnight culture of BL21∆ompA(pET-fHbpvIII). The culture was grown and protein expression induced with IPTG as described above. Bacterial cells from 1 ml culture were harvested by centrifugation at 6,000 g for 5 min at 4°C and re-suspended in 4% formaldehyde solution, incubated 15 min at 4°C and then centrifuged at 6,000 g for 5 min. Then samples were washed three times with 1 ml PBS, and incubated in 1 ml of blocking buffer (0.1% BSA, 10% normal goat serum in PBS) 20 min at RT. Subsequently, the bacterial suspension was incubated with rabbit polyclonal anti-EGFRvIIIpep antibodies (1 µg/ml) and mouse anti-LPS mAb (1 µg/ml) (Hycult Biotech, USA), for 1 h at RT. After two washes with 0.1% BSA in PBS, bacteria were incubated for 20 min at RT with goat anti-rabbit IgG, Alexa Fluor 594 conjugated-antibodies (Molecular Probes) and goat anti-mouse IgG, Alexa Fluor 488 conjugated-antibodies (Molecular Probes) at 1:400 final dilution. Labeled bacteria were washed twice with 0.1% BSA in PBS, and allowed to adhere to poly-lysine slides (Thermo Scientific) for 20 min at RT. Slides were mounted with ProLong Gold antifade reagent (Thermo Scientific). Confocal microscopy analysis was performed with a Leica SP5 microscope and images were obtained using Leica LASAF.

TEM Analysis

Outer membrane vesicles purified from *E. coli* BL21 Δ ompA(pET-Nm-fHbpvIII) strain were visualized using Immuno TEM. Briefly, a 5-µl aliquot of purified OMVs preparation at a final concentration of 20 ng/µl was applied to 200-square mesh nickel grids coated with a thin carbon film (Agar Scientific) and let stand for 3 min. The samples were then blocked in 0.5% BSA in PBS for 1 h at RT. Subsequently, the samples were incubated with primary rabbit anti-EGFRvIIIpep antibodies for 1 h at RT. Grids were washed three times in blocking buffer and incubated with 5-nm gold-labeled anti-rabbit secondary antibody (BB International, Madison, WI, USA) for 1 h at RT. Immunostained OMVs were then negatively stained in 1% phosphotungstic acid and visualized with a Tecnai G2 Spirit Transmission Electron Microscope operating at 100 kV. Images were collected at 87,000× magnification with a CCD camera Morada 2kx4k.

Vaccine Immunogenicity and Tumor Challenge in C57BL/6 Mice Immunization with OMVs from BL21*△ompA* (pET-Nm-fHbp-vIII) Strain

C57BL/6 mice (16 mice/group) were vaccinated on day 0, 14, and 28 with 20 μg of either "empty" OMVs [derived from BL21Δ*ompA* (pET21b+) strain] or 20 μg of Nm-fHbpvIII-OMVs [derived from BL21Δ*ompA*(pET-Nm-fHbp-vIII) strain] formulated in PBS. At day 35, 0.5×10^5 B16F10EGFRvIII cells were subcutaneously (s.c.) injected in each animal and tumor growth was measured with a caliper every 3 days over a period of 30 days. For ethical reasons, mice were euthanized when tumors reached a size of 1,500 mm³.

Immunization with Nm-fHbp-vIII-OMVs Combined with B16-M30 Peptide

C57BL/6 mice (eight mice/group) were vaccinated on day 0, 14, and 28 with 20 μ g "empty" OMVs, 20 μ g Nm-fHbp-vIII-OMVs, 100 μ g of synthetic B16-M30 peptide absorbed to 20 μ g Nm-fHbp-vIII-OMVs, or 20 μ g Nm-fHbp-M30-vIII-OMVs. At day 35, 0.5 \times 10⁵ B16F10EGFRvIII cells were s.c. injected in each animal and tumor growth was followed as descried above.

Analysis of Anti-EGFRvIII Antibodies in Immunized Animals

Anti-EGFRvIII antibodies were measured by ELISA. Amino plates (Thermo Fisher) were coated with synthetic EGFRvIII peptide (0.5 µg/well) and incubated overnight at 4°C. The day after, plates were saturated with a solution of 1% BSA in PBS (200 µl per well) for 1 h at 37°C. Mice sera were threefold serially diluted in PBS supplemented with 0.05% tween (PBST) and 0.1% BSA. After three washes with PBST, 100 µl of each serum dilution were dispensed in plate wells. As positive control, Anti-EGFRvIII rabbit serum from animals immunized with KLH-conjugated LEEKKGNYVVTDH (EGFRvIII) peptide was used. After 2 h incubation at 37°C, wells were washed three times with PBST and then incubated 30 min at 37°C with mouse anti-rabbit alkaline phosphatase-conjugate antibodies at a final dilution of 1:2,000. After three washes with PBST, 100 µl of alkaline phosphatase substrate (Sigma Aldrich) were added to each well and plates were maintained at RT in the dark for 30 min. Finally absorbance was read at 405 nm using the M2 Spectramax Reader plate instrument.

T Cell Analysis

At the end of the tumor challenge studies described above (30 days from tumor cell administration) mice were sacrificed and spleens collected in 5 ml DMEM high glucose (GIBCO). Alternatively, mice were immunized twice i.p. at days 0 and 7 with either 20 μ g "Empty" OMVs + 100 mg M30pep or 20 μ g Nm-fHbp-M30-vIII-OMVs. Five days after the second immunization, mice were sacrificed and spleens collected. Spleens were then homogenized and splenocytes filtered using a Cell Strainer 70 μ m. After centrifugation at 400 *g* for 7 min, splenocytes were re-suspended in PBS and aliquoted in a 96-well plate at a concentration of 1 × 10⁶ cells per well. Cells were stimulated with 2 mg/ml of an unrelated peptide (negative control), or 2 mg/ml of B16-M30 peptide. As positive control, cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 0.5 mg/ml) and Ionomycin (1 mg/ml). After 2 h of stimulation at RT, Brefeldin A [Beckton Dickenson (BD)] was added to each well and cells incubated for 4 h at 37°C. After two washes with PBS, NearIRDead cell staining reaction mixture (Thermo Fisher) was incubated with the splenocytes for 20 min at RT in the dark. After two washes with PBS and permeabilization and fixing with Cytofix/Cytoperm (BD) using the manufacturer's protocol, splenocytes were stained with a mix of the following fluorescent-labeled antibodies: anti CD3-APC (BioLegend), anti-CD4-BV510 (BioLegend), anti-CD8-PECF594 (BD), and IFN- γ -BV785 (BioLegend). Samples were analyzed on a BD FACSCanto II using FlowJo software. Graphs were processed with Prism 5.0 software (Graphpad). Statistical analysis and differences in means between two groups were calculated with a *t*-test calculator carried out using GraphPad Prism 5.03 (n.s.: *P* > 0.0.05, **P* < 0.01, ****P* < 0.001).

Analysis of TILs

Tumor-infiltrating lymphocytes were isolated from subcutaneous B16F10EGFRvIII tumors taken from sacrificed mice. At least two tumors per group were collected and minced into pieces of 1-2 mm of diameter using a sterile scalpel. Tumor samples were then transferred into 15-ml tubes containing 5 ml of collagenase solution (Collagenase Type 3,200 U/ml, Collagenase Type 4,200 U/ml) diluted in HBSS with 3 mM CaCl₂ and incubated under agitation for 2 h at 37°C. The resulting cell suspensions were filtered through a Cell Strainer 70 µm, washed twice with PBS and 1×10^6 cells were dispensed in a 96-well plate. Then, cells were incubated with NearIRDead cell staining Kit (Thermo Fisher) 20 min on ice in the dark. After two washes with PBS, samples were stained with the following mixture of fluorescentlabeled antibodies (BD): anti-GR1 (BV605), anti-CD11b-BV480, anti-CD45-BV786, anti-CD3-BV421, anti-CD4-PE, anti-CD8-PECF594, and anti-CD25-APC. The samples were then incubated 1 h at RT. After two washes with PBS, Cytofix/Cytoperm (BD) was added to each well and incubated 20 min on ice in the dark. After two washes with PBS, cells were stained with anti-Foxp3-A488 (BD) antibodies diluted in Permwash 1× buffer 20 min at RT in the dark. Finally, samples were washed two times with 1% BSA in PBS and analyzed on a BD FACSCanto II as described above.

ETHICS STATEMENT

Mice were monitored twice per day to evaluate early signs of pain and distress, such as respiration rate, posture, and loss of weight (more than 20%) according to humane end points. Animals showing such conditions were anesthetized and subsequently sacrificed in accordance with experimental protocols, which were reviewed and approved by the Animal Ethical Committee of Toscana Life Sciences Foundation and by the Italian Ministry of Health.

AUTHOR CONTRIBUTIONS

GG: design of experimental plan and manuscript preparation. AG: experimental design, strain Engineering, analysis of immune responses, and animal studies. MP: analysis of Immune responses, FACS analysis, T cell analysis, and confocal microsacopy analysis. MT and IZ: animal studies. LG, EC, LFantappiè, SI, FZ, and CI: strain engineering and OMV preparation and characterization. LFagnocchi and EK: strain fermentation and OMV purification and characterization. ST and CS: FACS analysis of T cell population. FG and IF: electron microscopy analysis of OMVs.

ACKNOWLEDGMENTS

The authors are deeply indebted to Prof. J. H. Sampson (Duke University Medical Center, Durham, NC, USA) for providing the B16F10EGFRvIII cellline and to Dr. Stefano Prete, Dr. Silvia Valensin, and Dr. Erika Bellini for their expert support in animal studies.

FUNDING

This work has been supported by the Advanced ERC grant OMVac 340915 assigned to GG.

REFERENCES

- Klebanoff CA, Acquavella N, Yu Z, Restifo NP. Therapeutic cancer vaccines: are we there yet? *Immunol Rev* (2011) 239:27–44. doi:10.1111/j. 1600-065X.2010.00979.x
- Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA Jr, Kinzler KW. Cancer genome landscapes. *Science* (2013) 339:1546–58. doi:10.1126/science.1235122
- Rosenberg SA, Restifo NP. Adoptive T cell transfer as personalized immunotherapy for human cancer. *Science* (2015) 348:62–8. doi:10.1126/science. aaa4967
- Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, et al. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science* (2015) 348(6230):124–8. doi:10.1126/science.aaa1348
- Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N Engl J Med* (2014) 371:2189–99. doi:10.1056/NEJMoa1406498
- Van Allen EM, Miao D, Schilling B, Shukla SA, Blank C, Zimmer L, et al. Genomic correlates of response to CTLA4 blockade in metastatic melanoma. *Science* (2014) 350:207–11. doi:10.1126/science.aad0095
- Kreiter S, Vormehr M, van de Roemer N, Diken M, Löwer M, Diekmann J, et al. Mutant MHC class II epitopes drive therapeutic immune responses to cancer. *Nature* (2015) 520:692–6. doi:10.1038/nature14426
- Kranz LM, Diken M, Haas H, Kreiter S, Loquai C, Reuter KC, et al. Systemic RNA delivery to dendritic cells exploits antiviral defence for cancer immunotherapy. *Nature* (2016) 534:396–401. doi:10.1038/ nature18300
- 9. Beveridge TJ. Structures of gram-negative cell walls and their derived membrane vesicles. *J Bacteriol* (1999) 181:4725–33.
- Mayrand D, Grenier D. Biological activities of outer membrane vesicles. *Can J Microbiol* (1989) 35:607–13. doi:10.1139/m89-097
- Kulp A, Kuehn MJ. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annu Rev Microbiol* (2010) 64:163–84. doi:10.1146/ annurev.micro.091208.073413
- Ellis TN, Kuehn MJ. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiol Mol Biol Rev* (2010) 74:81–94. doi:10.1128/ MMBR.00031-09
- Moshiri A, Dashtbani-Roozbehani A, Najar Peerayeh S, Siadat SD. Outer membrane vesicle: a macromolecule with multifunctional activity. *Hum Vaccin Immunother* (2012) 8:953–5. doi:10.4161/hv.20166
- Ellis TN, Leiman SA, Kuehn MJ. Naturally produced outer membrane vesicles from *Pseudomonas aeruginosa* elicit a potent innate immune response via combined sensing of both lipopolysaccharide and protein components. *Infect Immun* (2010) 78:3822–31. doi:10.1128/IAI.00433-10

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fonc.2017.00253/ full#supplementary-material.

FIGURE S1 | Flow cytometry gating strategy and analysis of tumor cell populations. Cells (1×10^6) were isolated after collagenase treatment from frozen tumors collected from one mouse immunized with "Empty" OMVs (**A**) and one mouse immunized with Nm-fHbpvIII OMVs (**B**). A first selection was made based on NearIRDead cell staining and only alive cells were included in the analysis. Subsequently, a homogeneous population of single cells was selected according to morphological parameters. The percentage of CD8+ and CD4+ T cells in each tumor was calculated from the CD45+/CD8+ and CD45+/CD4+ double positive cell populations, respectively. The double positive cells CD45+/CD4+ were subsequently selected for Treg analysis using anti-Foxp3+ antibodies. Finally, MDSCs were identified by selecting the CD45+ cell population and analyzing their positivity to CD11b and Gr1 staining.

- Kaparakis M, Turnbull L, Carneiro L, Firth S, Coleman HA, Parkington HC, et al. Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells. *Cell Microbiol* (2010) 12:372–85. doi:10.1111/j.1462-5822.2009. 01404.x
- Chen DJ, Osterrieder N, Metzger SM, Buckles E, Doody AM, DeLisa MP, et al. Delivery of foreign antigens by engineered outer membrane vesicle vaccines. *Proc Natl Acad Sci U S A* (2010) 107:3099–104. doi:10.1073/pnas. 0805532107
- Kesty NC, Kuehn MJ. Incorporation of heterologous outer membrane and periplasmic proteins into *Escherichia coli* outer membrane vesicles. *J Biol Chem* (2004) 279:2069–76. doi:10.1074/jbc.M307628200
- Schroeder J, Aebischer T. Recombinant outer membrane vesicles to augment antigen-specific live vaccine responses. *Vaccine* (2009) 27:6748–54. doi:10.1016/j.vaccine.2009.08.106
- Fantappiè L, de Santis M, Chiarot E, Carboni F, Bensi G, Jousson O, et al. Antibody-mediated immunity induced by engineered *Escherichia coli* OMVs carrying heterologous antigens in their lumen. *J Extracell Vesicles* (2014) 3:24015. doi:10.3402/jev.v3.24015
- Fantappiè L, Irene C, De Santis M, Armini A, Gagliardi A, Tomasi M, et al. Some Gram-negative lipoproteins keep their surface topology when transplanted from one species to another and deliver foreign polypeptides to the bacterial surface. *Mol Cell Proteomics* (2017) 16(7):1348–64. doi:10.1074/mcp. M116.065094
- Granoff D. Review of meningococcal group B vaccines. Clin Infect Dis (2010) 50:S54–65. doi:10.1086/648966
- 22. Ferrari G, Garaguso I, Adu-Bobie J, Doro F, Taddei AR, Biolchi A, et al. Outer membrane vesicles from group B *Neisseria meningitidis* delta gna33 mutant: proteomic and immunological comparison with detergent-derived outer membrane vesicles. *Proteomics* (2006) 6:1856–66. doi:10.1002/ pmic.200500164
- Bernadac A, Gavioli M, Lazzaroni JC, Raina S, Lloubès R. Escherichia coli tol-pal mutants form outer membrane vesicles. J Bacteriol (1998) 180:4872–8.
- 24. Deatherage BL, Lara JC, Bergsbaken T, Rassoulian Barrett SL, Lara S, Cookson BT. Biogenesis of bacterial membrane vesicles. *Mol Microbiol* (2009) 72:1395–407. doi:10.1111/j.1365-2958.2009.06731.x
- McBroom AJ, Kuehn MJ. Release of outer membrane vesicles by Gramnegative bacteria is a novel envelope stress response. *Mol Microbiol* (2007) 63:545–58. doi:10.1111/j.1365-2958.2006.05522.x
- Berlanda Scorza F, Colucci AM, Maggiore L, Sanzone S, Rossi O, Ferlenghi I, et al. High yield production process for *Shigella* outer membrane particles. *PLoS One* (2012) 7:e35616. doi:10.1371/journal.pone.0035616
- Humphrey PA, Wong AJ, Vogelstein B, Zalutsky MR, Fuller GN, Archer GE, et al. Anti-synthetic peptide antibody reacting at the fusion junction of deletion-mutant epidermal growth factor receptors in human glioblastoma. *Proc Natl Acad Sci U S A* (1990) 87:4207–11. doi:10.1073/pnas.87.11.4207

- Heimberger AB, Crotty LE, Archer GE, Hess KR, Wikstrand CJ, Friedman AH, et al. Epidermal growth factor receptor VIII peptide vaccination is efficacious against established intracerebral tumors. *Clin Cancer Res* (2003) 9(11):4247–52.
- Del Vecchio CA, Li G, Wong AJ. Targeting EGF receptor variant III: tumor-specific peptide vaccination for malignant gliomas. *Expert Rev Vaccines* (2012) 11:133–44. doi:10.1586/erv.11.177
- Gerritzen MJH, Martens DE, Wijffels RH, van der Pol L, Stork M. Bioengineering bacterial outer membrane vesicles as vaccine platform. *Biotechnol Adv* (2017) 35(5):565–74. doi:10.1016/j.biotechadv.2017.05.003
- Elsamadicy AA, Chongsathidkiet P, Desai R, Woroniecka K, Farber SH, Fecci PE, et al. Prospect of rindopepimut in the treatment of glioblastoma. *Expert Opin Biol Ther* (2017) 17:507–13. doi:10.1080/14712598.2017.1299705
- 32. Alaniz RC, Deatherage BL, Lara JC, Cookson BT. Membrane vesicles are immunogenic facsimiles of *Salmonella typhimurium* that potently activate dendritic cells, prime B and T cell responses, and stimulate protective immunity in vivo. *J Immunol* (2007) 179:7692–701. doi:10.4049/jimmunol.179.11.7692

 Rosenthal JA, Huang CJ, Doody AM, Leung T, Mineta K, Feng DD, et al. Mechanistic insight into the TH1-biased immune response to recombinant subunit vaccines delivered by probiotic bacteria-derived outer membrane vesicles. *PLoS One* (2014) 9:e112802. doi:10.1371/journal.pone.0112802

Conflict of Interest Statement: ST, CS, FG, and IF are employees of GSK (Siena, Italy). The Company has not financially supported the activities reported in the manuscript and has not expressed any commercial interest in the project.

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Synthetic Gene Expression Circuits for Designing Precision Tools in Oncology

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Precision medicine in oncology needs to enhance its capabilities to match diagnostic and therapeutic technologies to individual patients. Synthetic biology streamlines the design and construction of functionalized devices through standardization and rational engineering of basic biological elements decoupled from their natural context. Remarkable improvements have opened the prospects for the availability of synthetic devices of enhanced mechanism clarity, robustness, sensitivity, as well as scalability and portability, which might bring new capabilities in precision cancer medicine implementations. In this review, we begin by presenting a brief overview of some of the major advances in the engineering of synthetic genetic circuits aimed to the control of gene expression and operating at the transcriptional, post-transcriptional/translational, and post-translational levels. We then focus on engineering synthetic circuits as an enabling methodology for the successful establishment of precision technologies in oncology. We describe significant advancements in our capabilities to tailor synthetic genetic circuits to specific applications in tumor diagnosis, tumor cell- and gene-based therapy, and drug delivery.

OPEN ACCESS Edited by:

Scott Bultman, University of North Carolina at Chapel Hill, United States

Reviewed by:

Giovanni Sorrentino, École Polytechnique Fédérale de Lausanne, Switzerland Hatem E. Sabaawy, Rutgers University, The State University of New Jersey, United States

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Specialty section:

This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Cell and Developmental Biology

> Received: 18 June 2017 Accepted: 16 August 2017 Published: 28 August 2017

Citation:

Re A (2017) Synthetic Gene Expression Circuits for Designing Precision Tools in Oncology. Front, Cell Dev. Biol. 5:77. doi: 10.3389/fcell.2017.00077

Keywords: synthetic circuit, biological engineering, synthetic biology, precision medicine, tumor diagnosis, tumor therapy, drug delivery, drug discovery

INTRODUCTION

Synthetic biology builds on the transformative assertion that engineering approaches could be used to elucidate design principles of cellular systems and to implement synthetic digital and analog subsystems for a variety of end settings including health applications (Lienert et al., 2014). Since its beginning as a formalized engineering paradigm, which could be envisioned near the turn of the century when bacterial cells were programmed with basic genetic circuits (Gardner et al., 2000; Cameron et al., 2014), synthetic biology has provided a rigorous mechanistic foundation extremely helpful to quantitatively characterize the basic functions that are performed by the simple parts of a system and that collectively dictate the emergence of natural and human-defined phenotypes (Mukherji and van Oudenaarden, 2009; Elowitz and Lim, 2010). Nowadays, synthetic biology has greatly expanded in outlook, arising expectations, and stream of thought owing to the increasing intensive convergence of multifaceted engineering, life science, and biotechnology subfields.

The rapid progresses ensued from basic and applied synthetic biology research hold great promise in many contexts of substantial scientific and economic interest. The objective of this review is to reflect on the applications relevant to develop solutions to some of the challenges put forward by precision oncology. The precision paradigm that is being variously adopted by oncology

refers both to the chances for enhanced resolution and clarity in tumor identification as well as to the implementation of therapeutic interventions that could be set up on individual case basis (Jain, 2013; Kis et al., 2015). In this text, we provide an overview of synthetic genetic circuits engineering that apply to precision oncology and take advantage of the tight molecular control operating at multiple levels of gene expression (Vazquez-Anderson and Contreras, 2013; Fern and Schulman, 2017), through signal amplification, feedback, oscillatory, and logic capabilities (Wang et al., 2013; Lienert et al., 2014). Specifically, we show that engineered gene regulatory circuits are widening the assays available to report on tumor state and anti-tumor drug responses as well as to devise localized therapeutic options; for instance, increasingly advanced studies are being published on engineering cell classifiers (Morel et al., 2016; Mohammadi et al., 2017) and synthetic constructs for local payload delivery (Wagner et al., 2016). Furthermore, multiple gene-and cell-based therapy choices enhanced by synthetic biology applications are here described (Lim and June, 2017).

A great deal of efforts has been applied to investigate the rules of gene expression by precise measurements afforded by artificially constructed systems (Mukherji and van Oudenaarden, 2009). Much of the early contributions have focused on detailed and quantitative views of transcriptional regulation (Hockenberry and Jewett, 2012), and proceeded in tandem with experimental breakthroughs such as the use of combinatorial promoter libraries (Gertz et al., 2009). Nevertheless, substantial progress has also been achieved in ascertaining other regulatory mechanisms including post-transcriptional, translational, and post-translational modifications (Isaacs et al., 2004; Grilly et al., 2007). Almost all of these regulatory mechanisms are applicable to design gene regulatory platforms with controllable and predictable behaviors. Building on natural examples of regulatory circuits known to tune transcriptional and post-transcriptional activity (Cora et al., 2017), synthetic devices have demonstrated to modulate malignant phenotypes. Interesting examples here include synthetically engineered microRNAs targeting the MYC proto-oncogene (c-Myc) gene, which were shown to inhibit proliferation and induce apoptosis in bladder cancer cells (Fu et al., 2015), and the usage of aptamers to induce tumor cell death by destabilizing the apoptosis regulator bcl-2 (Soundararajan et al., 2008).

While the approaches to design the synthetic biological circuits that will be described could greatly vary, it is clear that abstraction, standardization (Galdzicki et al., 2014), and modularity (Endy, 2005) have been essential to formalize the design of such a broad range of gene expression systems and to handle biological complexity. Such principles lie behind many synthetic circuits to develop diagnostic and therapeutic tools, where basic parts such as promoters, gene coding sequences, terminators, and ribosome binding sites are assembled into modules such as toggle switches (Gardner et al., 2000; Niederholtmeyer et al., 2013) oscillators, and cascades (Davidsohn et al., 2015) to create predictable and continuously more sophisticated functionalities. The achievement of general and scalable systems (Weinberg et al., 2017) capable of sensing, reacting to, and controlling multiple component activities *in vivo*

have required advanced programming paradigms to overcome barriers such as metabolic load (Weinberg et al., 2017), crosstalk (Huh et al., 2013; Kosuri et al., 2013; Trosset and Carbonell, 2013; Brewster et al., 2014), resource sharing (Cardinale et al., 2013; Segall-Shapiro et al., 2014), and gene expression noise (An and Chin, 2009) and thus to grant stability, robustness, and reliability of the engineered systems (Green et al., 2017).

The review is structured in two main sections. The former section summarizes engineering principles that are being applied to devise synthetic genetic circuits. Here, molecular tools exploiting transcriptional, post-transcriptional/translational, and post-translational control mechanisms of gene expression are discussed in separate subsections. The latter section describes specific areas of diagnostic and therapeutic technologies within the precision oncology enterprise where the potential of synthetic biology applications sits at the vanguard.

FROM GENE SWITCHES TO COMPUTING DEVICES

Biological engineering has enlarged the molecular tool set available to customize multicomponent constructs with increasingly varied and improved options for controlling gene expression. In particular, a great deal of design effort on synthetic gene switches has allowed to engineer cells with the capacity to sense, process, and switch gene expression state in response to intra- and extracellular signals. Engineering such sensingactuating constructs involves linking a sensor part that detects the ligand to an actuator part that controls gene expression. The molecular design principles that have been used to customize synthetic gene switches differ according to the gene expression stage at which the switch is applied as well as on the distinctive properties that come with the choice of the switch constitutive parts (**Figure 1**).

Tools for Transcriptional Control

Circuits based on transcriptional control make up the largest number of synthetic circuits and share a common design, where an actuator part enabling positive or negative regulation of transcription is connected with a DNA-binding part that recognizes a promoter DNA sequence. Upon binding of a ligand, a sensor part triggers the activity of this complex through tethering or allosteric mechanisms (Ausländer and Fussenegger, 2013). While native transcription factors have come a long way in synthetic biology applications, it was not until the arrival of programmable transcription factors that it was possible to enhance the engineering capabilities of human-defined transcriptional switches. For example, Zinc-Finger (ZF)-containing factors (Khalil et al., 2012), Transcription Activator-Like Effectors (TALEs; Sanjana et al., 2012; Li et al., 2015), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-based regulators (Bikard et al., 2013; Qi et al., 2013; Ferry et al., 2017) can be engineered to bind to specific DNA sequences of interest. Each class of TFs comes with advantages and disadvantages and is ideally suited to different applications (Jain, 2013). Major limitations



in the application of ZF-containing factors on synthetic circuits are their limited modularity and the lack of specificity of some ZF domains. TALEs are more straightforward to design than ZFs even though they pose challenges to cloning and delivery into host genomes. The CRISPR-based regulators are easier to construct than TALEs which, nonetheless, perform better for the construction of layered circuits (Lebar and Jerala, 2016). The plasmid pT181 antisense-RNA-mediated transcription attenuation platform is well established to control transcription through RNA-RNA interactions (Lucks et al., 2011).

Tools for Post-transcriptional Control

Due to its functional diversity, RNA is an advantageous substrate for information sensing, processing, and computation functions. Furthermore, the transient nature of RNA is appealing for applications where safety is a primary concern, since RNA-mediated circuits do not leave a long-term genetic footprint. RNA-based sensing-actuation switches are generally composed of highly folded sensor RNAs (aptamers) that, through conformational changes induced by the binding of small molecules or proteins, regulate the activity of RNA actuators that can operate *in cis* or *in trans.* Switches can sometimes rely on a transmitter part to transduce information between the sensor and actuator (Ogawa and Maeda, 2008). Aptamers have been engineered to respond predominantly to small molecules and

nucleic acids (Werstuck and Green, 1998; Win et al., 2009; Shen et al., 2015) with extreme specificity whereas aptamers sensing proteins are far less intensely exploited (Culler et al., 2010).

Actuation can occur through diverse mechanisms including splicing, stability, translation, and mRNA localization. Owing to the known impact of ribonucleases (RNases) on RNA maturation and stability, aptamers have often been combined with RNA substrates for RNase activities (Vazquez-Anderson and Contreras, 2013; Comeau et al., 2016). Many RNA-based devices combine aptamers with catalytic actuators such as selfcleaving ribozymes to achieve flexible regulatory properties to fit application-specific performance requirements (Win and Smolke, 2007; Chen et al., 2010; Ketzer et al., 2012). Aptamers were also used with RNA interference substrates to control target mRNA silencing by regulating Drosha processing of pri-miRNAs (Beisel et al., 2011) or Dicer processing of small hairpin RNAs in response to endogenous signals (Saito et al., 2011). Furthermore, siRNAs and miRNAs have been shown to provide valuable options to implement Boolean logic frameworks (Rinaudo et al., 2007; Xie et al., 2011; Schreiber et al., 2016). A variety of switches have been developed to regulate translation of an open reading frame in response to the binding between the aptamer and small molecule (Stoltenburg et al., 2007; Wroblewska et al., 2015) or protein ligand (Hanson et al., 2003; Win and Smolke, 2007). Translation-control switches mainly affect translation initiation, such as the translational repression/activation switches consisting

of the ribosomal protein L7Ae and its box C/D kink-turn binding RNA motif (Saito et al., 2010, 2011). Furthermore, engineered systems can repress and/or activate translation by inducing conformational changes in nascent structured mRNA that modulate the access of the translational machinery to ribosome binding sites (Isaacs et al., 2004; Salis et al., 2009). Enhancement of protein synthesis has been recently achieved by the use of natural and synthetic antisense long non-coding RNAs (Yao et al., 2015) which were named SINEUPs due to the requisite of the inverted SINEB2 sequence to UP-regulate gene-specific translation (Zucchelli et al., 2015). Finally, engineering upstream Open Reading Frames (uORFs), whose regulatory potential is increasingly being appreciated (Re et al., 2016), is predictably an additional exploitable tool for protein manufacturing (Ferreira et al., 2013). Finally, RNA-based devices have been built to enhance gene regulatory activities through co-localization (Lee et al., 1999).

Artificial signal cascades can be constructed by combining multiple regulators, examples of which are inverter modules for synthetic translational switches (Endo et al., 2013). Programming Boolean operators for translational regulation has also been allowed by rationally designed variants of the RNA-IN-RNA-OUT antisense RNA-mediated translation system (Mutalik et al., 2012) as well as by the design of multiple orthogonal ribosomemRNA pairs (Rackham and Chin, 2005), which were also implemented to synthesize orthogonal transcription-translation networks (An and Chin, 2009).

Tools for Post-translational Control

Synthetic switches have been designed that control protein activity by altering protein stability, which for instance is obtained by temporarily tagging proteins with a degradation signal, which guides the protein to the endogenous ubiquitinproteasome system (Los et al., 2008; Collins et al., 2017). Efforts to engineer phosphorylation-mediated circuitry have been undertaken to rewire and construct MAP kinase circuits (Bashor et al., 2008; Wei et al., 2012; Ryu and Park, 2015). Additionally, the ability of inteins to form and cleave specific peptide bonds is extensively exploited to implement sensors of protein-protein interactions and small molecules, to realize synthetic circuits to deliver CRISPR-Cas9 system components (Truong et al., 2015) and to implement logic gates (Schaerli et al., 2014). Further efforts are ongoing to engineer and characterize synthetic compartmentalization approaches providing veritable solutions to implement modularity in synthetic devices (Chen and Silver, 2012).

SYNTHETIC CIRCUIT-BASED TOOLS FOR PRECISION MEDICINE IN ONCOLOGY

We outline synthetic biology applications which are expanding existing options in cancer diagnosis, cancer therapeutics, and for pharmaceutical compound screening (**Figure 1**).

Tumor Diagnosis

Precise cell state discrimination is essential for *in vivo* targeting of cancer cells. Medical diagnosis based on individual elements

is unavoidably thwarted by lack of specificity and sensitivity. Therefore, diagnostic algorithms are being formalized using combinatorial Boolean logic to perform integrated detection and analysis of multiple signals in living cells (Rubens et al., 2016; Schreiber et al., 2016). Expression profiles are widely used to drive decision-making circuits such as the multi-input RNAi-based logic circuit identifying specific cancer cells (Xie et al., 2011). The cancer classifier circuit implemented in this study selectively triggers either a fluorescent reporter or apoptosis in HeLa cells. More precisely, this circuit integrates sensory information from six endogenous microRNAs to determine whether a cell matches a pathological reference pattern characteristic of the HeLa cervical cancer cell line and, if so, produces an apoptotic response. Early efforts to develop bio-based computing capabilities such as counting (Friedland et al., 2009) and memory storage (Siuti et al., 2013) lead to the notion that bacterial cells could become diagnostic indicators for recording exposure events (Cronin et al., 2012). In one of such studies, probiotic bacteria were transformed with a dual-stabilized, high-expression lacZ vector, and an integrated luxCDABE cassette endowing luminescent visualization in order to target, visualize, and diagnose liver metastasis (Danino et al., 2015). A recent study brought whole-cell biosensor closer to clinical requirements by configuring digital amplifying genetic switches, based on transistor-like three terminal devices (Bonnet et al., 2013), to actuate logic gates in bacterial chasses (Courbet et al., 2015). Here, digital amplifying switches are used in Boolean logic gates to perform complex signal processing tasks such as multiplexed detection of clinically relevant markers, signal digitization, and amplification along with storage of the medically informed outcome in a stable DNA register for a posteriori interrogation. Standardized devices for cancer diagnosis require a great deal of fine-tuning efforts to make combinatorial logic gates to perform as intended. Therefore, progressively advanced studies are being reported, opening interesting avenues to the automation of combinatorial circuit engineering (Ausländer et al., 2012; Nielsen et al., 2016; Weinberg et al., 2017). Even so, there are cumbersome problems that still need to be dealt with. Despite the breadth and depth described above, it is difficult to control the trade-off between specificity and sensitivity achieved by expression-based cell classifier designs, the changes in constructs performance dependent on genetic context, space and time as well as the possible toxicity induced by regulators overexpression. Balancing these problems must be addressed in order to allow synthetic gene constructs to become part of a personalized cancer therapy toolbox.

Tumor Therapy

Synthetic biology is primed to provide the conceptual framework and genetic tools necessary to enhance cell- (Fischbach et al., 2013) and gene- (Costales et al., 2017) based therapeutics.

Cell-Based Therapeutics

Immunotherapy has shown great promise for eradicating tumor in clinical trials. Much of the current success derives from therapies based on engineering T cell receptors (TCRs) and chimeric antigen receptors (Wilkie et al., 2012; Kloss et al., 2013; Duong et al., 2015; CARs), that consist of a cancer antigen-specific single-chain variable fragment (scFv) fused to a T cell signaling domain that triggers activation and proliferation. Nowadays, synthetic sensors, switches, and circuits are primed to improve T cell therapy efficacy and meet safety concerns (e.g., discriminative capacity between tumors and vital organs and potential adverse side effects) by providing inducible control over the specificity, localization, duration, and extent of T cell activities.

Receptor systems

One of the most important challenges is represented by cell specificity. A powerful way to enhance on-target activity of therapeutic T cells is to engineer combinatorial receptor systems such as dual receptor AND-gate T cells (Roybal et al., 2016). In the antibody-coupled T cell receptor (ACTR) system, the scFv is replaced with the extracellular portion of CD16, a receptor that binds to the constant fragment of antibodies so that any relevant cancer-specific antibody can, in principle, be administered upon antigen binding (Kudo et al., 2014). Another major concern is the potential risk of unpredictable therapeutic effect. To enhance controllability, the recent GoCAR-T system incorporates a switch that activates CAR T cells when it is triggered not only by the target antigen expressed on the surface of the cancer cells but also by controlled administration of the drug rimiducid (Foster et al., 2017).

Control switches and circuits

T cell therapies could meet safety concerns if it were possible to eliminate quickly the engineered cells upon adverse side effects. Drug-inducible kill switches are an interesting development to achieve this goal. A recent example employs an inducible caspase 9 in conjunction with a CD20-specific CAR to test *in vivo* its potential to remove CAR-bearing T cells (Budde et al., 2013). Another study proposed to fuse caspase 9 to a modified FKbinding protein in order to allow conditional dimerization. This construct was proven to lead to cell death when exposed to a dimerizing small molecule (Di Stasi et al., 2011).

The design of negative feedback loops and inducible pause switches is proving a useful alternative to T cell elimination by modulating the immune response amplitude and timing. These circuits exploit the ability of bacterial virulence effector proteins to evade the immune response. The former type creates a negative feedback loop by expressing these proteins under the control of a T cell activation responsive promoter (Wei et al., 2012). The latter type of circuits pauses T cell activation by expressing bacterial virulence proteins under the control of a tetracycline inducible promoter. Indeed, adding the drug leads to the expression of the effector proteins, which in turn stop cell activation until the drug is removed (Wei et al., 2012).

Finally, a potent tool to regulate the therapy safety and efficacy is provided by growth switches (Chen et al., 2010). Here, a ribozyme drives self-cleavage of the cytokine transcript and leads to cytokine expression shut off; adding a proper drug prevents self-cleavage so that cytokines are expressed and lead to T cell proliferation.

Gene-Based Therapeutics

Gene circuit engineering has greatly improved our ability to programme genes involved in tumor origin and progress. For instance, some high-affinity RNA aptamers against PPAR- δ , a lipid-sensing nuclear receptor involved in cancer (Kwak et al., 2009), β -catenin (Lee et al., 2006), and nucleolin (Soundararajan et al., 2008), could lead to reduction of tumor-forming potential. Furthermore, a computational workflow, that selects RNA motif-small molecule binding interactions by library-vs.-library screening (2DCS) and then mines them against RNA folds in the transcriptome, allowed to identify a small molecule inhibitor of an oncogenic non-coding RNA (Velagapudi et al., 2017). SiRNAs can also specifically bind to target genes but their application can be limited by the absence of effective vehicles. For this purpose, several studies have proposed the use of aptamers in siRNA expressing constructs as vehicles (Tai and Gao, 2016).

Drug Delivery

Today nanobiotechnology provides extremely versatile options to address the localized delivery of genetically encoded tools such as virus-based vectors modified to carry engineered payloads (Ryan et al., 2004; Li et al., 2005), oncolytic viruses exploiting dual promoter logics (Nissim and Bar-Ziv, 2010), and nanoparticle-aptamer bioconjugates (Farokhzad et al., 2006). Recently, (Douglas et al., 2012) described a shape-switching device for targeted transport of signaling molecules. The robotic DNA device consists of a barrel provided with DNA aptamer-based locks that open in response to the binding of cell type-specific antigen keys. Vibrant developments greatly enhance and wide the range of applicable dynamic DNA and RNA-based nanoparticles (Afonin et al., 2013; Edwardson et al., 2016) besides opening newer avenue to conjugate inter-dependent nanoparticles (Halman et al., 2017). Polymer materials responsive to external signals (Stuart et al., 2010) such as nanogels conjugated to ligands recognized by cell specific receptors (Oishi et al., 2007), virus-mimetic nanogels (Lee et al., 2008), and hydrogels based on ligand-responsive DNA-protein interactions (Christen et al., 2011) demonstrate the essential progress in the area. In the future, nanorobots could be routed toward the tumor by exploiting the tumor-homing ability of self-propelled bacteria, similar to a recent study (Katuri et al., 2017). Biological vesicles derived from mammalian cells have also attracted much attention for in vivo delivery (Yoo et al., 2011). In particular, exosomes (Wang et al., 2016) have been engineered to deliver chemotherapeutics to tumor tissue in mouse models for cancer (Tian et al., 2014).

Drug Discovery

Synthetic biology is helping to address previously unfeasible challenges the field of drug discovery. Progress on design of synthetic genetic circuits (Carbonell et al., 2014; Trosset and Carbonell, 2015) has opened the possibility of their use not only for production of drugs (Breitling and Takano, 2015) but also for the development of platforms for identification and validation of drug targets (Firman et al., 2012; Kasap et al., 2014) as well as for phenotypic cell-based screening approaches (Duportet et al., 2014) such as the screening for anti-cancer drugs presented

in Gonzalez-Nicolini et al. (2004), that discriminates between proliferation competent and mitotically inert cells and eliminates preferentially neoplastic ones. With this purpose, (Gonzalez-Nicolini et al., 2004) engineered a transgenic CHO-K1-derived cell line to enable G1-specific growth arrest conditioned on the tetracycline responsive overexpression of the human cyclindependent kinase inhibitor p27. Another study applied a onebead-two-compound (OB2C) cell-based screening approach for the discovery of synthetic molecules that can interact with cellular receptors as well as enhance or inhibit downstream cell signaling (Kumaresan et al., 2011). The primary innovation of this system is represented by the usage of beads provided with two chemical molecules on the surface and a chemical tag to probe cellular responses. When cells are incubated with the OB2C library, a cell adhesion ligand captures live cells on each bead in the library. The bound cells can interface with the tethered OB2C library compounds and then be probed for specific cellular signaling pathways such as leukemic cell death responses (Kumaresan et al., 2011). Largely because of similar progresses in conceptual design and technologies, synthetic biology is being employed as a powerful way to identify drug mechanisms of action and to accelerate the development of drug combination-based approaches (Chandrasekaran et al., 2016).

REFERENCES

- Afonin, K. A., Viard, M., Martins, A. N., Lockett, S. J., Maciag, A. E., Freed, E. O., et al. (2013). Activation of different split functionalities on re-association of RNA-DNA hybrids. *Nat. Nanotechnol.* 8, 296–304. doi: 10.1038/nnano.2013.44
- An, W., and Chin, J. W. (2009). Synthesis of orthogonal transcriptiontranslation networks. *Proc. Natl. Acad. Sci. U.S.A.* 106, 8477–8482. doi: 10.1073/pnas.0900267106
- Ausländer, S., Ausländer, D., Müller, M., Wieland, M., and Fussenegger, M. (2012). Programmable single-cell mammalian biocomputers. *Nature* 487, 123–127. doi: 10.1038/nature11149
- Ausländer, S., and Fussenegger, M. (2013). From gene switches to mammalian designer cells: present and future prospects. *Trends Biotechnol.* 31, 155–168. doi: 10.1016/j.tibtech.2012.11.006
- Bashor, C. J., Helman, N. C., Yan, S., and Lim, W. A. (2008). Using engineered scaffold interactions to reshape MAP kinase pathway signaling dynamics. *Science* 319, 1539–1543. doi: 10.1126/science.1151153
- Beisel, C. L., Chen, Y. Y., Culler, S. J., Hoff, K. G., and Smolke, C. D. (2011). Design of small molecule-responsive microRNAs based on structural requirements for Drosha processing. *Nucleic Acids Res.* 39, 2981–2994. doi: 10.1093/nar/gkq954
- Bikard, D., Jiang, W., Samai, P., Hochschild, A., Zhang, F., and Marraffini, L. A. (2013). Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. *Nucleic Acids Res.* 41, 7429–7437. doi: 10.1093/nar/gkt520
- Bonnet, J., Yin, P., Ortiz, M. E., Subsoontorn, P., and Endy, D. (2013). Amplifying genetic logic gates. *Science* 340, 599–603. doi: 10.1126/science.1232758
- Breitling, R., and Takano, E. (2015). Synthetic biology advances for pharmaceutical production. *Curr. Opin. Biotechnol.* 35, 46–51. doi: 10.1016/j.copbio.2015.02.004
- Brewster, R. C., Weinert, F. M., Garcia, H. G., Song, D., Rydenfelt, M., and Phillips, R. (2014). The transcription factor titration effect dictates level of gene expression. *Cell* 156, 1312–1323. doi: 10.1016/j.cell.2014.02.022
- Budde, L. E., Berger, C., Lin, Y., Wang, J., Lin, X., Frayo, S. E., et al. (2013). Combining a CD20 chimeric antigen receptor and an inducible caspase 9 suicide switch to improve the efficacy and safety of T cell adoptive immunotherapy for lymphoma. *PLoS ONE* 8:e82742. doi: 10.1371/journal.pone.0082742

CONCLUSIONS

In this review, we focus on advances in biological engineering which stimulated the development of innovative approaches for precision intervention in oncology. The growing contribution of synthetic biology to drug discovery as well as the widening availability of synthetic circuits, which are already being used in different human compatible cell types and animal models for safe operation of gene- and cell-based therapies, demonstrate the potential of future approaches integrating systems and synthetic biology tools to precisely match therapies to individual cancer patients.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

FUNDING

This manuscript has been funded by the Istituto Italiano di Tecnologia.

- Cameron, D. E., Bashor, C. J., and Collins, J. J. (2014). A brief history of synthetic biology. *Nat. Rev. Microbiol.* 12, 381–390. doi: 10.1038/nrmicro3239
- Carbonell, P., Parutto, P., Baudier, C., Junot, C., and Faulon, J-L. (2014). Retropath: automated pipeline for embedded metabolic circuits. *ACS Synth. Biol.* 3, 565–577. doi: 10.1021/sb4001273
- Cardinale, S., Joachimiak, M. P., and Arkin, A. P. (2013). Effects of genetic variation on the *E.* coli host-circuit interface. *Cell Rep.* 4, 231–237. doi: 10.1016/j.celrep.2013.06.023
- Chandrasekaran, S., Cokol-Cakmak, M., Sahin, N., Yilancioglu, K., Kazan, H., Collins, J. J., et al. (2016). Chemogenomics and orthology-based design of antibiotic combination therapies. *Mol. Syst. Biol.* 12:872. doi: 10.15252/msb.20156777
- Chen, A. H., and Silver, P. A. (2012). Designing biological compartmentalization. *Trends Cell Biol.* 22, 662–670. doi: 10.1016/j.tcb.2012.07.002
- Chen, Y. Y., Jensen, M. C., and Smolke, C. D. (2010). Genetic control of mammalian T-cell proliferation with synthetic RNA regulatory systems. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8531–8536. doi: 10.1073/pnas.10017 21107
- Christen, E. H., Karlsson, M., Kämpf, M. M., Schoenmakers, R., Gübeli, R. J., Wischhusen, H. M., et al. (2011). Conditional DNA-protein interactions confer stimulus-sensing properties to biohybrid materials. *Adv. Funct. Mater.* 21, 2861–2867. doi: 10.1002/adfm.201100731
- Collins, I., Wang, H., Caldwell, J. J., and Chopra, R. (2017). Chemical approaches to targeted protein degradation through modulation of the ubiquitin-proteasome pathway. *Biochem. J.* 474, 1127–1147. doi: 10.1042/BCJ20160762
- Comeau, M.-A., Lafontaine, D. A., and Abou Elela, S. (2016). The catalytic efficiency of yeast ribonuclease III depends on substrate specific product release rate. *Nucleic Acids Res.* 44, 7911–7921. doi: 10.1093/nar/gkw507
- Cora, D., Re, A., Caselle, M., and Bussolino, F. (2017). MicroRNA-mediated regulatory circuits: outlook and perspectives. *Phys. Biol.* 14:045001. doi: 10.1088/1478-3975/aa6f21
- Costales, M. G., Haga, C. L., Velagapudi, S. P., Childs-Disney, J. L., Phinney, D. G., and Disney, M. D. (2017). Small molecule inhibition of microRNA-210 reprograms an oncogenic hypoxic circuit. J. Am. Chem. Soc. 139, 3446–3455. doi: 10.1021/jacs.6b11273
- Courbet, A., Endy, D., Renard, E., Molina, F., and Bonnet, J. (2015). Detection of pathological biomarkers in human clinical samples via

amplifying genetic switches and logic gates. *Sci. Transl. Med.* 7:289ra83. doi: 10.1126/scitranslmed.aaa3601

- Cronin, M., Akin, A. R., Collins, S. A., Meganck, J., Kim, J.-B., Baban, C. K., et al. (2012). High resolution *in vivo* bioluminescent imaging for the study of bacterial tumour targeting. *PLoS ONE* 7:e30940. doi: 10.1371/journal.pone.0030940
- Culler, S. J., Hoff, K. G., and Smolke, C. D. (2010). Reprogramming cellular behavior with RNA controllers responsive to endogenous proteins. *Science* 330, 1251–1255. doi: 10.1126/science.1192128
- Danino, T., Prindle, A., Kwong, G. A., Skalak, M., Li, H., Allen, K., et al. (2015). Programmable probiotics for detection of cancer in urine. *Sci. Transl. Med.* 7:289ra84. doi: 10.1126/scitranslmed.aaa3519
- Davidsohn, N., Beal, J., Kiani, S., Adler, A., Yaman, F., Li, Y., et al. (2015). Accurate predictions of genetic circuit behavior from part characterization and modular composition. ACS Synth. Biol. 4, 673–681. doi: 10.1021/sb500263b
- Di Stasi, A., Tey, S.-K., Dotti, G., Fujita, Y., Kennedy-Nasser, A., Martinez, C., et al. (2011). Inducible apoptosis as a safety switch for adoptive cell therapy. *N. Engl. J. Med.* 365, 1673–1683. doi: 10.1056/NEJMoa1106152
- Douglas, S. M., Bachelet, I., and Church, G. M. (2012). A logic-gated nanorobot for targeted transport of molecular payloads. *Science* 335, 831–834. doi: 10.1126/science.1214081
- Duong, C. P. M., Yong, C. S. M., Kershaw, M. H., Slaney, C. Y., and Darcy, P. K. (2015). Cancer immunotherapy utilizing gene-modified T cells: from the bench to the clinic. *Mol. Immunol.* 67, 46–57. doi: 10.1016/j.molimm.2014.12.009
- Duportet, X., Wroblewska, L., Guye, P., Li, Y., Eyquem, J., Rieders, J., et al. (2014). A platform for rapid prototyping of synthetic gene networks in mammalian cells. *Nucleic Acids Res.* 42, 13440–13451. doi: 10.1093/nar/gku1082
- Edwardson, T. G. W., Lau, K. L., Bousmail, D., Serpell, C. J., and Sleiman, H. F. (2016). Transfer of molecular recognition information from DNA nanostructures to gold nanoparticles. *Nat. Chem.* 8, 162–170. doi: 10.1038/nchem.2420
- Elowitz, M., and Lim, W. A. (2010). Build life to understand it. *Nature* 468, 889–890. doi: 10.1038/468889a
- Endo, K., Hayashi, K., Inoue, T., and Saito, H. (2013). A versatile cis-acting inverter module for synthetic translational switches. *Nat. Commun.* 4:2393. doi: 10.1038/ncomms3393
- Endy, D. (2005). Foundations for engineering biology. Nature 438, 449–453. doi: 10.1038/nature04342
- Farokhzad, O. C., Cheng, J., Teply, B. A., Sherifi, I., Jon, S., Kantoff, P. W., et al. (2006). Targeted nanoparticle-aptamer bioconjugates for cancer chemotherapy *in vivo. Proc. Natl. Acad. Sci. U.S.A.* 103, 6315–6320. doi: 10.1073/pnas.0601755103
- Fern, J., and Schulman, R. (2017). Design and characterization of DNA stranddisplacement circuits in serum-supplemented cell medium. ACS Synth. Biol. doi: 10.1021/acssynbio.7b00105. [Epub ahead of print].
- Ferreira, J. P., Overton, K. W., and Wang, C. L. (2013). Tuning gene expression with synthetic upstream open reading frames. *Proc. Natl. Acad. Sci. U.S.A.* 110, 11284–11289. doi: 10.1073/pnas.1305590110
- Ferry, Q. R. V., Lyutova, R., and Fulga, T. A. (2017). Rational design of inducible CRISPR guide RNAs for de novo assembly of transcriptional programs. *Nat. Commun.* 8:14633. doi: 10.1038/ncomms14633
- Firman, K., Evans, L., and Youell, J. (2012). A synthetic biology project developing a single-molecule device for screening drug-target interactions. *FEBS Lett.* 586, 2157–2163. doi: 10.1016/j.febslet.2012.01.057
- Fischbach, M. A., Bluestone, J. A., and Lim, W. A. (2013). Cell-based therapeutics: the next pillar of medicine. *Sci. Transl. Med.* 5:179ps7. doi: 10.1126/scitranslmed.3005568
- Foster, A.E., Mahendravada, A., Shinners, N. P., Chang, W.-C., Crisostomo, J., Lu, A., et al. (2017). Regulated expansion and survival of chimeric antigen receptormodified t cells using small molecule-dependent inducible MyD88/CD40. *Mol. Ther.* doi: 10.1016/j.ymthe.2017.06.014. [Epub ahead of print].
- Friedland, A. E., Lu, T. K., Wang, X., Shi, D., Church, G., and Collins, J. J. (2009). Synthetic gene networks that count. *Science* 324, 1199–1202. doi: 10.1126/science.1172005
- Fu, X., Liu, Y., Zhuang, C., Liu, L., Cai, Z., and Huang, W. (2015). Synthetic artificial microRNAs targeting UCA1-MALAT1 or c-Myc inhibit malignant phenotypes of bladder cancer cells T24 and 5637. *Mol. Biosyst.* 11, 1285–1289. doi: 10.1039/C5MB00127G

- Galdzicki, M., Clancy, K. P., Oberortner, E., Pocock, M., Quinn, J. Y., Rodriguez, C. A., et al. (2014). The Synthetic Biology Open Language (SBOL) provides a community standard for communicating designs in synthetic biology. *Nat. Biotechnol.* 32, 545–550. doi: 10.1038/nbt.2891
- Gardner, T. S., Cantor, C. R., and Collins, J. J. (2000). Construction of a genetic toggle switch in *Escherichia coli*. Nature 403, 339–342. doi: 10.1038/35002131
- Gertz, J., Siggia, E. D., and Cohen, B. A. (2009). Analysis of combinatorial cis-regulation in synthetic and genomic promoters. *Nature* 457, 215–218. doi: 10.1038/nature07521
- Gonzalez-Nicolini, V., Fux, C., and Fussenegger, M. (2004). A novel mammalian cell-based approach for the discovery of anticancer drugs with reduced cytotoxicity on non-dividing cells. *Invest. New Drugs* 22, 253–262. doi: 10.1023/B:DRUG.0000026251.00854.77
- Green, A. A., Kim, J., Ma, D., Silver, P. A., Collins, J. J., and Yin, P. (2017). Complex cellular logic computation using ribocomputing devices. *Nature* 548, 117–121. doi: 10.1038/nature23271
- Grilly, C., Stricker, J., Pang, W. L., Bennett, M. R., and Hasty, J. (2007). A synthetic gene network for tuning protein degradation in *Saccharomyces cerevisiae*. *Mol. Syst. Biol.* 3:127. doi: 10.1038/msb4100168
- Halman, J. R., Satterwhite, E., Roark, B., Chandler, M., Viard, M., Ivanina, A., et al. (2017). Functionally-interdependent shape-switching nanoparticles with controllable properties. *Nucleic Acids Res.* 45, 2210–2220. doi: 10.1093/nar/gkx008
- Hanson, S., Berthelot, K., Fink, B., McCarthy, J. E. G., and Suess, B. (2003). Tetracycline-aptamer-mediated translational regulation in yeast. *Mol. Microbiol.* 49, 1627–1637. doi: 10.1046/j.1365-2958.2003.03656.x
- Hockenberry, A. J., and Jewett, M. C. (2012). Synthetic in vitro circuits. Curr. Opin. Chem. Biol. 16, 253–259. doi: 10.1016/j.cbpa.2012.05.179
- Huh, J. H., Kittleson, J. T., Arkin, A. P., and Anderson, J. C. (2013). Modular design of a synthetic payload delivery device. ACS Synth. Biol. 2, 418–424. doi: 10.1021/sb300107h
- Isaacs, F. J., Dwyer, D. J., Ding, C., Pervouchine, D. D., Cantor, C. R., and Collins, J. J. (2004). Engineered riboregulators enable post-transcriptional control of gene expression. *Nat. Biotechnol.* 22, 841–847. doi: 10.1038/nbt986
- Jain, K. K. (2013). Synthetic biology and personalized medicine. *Med. Princ. Pract.* 22, 209–219. doi: 10.1159/000341794
- Kasap, C., Elemento, O., and Kapoor, T. M. (2014). DrugTargetSeqR: a genomicsand CRISPR-Cas9-based method to analyze drug targets. *Nat. Chem. Biol.* 10, 626–628. doi: 10.1038/nchembio.1551
- Katuri, J., Ma, X., Stanton, M. M., and Sánchez, S. (2017). Designing microand nanoswimmers for specific applications. Acc. Chem. Res. 50, 2–11. doi: 10.1021/acs.accounts.6b00386
- Ketzer, P., Haas, S. F., Engelhardt, S., Hartig, J. S., and Nettelbeck, D. M. (2012). Synthetic riboswitches for external regulation of genes transferred by replication-deficient and oncolytic adenoviruses. *Nucleic Acids Res.* 40:e167. doi: 10.1093/nar/gks734
- Khalil, A. S., Lu, T. K., Bashor, C. J., Ramirez, C. L., Pyenson, N. C., Joung, J. K., et al. (2012). A synthetic biology framework for programming eukaryotic transcription functions. *Cell* 150, 647–658. doi: 10.1016/j.cell.2012.05.045
- Kis, Z., Pereira, H. S., Homma, T., Pedrigi, R. M., and Krams, R. (2015). Mammalian synthetic biology: emerging medical applications. J. R. Soc. interf. 12:20141000. doi: 10.1098/rsif.2014.1000
- Kloss, C. C., Condomines, M., Cartellieri, M., Bachmann, M., and Sadelain, M. (2013). Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells. *Nat. Biotechnol.* 31, 71–75. doi: 10.1038/nbt.2459
- Kosuri, S., Goodman, D. B., Cambray, G., Mutalik, V. K., Gao, Y., Arkin, A. P., et al. (2013). Composability of regulatory sequences controlling transcription and translation in *Escherichia coli. Proc. Natl. Acad. Sci. U.S.A.* 110, 14024–14029. doi: 10.1073/pnas.1301301110
- Kudo, K., Imai, C., Lorenzini, P., Kamiya, T., Kono, K., Davidoff, A. M., et al. (2014). T lymphocytes expressing a CD16 signaling receptor exert antibody-dependent cancer cell killing. *Cancer Res.* 74, 93–103. doi: 10.1158/0008-5472.CAN-13-1365
- Kumaresan, P. R., Wang, Y., Saunders, M., Maeda, Y., Liu, R., Wang, X., et al. (2011). Rapid discovery of death ligands with one-bead-twocompound combinatorial library methods. ACS Comb. Sci. 13, 259–264. doi: 10.1021/co100069t

- Kwak, H., Hwang, I., Kim, J. H., Kim, M. Y., Yang, J. S., and Jeong, S. (2009). Modulation of transcription by the peroxisome proliferator-activated receptor delta–binding RNA aptamer in colon cancer cells. *Mol. Cancer Ther.* 8, 2664–2673. doi: 10.1158/1535-7163.MCT-09-0214
- Lebar, T., and Jerala, R. (2016). Benchmarking of TALE- and CRISPR/dCas9-based transcriptional regulators in mammalian cells for the construction of synthetic genetic circuits. ACS Synth. Biol. 5, 1050–1058. doi: 10.1021/acssynbio.5b00259
- Lee, E. S., Kim, D., Youn, Y. S., Oh, K. T., and Bae, Y. H. (2008). A virus-mimetic nanogel vehicle. Angew. Chem. Int. Ed. Engl. 47, 2418–2421. doi: 10.1002/anie.200704121
- Lee, H. K., Choi, Y. S., Park, Y. A., and Jeong, S. (2006). Modulation of oncogenic transcription and alternative splicing by beta-catenin and an RNA aptamer in colon cancer cells. *Cancer Res.* 66, 10560–10566. doi: 10.1158/0008-5472.CAN-06-2526
- Lee, N. S., Bertrand, E., and Rossi, J. (1999). mRNA localization signals can enhance the intracellular effectiveness of hammerhead ribozymes. *RNA* 5, 1200–1209. doi: 10.1017/S1355838299990246
- Li, Y., Idamakanti, N., Arroyo, T., Thorne, S., Reid, T., Nichols, S., et al. (2005). Dual promoter-controlled oncolytic adenovirus CG5757 has strong tumor selectivity and significant antitumor efficacy in preclinical models. *Clin. Cancer Res.* 11, 8845–8855. doi: 10.1158/1078-0432.CCR-05-1757
- Li, Y., Jiang, Y., Chen, H., Liao, W., Li, Z., Weiss, R., et al. (2015). Modular construction of mammalian gene circuits using TALE transcriptional repressors. *Nat. Chem. Biol.* 11, 207–213. doi: 10.1038/nchembio.1736
- Lienert, F., Lohmueller, J. J., Garg, A., and Silver, P. A. (2014). Synthetic biology in mammalian cells: next generation research tools and therapeutics. *Nat. Rev. Mol. Cell Biol.* 15, 95–107. doi: 10.1038/nrm3738
- Lim, W. A., and June, C. H. (2017). The principles of engineering immune cells to treat cancer. *Cell* 168, 724–740. doi: 10.1016/j.cell.2017.01.016
- Los, G. V., Encell, L. P., McDougall, M. G., Hartzell, D. D., Karassina, N., Zimprich, C., et al. (2008). HaloTag: a novel protein labeling technology for cell imaging and protein analysis. ACS Chem. Biol. 3, 373–382. doi: 10.1021/cb 800025k
- Lucks, J. B., Qi, L., Mutalik, V. K., Wang, D., and Arkin, A. P. (2011). Versatile RNA-sensing transcriptional regulators for engineering genetic networks. *Proc. Natl. Acad. Sci. U.S.A.* 108, 8617–8622. doi: 10.1073/pnas.1015741108
- Mohammadi, P., Beerenwinkel, N., and Benenson, Y. (2017). Automated design of synthetic cell classifier circuits using a two-step optimization strategy. *Cell Syst.* 4, 207.e14–218.e14. doi: 10.1016/j.cels.2017.01.003
- Morel, M., Shtrahman, R., Rotter, V., Nissim, L., and Bar-Ziv, R. H. (2016). Cellular heterogeneity mediates inherent sensitivity-specificity tradeoff in cancer targeting by synthetic circuits. *Proc. Natl. Acad. Sci. U.S.A.* 113, 8133–8138. doi: 10.1073/pnas.1604391113
- Mukherji, S., and van Oudenaarden, A. (2009). Synthetic biology: understanding biological design from synthetic circuits. *Nat. Rev. Genet.* 10, 859–871. doi: 10.1038/nrg2697
- Mutalik, V. K., Qi, L., Guimaraes, J. C., Lucks, J. B., and Arkin, A. P. (2012). Rationally designed families of orthogonal RNA regulators of translation. *Nat. Chem. Biol.* 8, 447–454. doi: 10.1038/nchembio.919
- Niederholtmeyer, H., Stepanova, V., and Maerkl, S. J. (2013). Implementation of cell-free biological networks at steady state. *Proc. Natl. Acad. Sci. U.S.A.* 110, 15985–15990. doi: 10.1073/pnas.1311166110
- Nielsen, A. A. K., Der, B. S., Shin, J., Vaidyanathan, P., Paralanov, V., Strychalski, E. A., et al. (2016). Genetic circuit design automation. *Science* 352:aac7341. doi: 10.1126/science.aac7341
- Nissim, L., and Bar-Ziv, R. H. (2010). A tunable dual-promoter integrator for targeting of cancer cells. *Mol. Syst. Biol.* 6:444. doi: 10.1038/msb.2010.99
- Ogawa, A., and Maeda, M. (2008). An artificial aptazyme-based riboswitch and its cascading system in *E. coli. Chembiochem* 9, 206–209. doi: 10.1002/cbic.200700478
- Oishi, M., Hayashi, H., Iijima, M., and Nagasaki, Y. (2007). Endosomal release and intracellular delivery of anticancer drugs using pH-sensitive PEGylated nanogels. J. Mater. Chem. 17, 3720–3725. doi: 10.1039/B706973A
- Qi, L. S., Larson, M. H., Gilbert, L. A., Doudna, J. A., Weissman, J. S., Arkin, A. P., et al. (2013). Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 152, 1173–1183. doi: 10.1016/j.cell.2013.02.022

- Rackham, O., and Chin, J. W. (2005). A network of orthogonal ribosome x mRNA pairs. Nat. Chem. Biol. 1, 159–166. doi: 10.1038/nchembio719
- Re, A., Waldron, L., and Quattrone, A. (2016). Control of gene expression by RNA binding protein action on alternative translation initiation sites. *PLoS Comput. Biol.* 12:e1005198. doi: 10.1371/journal.pcbi.1005198
- Rinaudo, K., Bleris, L., Maddamsetti, R., Subramanian, S., Weiss, R., and Benenson, Y. (2007). A universal RNAi-based logic evaluator that operates in mammalian cells. *Nat. Biotechnol.* 25, 795–801. doi: 10.1038/nbt1307
- Roybal, K. T., Rupp, L. J., Morsut, L., Walker, W. J., McNally, K. A., Park, J. S., et al. (2016). Precision tumor recognition by T Cells with combinatorial antigen-sensing circuits. *Cell* 164, 770–779. doi: 10.1016/j.cell.2016.01.011
- Rubens, J. R., Selvaggio, G., and Lu, T. K. (2016). Synthetic mixed-signal computation in living cells. Nat. Commun. 7:11658. doi: 10.1038/ncomms11658
- Ryan, P. C., Jakubczak, J. L., Stewart, D. A., Hawkins, L. K., Cheng, C., Clarke, L. M., et al. (2004). Antitumor efficacy and tumor-selective replication with a single intravenous injection of OAS403, an oncolytic adenovirus dependent on two prevalent alterations in human cancer. *Cancer Gene Ther.* 11, 555–569. doi: 10.1038/sj.cgt.7700735
- Ryu, J., and Park, S-H. (2015). Simple synthetic protein scaffolds can create adjustable artificial MAPK circuits in yeast and mammalian cells. *Sci Signal.* 8:ra66. doi: 10.1126/scisignal.aab3397
- Saito, H., Fujita, Y., Kashida, S., Hayashi, K., and Inoue, T. (2011). Synthetic human cell fate regulation by protein-driven RNA switches. *Nat. Commun.* 2:160. doi: 10.1038/ncomms1157
- Saito, H., Kobayashi, T., Hara, T., Fujita, Y., Hayashi, K., Furushima, R., et al. (2010). Synthetic translational regulation by an L7Ae-kink-turn RNP switch. *Nat. Chem. Biol.* 6, 71–78. doi: 10.1038/nchembio.273
- Salis, H. M., Mirsky, E. A., and Voigt, C. A. (2009). Automated design of synthetic ribosome binding sites to control protein expression. *Nat. Biotechnol.* 27, 946–950. doi: 10.1038/nbt.1568
- Sanjana, N. E., Cong, L., Zhou, Y., Cunniff, M. M., Feng, G., and Zhang, F. (2012). A transcription activator-like effector toolbox for genome engineering. *Nat. Protoc.* 7, 171–192. doi: 10.1038/nprot.2011.431
- Schaerli, Y., Gili, M., and Isalan, M. (2014). A split intein T7 RNA polymerase for transcriptional AND-logic. *Nucleic Acids Res.* 42, 12322–12328. doi: 10.1093/nar/gku884
- Schreiber, J., Arter, M., Lapique, N., Haefliger, B., and Benenson, Y. (2016). Modelguided combinatorial optimization of complex synthetic gene networks. *Mol. Syst. Biol.* 12:899. doi: 10.15252/msb.20167265
- Segall-Shapiro, T. H., Meyer, A. J., Ellington, A. D., Sontag, E. D., and Voigt, C. A. (2014). A "resource allocator" for transcription based on a highly fragmented T7 RNA polymerase. *Mol. Syst. Biol.* 10:742. doi: 10.15252/msb.20145299
- Shen, S., Rodrigo, G., Prakash, S., Majer, E., Landrain, T. E., Kirov, B., et al. (2015). Dynamic signal processing by ribozyme-mediated RNA circuits to control gene expression. *Nucleic Acids Res.* 43, 5158–5170. doi: 10.1093/nar/gkv287
- Siuti, P., Yazbek, J., and Lu, T. K. (2013). Synthetic circuits integrating logic and memory in living cells. Nat. Biotechnol. 31, 448–452. doi: 10.1038/nbt.2510
- Soundararajan, S., Chen, W., Spicer, E. K., Courtenay-Luck, N., and Fernandes, D. J. (2008). The nucleolin targeting aptamer AS1411 destabilizes Bcl-2 messenger RNA in human breast cancer cells. *Cancer Res.* 68, 2358–2365. doi: 10.1158/0008-5472.CAN-07-5723
- Stoltenburg, R., Reinemann, C., and Strehlitz, B. (2007). SELEX–a (r)evolutionary method to generate high-affinity nucleic acid ligands. *Biomol. Eng.* 24, 381–403. doi: 10.1016/j.bioeng.2007.06.001
- Stuart, M. A. C., Huck, W. T. S., Genzer, J., Müller, M., Ober, C., Stamm, M., et al. (2010). Emerging applications of stimuli-responsive polymer materials. *Nat. Mater.* 9, 101–113. doi: 10.1038/nmat2614
- Tai, W., and Gao, X. (2016). Functional peptides for siRNA delivery. Adv. Drug Deliv. Rev. 110–111, 157–168. doi: 10.1016/j.addr.2016.08.004
- Tian, Y., Li, S., Song, J., Ji, T., Zhu, M., Anderson, G. J., et al. (2014). A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. *Biomaterials* 35, 2383–2390. doi: 10.1016/j.biomaterials.2013.11.083
- Trosset, J.-Y., and Carbonell, P. (2013). Synergistic synthetic biology: units in concert. Front. Bioeng. Biotechnol. 1:11. doi: 10.3389/fbioe.2013.00011
- Trosset, J.-Y., and Carbonell, P. (2015). Synthetic biology for pharmaceutical drug discovery. Drug Des. Devel. Ther. 9, 6285–6302. doi: 10.2147/DDDT.S58049

- Truong, D-J. J., Kühner, K., Kühn, R., Werfel, S., Engelhardt, S., Wurst, W., et al. (2015). Development of an intein-mediated split-Cas9 system for gene therapy. *Nucleic Acids Res.* 43, 6450–6458. doi: 10.1093/nar/gkv601
- Vazquez-Anderson, J., and Contreras, L. M. (2013). Regulatory RNAs: charming gene management styles for synthetic biology applications. *RNA Biol.* 10, 1778–1797. doi: 10.4161/rna.27102
- Velagapudi, S. P., Luo, Y., Tran, T., Haniff, H. S., Nakai, Y., Fallahi, M., et al. (2017). Defining RNA-Small Molecule Affinity Landscapes Enables Design of a Small Molecule Inhibitor of an Oncogenic Noncoding, RNA. ACS Cent. Sci. 3, 205–216. doi: 10.1021/acscentsci.7b00009
- Wagner, H. J., Sprenger, A., Rebmann, B., and Weber, W. (2016). Upgrading biomaterials with synthetic biological modules for advanced medical applications. Adv. Drug Deliv. Rev. 105, 77–95. doi: 10.1016/j.addr.2016.05.004
- Wang, J., Zheng, Y., and Zhao, M. (2016). Exosome-based cancer therapy: implication for targeting cancer stem cells. *Front. Pharmacol.* 7:533. doi: 10.3389/fphar.2016.00533
- Wang, Y.-H., Wei, K. Y., and Smolke, C. D. (2013). Synthetic biology: advancing the design of diverse genetic systems. Annu. Rev. Chem. Biomol. Eng. 4, 69–102. doi: 10.1146/annurev-chembioeng-061312-103351
- Wei, P., Wong, W. W., Park, J. S., Corcoran, E. E., Peisajovich, S. G., Onuffer, J. J., et al. (2012). Bacterial virulence proteins as tools to rewire kinase pathways in yeast and immune cells. *Nature* 488, 384–388. doi: 10.1038/nature 11259
- Weinberg, B. H., Pham, N. T. H., Caraballo, L. D., Lozanoski, T., Engel, A., Bhatia, S., et al. (2017). Large-scale design of robust genetic circuits with multiple inputs and outputs for mammalian cells. *Nat. Biotechnol.* 35, 453–462. doi: 10.1038/nbt.3805
- Werstuck, G., and Green, M. R. (1998). Controlling gene expression in living cells through small molecule-RNA interactions. *Science* 282, 296–298. doi: 10.1126/science.282.5387.296
- Wilkie, S., van Schalkwyk, M. C. I., Hobbs, S., Davies, D. M., van der Stegen, S. J. C., Pereira, A. C. P., et al. (2012). Dual targeting of ErbB2 and MUC1 in breast cancer using chimeric antigen receptors engineered to provide complementary signaling. J. Clin. Immunol. 32, 1059–1070. doi: 10.1007/s10875-012-9689-9

- Win, M. N., Liang, J. C., and Smolke, C. D. (2009). Frameworks for programming biological function through RNA parts and devices. *Chem. Biol.* 16, 298–310. doi: 10.1016/j.chembiol.2009.02.011
- Win, M. N., and Smolke, C. D. (2007). A modular and extensible RNA-based gene-regulatory platform for engineering cellular function. *Proc. Natl. Acad. Sci. U.S.A.* 104, 14283–14288. doi: 10.1073/pnas.0703961104
- Wroblewska, L., Kitada, T., Endo, K., Siciliano, V., Stillo, B., Saito, H., et al. (2015). Mammalian synthetic circuits with RNA binding proteins for RNA-only delivery. *Nat. Biotechnol.* 33, 839–841. doi: 10.1038/nbt.3301
- Xie, Z., Wroblewska, L., Prochazka, L., Weiss, R., and Benenson, Y. (2011). Multiinput RNAi-based logic circuit for identification of specific cancer cells. *Science* 333, 1307–1311. doi: 10.1126/science.1205527
- Yao, Y., Jin, S., Long, H., Yu, Y., Zhang, Z., Cheng, G., et al. (2015). RNAe: an effective method for targeted protein translation enhancement by artificial non-coding RNA with SINEB2 repeat. *Nucleic Acids Res.* 43:e58. doi: 10.1093/nar/gkv125
- Yoo, J.-W., Irvine, D. J., Discher, D. E., and Mitragotri, S. (2011). Bio-inspired, bioengineered and biomimetic drug delivery carriers. *Nat. Rev. Drug Discov.* 10, 521–535. doi: 10.1038/nrd3499
- Zucchelli, S., Cotella, D., Takahashi, H., Carrieri, C., Cimatti, L., Fasolo, F., et al. (2015). SINEUPs: a new class of natural and synthetic antisense long non-coding RNAs that activate translation. *RNA Biol.* 12, 771–779. doi: 10.1080/15476286.2015.1060395

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Mouse Hospital and Its Integration in Ultra-Precision **Approaches to Cancer Care**

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Precision medicine holds real promise for the treatment of cancer. Adapting therapeutic strategies so patients receive individualized treatment protocols, will transform how diseases like cancer are managed. Already, molecular profiling technologies have provided unprecedented capacity to characterize tumors, yet the ability to translate this to actionable outcome in the clinic is limited. To enable real time translation of personalized therapeutic approaches to patient care in a co-clinical manner will require the adoption and integration of approaches that facilitate modeling of patient disease. The Mouse Hospital represents an approach that is ideally suited to pre- and co-clinical evaluation of novel therapeutic strategies for clinical care. Patient derived xenograft (PDX) technologies and in situ tumor modeling approaches using genetically engineered mouse models (GEMMs) already have a proven capacity to mimic human tumor responses, and their application can deliver invaluable insights into appropriate clinical approaches for individual patients by mirroring human clinical trials using a Co-Clinical Trial project and Mouse Hospital infrastructure. Additionally, the integration of the Mouse Hospital with other emerging technologies for the application of precision medicines, including organoid technologies, provides a platform that enables medical centers to truly reap the benefits that precision medicine has to offer.

Keywords: mouse models, PDX, precision medicine, cancer, Mouse Hospital, Co-Clinical Trial

Precision medicine has long been lauded to deliver the cure and eradication of diseases such as cancer, tailoring treatments to the specific genetics and needs of the patient. Indeed, the technological advances that we have seen over the 20-30 years have allowed us to profile and characterize patients and tumors to an unprecedented level, enabling a detailed mapping of genomic alterations and characterization of mutations observed in disease. Yet, the translation of these findings and approaches to the care and treatment of individual patients falls far behind the trailblazing advances in the technology that individualizes tumors. Much of this lag in translation to the clinic, lies in the historical approaches and methods in place for the testing and clinical evaluation of agents to be brought to the clinic, with a lack of infrastructure to facilitate translational studies in academic medical centers.

Our lab has pioneered the development and implementation of The Mouse Hospital and Co-Clinical Trial Project (1-4). This concept offers a mechanism by which tailoring of treatments and design of patient specific therapies can be rapidly evaluated. The Mouse Hospital encapsulates an infrastructure by which mouse trials can be carried out in a manner that mimics human trials

OPEN ACCESS

Edited by:

Alessandro Quattrone, University of Trento, Italy

Reviewed by:

Hatem E. Sabaawy, Rutgers University, The State University of New Jersey, United States Lawrence Schook University of Illinois at Urbana-Champaign, United States

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Specialty section:

This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

> Received: 22 February 2018 Accepted: 06 August 2018 Published: 28 August 2018

Citation:

Clohessy JG and Pandolfi PP (2018) The Mouse Hospital and Its Integration in Ultra-Precision Approaches to Cancer Care. Front. Oncol. 8:340. doi: 10.3389/fonc.2018.00340

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and treatments. In this setting, resources including imaging, treatment and pathology mirror human resources, and are integrated with standardized operating procedures and ongoing training of technical staff to ensure best practice and provide a recognized "standard of care" in mice that mimics human treatments. This in turn enables Co-Clinical Trials to be carried out in mice, whereby concurrent human/mouse trials mimic and inform one another. However, such an approach requires a number of important considerations from a practical perspective (3), and its integration within the context of a clinical trial and translational framework to benefit patients requires careful consideration. Indeed, the variety of models and their application offer a number of different and unique approaches that can be adopted and tailored to patient needs, and should be considered in the context of other precision medicine based technologies that offer the potential to identify unique therapeutic protocols for patient treatments. Here we outline key elements of the Mouse Hospital and the Co-Clinical Trial approach that can meet the needs of precision medicine, and discuss the challenges facing these approaches that need to be met to facilitate routine incorporation and utilization to deliver superior cancer patient care.

MODELING PATIENTS IN MICE

For the purposes of modeling human cancer in mice, there are currently two predominant approaches utilized. One represents the growth and expansion of tumor tissue in immunocompromised mice in a patient derived xenograft (PDX) or avatar setting, while the other represents genetically engineered mouse models (GEMMs), whereby the mouse genome is engineered to harbor key genetic alterations to drive tumor development in situ for the purposes of following tumor initiation and progression (5, 6). PDX tumor models have the advantage of studying human tumors themselves, enabling the expansion and evaluation of multiple single agent and combination therapies, however their immune compromised state fails to fully recapitulate the tumor microenvironment within which tumors exist. Although GEMMs may not always fully recapitulate the full heterogeneity and complex genetics of human patients, they do have the advantage of their in situ localization, and enable study of evolution and immune related function on tumor growth, progression and response to therapy. This is of particular importance in the context of immune-therapies, which represent a rapidly growing area for therapeutic intervention in many cancer types, and where novel immune targeting therapies require pre-clinical evaluation (7). Indeed, although both PDX and GEMM models have provided important tools for the study of human cancer, there are key challenges that still need to be met in order to provide a more robust and useful platform for integration with clinical studies.

While PDX models offer the opportunity to uniquely match individual patients with mouse avatars for evaluation of drug response to their unique tumor, not all patient tumors grow and progress in a xenograft setting (8–10). In addition, orthotopic

vs. subcutaneous tumor implantation for development and propagation remains an issue of discussion (8, 10). Much of the work to date has focused on subcutaneous propagation of PDX tumors, providing easy access to follow tumor growth and monitor response to treatment, and while there is evidence that orthotopic propagation of PDX tumors may facilitate some tumor types, this frequently requires much greater technical expertise, and does not always lend easily to enrollment of tumors at similar stages, and the longitudinal monitoring of individual tumor types. More recently, evidence highlighting the limitations of PDX models to faithfully model human tumors has demonstrated that propagation of human tumors in mice can result in a distinct evolution of these tumors (11). Indeed, while this study noted that the degree of genetic instability between human tumors and PDX models shares similarities, the distinct copy number alterations (CNAs) that occur in the evolution of human vs. PDX tumors highlights how the murine environment promotes clonal selection distinct from that occurring in patients (11). This may have important implications for the reliability of PDX tumors as avatars for human disease and their use in co-clinical studies, particularly as arm-level CNAs can be associated with drug response, and clonal selection resulting from PDX propagation can impact CNAs present, and in turn influence therapeutic outcomes (11).

GEMM models have their own particular challenges and are somewhat limited by the number of genetic alterations and time required for development of tumors in vivo. This frequently prevents GEMM models from acting as individual patient avatars, but they approximate patients based on key genetic drivers or modifiers for a particular cancer type. However, the emerging role of immune cell types in cancer has highlighted the need for models to study and understand the relationship in cancer (7). Particularly in the context of therapy where agents targeting immune cells are emerging as key elements for cancer therapy, and increasing relevance of cancer vaccines in maintaining remission and preventing recurrent disease is gaining momentum (12-14). Indeed, GEMM models are also now highlighting how the genetics of the tumor can influence the immune landscape of tumors, and in doing so influence the tumor biology (15). However, adaption and refinement of GEMMs is required to better facilitate pre- and co-clinical trials in the context of the mouse hospital so as to provide a more off-the-shelf approach for their utilization and application to real-time patient trial integration. Abilities to more easily modify genomes utilizing CRISPR genome editing approaches are facilitating this transition, and new opportunities for the development of models and their application are emerging (16-20).

Thus, PDX and GEMMs models complement each other in what they have to offer the cancer patient. A combination of efforts that take into account the patients own tumor, with its heterogeneity and complex genetics, in addition to a more simplified and focused model that looks to the main genetic drivers to account for generalizations amongst tumor types, and that take advantage of both immune-compromised and -competent settings.

INTEGRATING MOUSE STUDIES WITH CLINICAL CARE

While efforts aimed at improving models to provide enhancement in their application to uncovering novel therapeutic approaches for cancer is ongoing, how these models inform patient care, and how they are integrated into the precision medicine framework is also of relevance (**Figure 1**). This requires integration of mouse modeling approaches with existing technologies that have been built to support patient care in the context of precision medicine. Of particular relevance are areas of cellular profiling related to DNA and RNA sequencing, proteomic, and metabolic analyses, as well as culture of primary tissue explants from cancer patients.

In the context of molecular profiling, advances in computational approaches to defining individual patient drug resistance or sensitivity have greatly improved. Large publicly available datasets that include The Cancer Genome Atlas (TCGA), Cancer Target Discovery and Development (CTD²) database (21, 22), and the Cancer Cell Line Encyclopedia (CCLE) (23) have already demonstrated both the ability of molecular profiling to identify and hone in on key networks and pathways that provide insights on heterogeneity of tumors and facilitate identification of tumor sub-types, as well as highlighting how a central repository for datasets can facilitate analysis. Enabling data to be stored and input in a central repository can greatly facilitate co-clinical efforts involving multi-center co-clinical trials. Such a resource can extend beyond data sharing and analysis to also include shared protocols, relevant metadata, tools, and greatly facilitate research through web accessibility. Indeed, relevant DNA or transcriptome profiling for patients and models can enable comparison with big-data repositories to identify similarities with tumors already demonstrated to be sensitive or resistant to known chemotherapies or targeted agents (24). Such approaches facilitate the identification of focused therapeutic options for patients, and enable identification of targeted agents appropriate for defined genetic cancer types. In adapting such approaches, the evaluation and divergence of PDX models in particular from the original primary tumor should be followed, comparing CNA and transcriptomic profiles to account for clonal selection through PDX propagation. Indeed, it is possible that computational approaches can "correct" for responses in such situations, providing a statistical framework to facilitate translation of PDX response and outcome in co-clinical studies, to account for such divergence.

In general, while the long latency to generate and propagate PDX models for co-clinical studies provides a challenge for real-time application in this setting, organoid technologies are emerging as an efficient method by which to rapidly grow and expand primary tumors in culture. Current efforts to characterize these cultures as tumor models has highlighted their potential in study and evaluation of their representative patients (25-28). The ability to grow these primary tumors in vitro, enables a more high-throughput screening of individual patient tumors for sensitivity to drugs already approved for clinical use or under clinical evaluation. In addition, human organoid cultures can be utilized for the development of PDX tumors in vivo. and organoids derived from mouse primary tissues and cell types can provide useful models for human cancer (29-31). Thus, organoid approaches can facilitate with evaluation of tumor sensitivity to therapeutic agents, and enable the testing of



FIGURE 1 The precision medicine framework integrating The Mouse Hospital. The Mouse Hospital ideally is integrated in an ultra-precision medicine framework whereby data collected from computational and molecular profiling of patient tumors, in addition to screening and characterization of primary cancer organoid cultures, enable prioritization of novel therapeutic strategies for *in vivo* pre- and co-clinical evaluation. The integration of each of these approaches provides a comprehensive platform that can deliver actionable therapeutic strategies for individual patients that goes above and beyond what is currently available based on targeted genomic sequencing.

multiple combinations of therapeutic agents to identify potential therapeutic strategies for the treatment of individual cancers.

Combining these approaches as first line co-clinical efforts aids in optimally integrating mouse model approaches for precision medicine. Indeed, such *in silico* and *in vitro* analysis enables a well-defined prioritization of therapeutic strategies that can be evaluated and validated *in vivo*. This streamlines the use and application of mouse modeling approaches for translation of novel therapeutic strategies to the clinic, and enhances the effectiveness of *in vivo* translation. Such a pipeline represents an attractive model for the execution of precision medicine for cancer patients, going beyond a simple genetic or transcriptome profiling approach to stratify patients for therapy, to providing an ultra-precision platform that tailors treatments to provide the most optimal therapeutic strategy.

How co-clinical and clinical efforts are integrated and inform one another is also of relevance. The use of patient material for mouse related studies in the context of preand co-clinical requires approvals from both Institutional Review Boards (IRB) and Institutional Animal Care and Use Committees (IACUC). Additionally, challenges surrounding patient privacy and how data generated and analyzed are stored needs to be carefully considered, and protocols implemented need to adhere to appropriate HIPPA guidelines if such studies are to directly impact patient care. This requires that institutional infrastructures be in place to ensure that data are properly protected and patient identification only accessible by appropriate clinical staff. Similarly, while more general studies carried out using GEMM models or de-identified PDX models within a co-clinical setting to evaluate response to novel therapeutic agents or combinations thereof, it is crucial that therapeutic response in these models be carefully correlated with relevant response in human patients as outlined below. This frequently requires that individual models are carefully optimized to ensure standardized application of the model to anticipate therapeutic outcome.

ULTRA-PRECISION MOUSE MODELS FOR CANCER CARE

Although efforts to utilize mouse models in such an integrated ultra-precision platform is an attractive approach to maximize efficacy of data generated from in vivo studies, and provide effective clinical approaches to treat cancer patients, it is essential that strict procedures and protocols are in place to ensure reproducibility and reliability across the platform (3, 32). Currently there are no clear guidelines for how mouse models should be integrated into translational studies that directly impact patient therapy, and several studies highlighting issues concerning reproducibility across academic research demonstrate the need for systems that ensure the reliability of such data. Thus, it is essential that standard operating procedures (SOPs) are generated and in place to provide appropriate quality systems founded on good laboratory practices (GLP) that include detailed protocols, reporting and archiving to ensure all relevant data are recorded for reference and repeatability. It is also important that quality assurance units be included as part of the systems in place to ensure conformation with GLP. Such a GLP approach ensures uniformity and consistency in the performance of relevant studies, and facilitates evaluation of systems in place by regulatory authorities. Indeed, the Organization for Economic Co-operation and Development (OECD) already provides guidelines for testing and evaluation of chemicals that can be readily adapted for co-clinical use (33, 34). In addition, it is essential that appropriate education and training are provided to those carrying out such studies, and that records and data are appropriately maintained and archived. Ultimately, pre- and co-clinical studies involving mice will be carried out in a Clinical Laboratory Improvement Amendments (CLIA) approved environment to facilitate the approval and translation of studies from the bench to the bedside.

It is also important that such approaches be considered and evaluated by internal review boards (IRB), who oversee and approve ongoing clinical trial protocols within the academic medical setting. The ability to inform patient care in real-time, through precision medicine approaches offers unique opportunities for cancer patients, and translating novel therapeutic strategies to the clinical for individual patients based on a cohort of pre- and co-clinical studies requires careful evaluation to ensure patients are protected and offered treatments that truly represent best-option strategies for their specific cancer. In translating these results, the ability to match or predict how response in mouse models equates to a response in human patients is of great importance. The use and application of RECIST (Response Evaluation Criteria In Solid Tumors) and irRECIST (immune-related RECIST) criteria in patients has become an essential set of rules that define patient response to treatment. Equating responses in GEMM and PDX models to appropriate RECIST responses in patients can require optimization and may be developed through iterative processes, but can dramatically improve the clinical relevance of co-clinical studies. This requires that pilot studies be carried out to properly establish an appropriate treatment regime corresponding to patient treatments, which almost invariably includes upfront standard of care therapies. It is therefore important not to jumpstart the process by solely evaluating experimental therapies, but always evaluating and correlating mouse model response to standard of care as appropriate for the relevant clinical trial. This in turn can set clear criteria for evaluating response and subsequently be utilized to support the use of such models in clinical trial protocols, again however, clear criteria and GLP approaches are necessary to ensure reproducibility and reliability as outlined above.

As part of such an approach, it is imperative that drugs and therapies used in the pre- and co-clinical setting mimic as closely as possible those that will ultimately be administered to patients. However, it may not always be the case that such agents can be easily assessed, particularly in the case of GEMMs. Many human specific therapies, including biologics or small molecule inhibitors, demonstrate limited cross-reactivity or specificity for mouse targets, and thus lack of efficacy in such models requires that mouse specific reagents be generated (35). However, the enrollment of mouse models for the purposes for testing of novel therapeutic approaches can greatly facilitate evaluation of both targeted agents, and evaluation of combinatorial therapies. This can greatly aid rapid stratification and testing of multiple therapeutic options, in turn tailoring therapies for patients. Similarly, it is important to consider dosing strategies for corresponding mouse and human trials, and integration of mouse models can provide insights on differential dosage as well as metronomic therapy approaches for clinical application. Indeed, a number of studies have demonstrated the usefulness of mouse models in optimizing dosing strategies for patients to deliver more effective responses to standard cancer therapies (36, 37).

FUTURE DIRECTIONS

As outlined above, despite extensive advances in technologies that support cancer patients and the ability to characterize their unique cancer, there is a critical need to go beyond utilization of this resource as simply a diagnostic or prognostic tool. While currently such data provides an actionable therapeutic option in limited cases, often reserved for specific targetable mutations, all too frequently much of the information gleaned provides little therapeutic value (38). Thus, the integration of such data with computational and molecular databases, combined

REFERENCES

- Lunardi A, Ala U, Epping MT, Salmena L, Clohessy JG, Webster KA, et al. A co-clinical approach identifies mechanisms and potential therapies for androgen deprivation resistance in prostate cancer. *Nat Genet.* (2013) 45:747–55. doi: 10.1038/ng.2650
- Lunardi A, Pandolfi PP. A co-clinical platform to accelerate cancer treatment optimization. *Trends Mol Med.* (2015) 21:1–5. doi: 10.1016/j.molmed.2014.10.008
- Clohessy JG, de Stanchina E. Infrastructure needs for translational integration of mouse and human trials. *Cold Spring Harb Protoc.* (2013) 2013:pdb.top078782–1114. doi: 10.1101/pdb.top078782
- Clohessy JG, Pandolfi PP. Mouse hospital and co-clinical trial project-from bench to bedside. *Nat Rev Clin Oncol.* (2015) 12:491–8. doi: 10.1038/nrclinonc.2015.62
- Thakur Das M, Pryer NK, Singh M. Mouse tumour models to guide drug development and identify resistance mechanisms. *J Pathol.* (2014) 232:103–11. doi: 10.1002/path.4285
- Day C-P, Merlino G, van Dyke T. Preclinical mouse cancer models: a maze of opportunities and challenges. *Cell* (2015) 163:39–53. doi: 10.1016/j.cell.2015.08.068
- Zitvogel L, Pitt JM, Daillère R, Smyth MJ, Kroemer G. Mouse models in oncoimmunology. Nat Rev Cancer (2016) 16:759–73. doi: 10.1038/nrc.2016.91
- Williams JA. Using PDX for preclinical cancer drug discovery: the evolving field. J Clin Med. (2018) 7:41. doi: 10.3390/jcm7030041
- Gao H, Korn JM, Ferretti S, Monahan JE, Wang Y, Singh M, et al. High-throughput screening using patient-derived tumor xenografts to predict clinical trial drug response. *Nat Med.* (2015) 21:1318–25. doi: 10.1038/nm.3954
- Byrne AT, Alférez DG, Amant F, Annibali D, Arribas J, Biankin AV, et al. Interrogating open issues in cancer precision medicine with patient-derived xenografts. *Nat Rev Cancer* (2017) 17:254–68. doi: 10.1038/nrc.2016.140
- Ben-David U, Ha G, Tseng Y-Y, Greenwald NF, Oh C, Shih J, et al. Patientderived xenografts undergo mouse-specific tumor evolution. *Nat Genet.* (2017) 49:1567–75. doi: 10.1038/ng.3967

with *in vitro* screening and characterization of primary disease utilizing organoid technologies, can be readily translated to the clinic through *in vivo* validation using mouse models (2, 4, 39). It is also of paramount importance to include global genomic and transcriptomic analysis toward more accurate predictions, as well as for the identification of novel mechanisms of resistance as recent studies indicate (40). Development of such a platform to integrate patient and mouse hospitals through co-clinical studies provides a clear pipeline for delivery of ultra-precision solutions for individual cancer patients (41). Such an approach requires careful organization and set-up to ensure such models provide accurate insights for development of patient care strategies and represent a key component of precision medicine centers of the future.

AUTHOR CONTRIBUTIONS

JGC and PPP conceived, researched, and co-wrote the manuscript.

ACKNOWLEDGMENTS

The authors thank PPP laboratory members for critical discussions. PPP is supported by an NIH NCI R35 grant CA197529.

- Rosenblatt J, Stone RM, Uhl L, Neuberg D, Joyce R, Levine JD, et al. Individualized vaccination of AML patients in remission is associated with induction of antileukemia immunity and prolonged remissions. *Sci Transl Med.* (2016) 8:368ra171. doi: 10.1126/scitranslmed.aag1298
- Ott PA, Hu Z, Keskin DB, Shukla SA, Sun J, Bozym DJ, et al. An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature* (2017) 547:217–21. doi: 10.1038/nature22991
- Sahin U, Derhovanessian E, Miller M, Kloke B-P, Simon P, Löwer M, et al. Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature* (2017) 547:222–6. doi: 10.1038/nature 23003
- Bezzi M, Seitzer N, Ishikawa T, Reschke M, Chen M, Wang G, et al. Diverse genetic-driven immune landscapes dictate tumor progression through distinct mechanisms. *Nat Med.* (2018) 18:884. doi: 10.1038/nm.4463
- Pelletier S, Gingras S, Green DR. Mouse genome engineering via CRISPR-Cas9 for study of immune function. *Immunity* (2015) 42:18–27. doi: 10.1016/j.immuni.2015.01.004
- Tothova Z, Krill-Burger JM, Popova KD, Landers CC, Sievers QL, Yudovich D, et al. Multiplex CRISPR/Cas9-based genome editing in human hematopoietic stem cells models clonal hematopoiesis and myeloid neoplasia. *Cell Stem Cell* (2017) 21:547–55.e8. doi: 10.1016/j.stem.2017.07.015
- Guernet A, Mungamuri SK, Cartier D, Sachidanandam R, Jayaprakash A, Adriouch S, et al. CRISPR-barcoding for intratumor genetic heterogeneity modeling and functional analysis of oncogenic driver mutations. *Mol Cell* (2016) 63:526–38. doi: 10.1016/j.molcel.2016.06.017
- Annunziato S, Kas SM, Nethe M, Yücel H, Del Bravo J, Pritchard C, et al. Modeling invasive lobular breast carcinoma by CRISPR/Cas9-mediated somatic genome editing of the mammary gland. *Genes Dev.* (2016) 30:1470– 80. doi: 10.1101/gad.279190.116
- Roper J, Tammela T, Akkad A, Almeqdadi M, Santos SB, Jacks T, et al. Colonoscopy-based colorectal cancer modeling in mice with CRISPR-Cas9 genome editing and organoid transplantation. *Nat Protoc.* (2018) 13:217–34. doi: 10.1038/nprot.2017.136
- 21. Basu A, Bodycombe NE, Cheah JH, Price EV, Liu K, Schaefer GI, et al. An interactive resource to identify cancer genetic and lineage

dependencies targeted by small molecules. *Cell* (2013) 154:1151-61. doi: 10.1016/j.cell.2013.08.003

- Rees MG, Seashore-Ludlow B, Cheah JH, Adams DJ, Price EV, Gill S, et al. Correlating chemical sensitivity and basal gene expression reveals mechanism of action. *Nat Chem Biol.* (2016) 12:109–16. doi: 10.1038/nchembio.1986
- Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* (2012) 483:603–7. doi: 10.1038/nature11003
- Iorio F, Knijnenburg TA, Vis DJ, Bignell GR, Menden MP, Schubert M, et al. A landscape of pharmacogenomic interactions in cancer. *Cell* (2016) 166:740–54. doi: 10.1016/j.cell.2016.06.017
- Huang L, Holtzinger A, Jagan I, BeGora M, Lohse I, Ngai N, et al. Ductal pancreatic cancer modeling and drug screening using human pluripotent stem cell- and patient-derived tumor organoids. *Nat Med.* (2015) 21:1364–71. doi: 10.1038/nm.3973
- Gao D, Vela I, Sboner A, Iaquinta PJ, Karthaus WR, Gopalan A, et al. Organoid cultures derived from patients with advanced prostate cancer. *Cell* (2014) 159:176–87. doi: 10.1016/j.cell.2014.08.016
- van de Wetering M, Francies HE, Francis JM, Bounova G, Iorio F, Pronk A, et al. Prospective derivation of a living organoid biobank of colorectal cancer patients. *Cell* (2015) 161:933–45. doi: 10.1016/j.cell.2015.03.053
- Sachs N, de Ligt J, Kopper O, Gogola E, Bounova G, Weeber F, et al. A living biobank of breast cancer organoids captures disease heterogeneity. *Cell* (2018) 172:373–386.e10. doi: 10.1016/j.cell.2017.11.010
- Duarte AA, Gogola E, Sachs N, Barazas M, Annunziato S, de Ruiter J, et al. BRCA-deficient mouse mammary tumor organoids to study cancer-drug resistance. *Nature Methods* (2018) 15:134–40. doi: 10.1038/nmeth.4535
- Fumagalli A, Suijkerbuijk SJE, Begthel H, Beerling E, Oost KC, Snippert HJ, et al. A surgical orthotopic organoid transplantation approach in mice to visualize and study colorectal cancer progression. *Nat Protoc.* (2018) 13:235– 47. doi: 10.1038/nprot.2017.137
- Taguchi A, Nishinakamura R. Higher-order kidney organogenesis from pluripotent stem cells. *Cell Stem Cell* (2017) 21:730–746.e6. doi: 10.1016/j.stem.2017.10.011
- Benjamin D, Mandel DR, Kimmelman J. Can cancer researchers accurately judge whether preclinical reports will reproduce? *PLoS Biol.* (2017) 15:e2002212. doi: 10.1371/journal.pbio.2002212
- OECD. Test No. 453: Combined Chronic Toxicity/Carcinogenicity Studies, OECD Guidelines for the Testing of Chemicals, Section 4. Paris: OECD Publishing (2018).

- OECD. Test No. 452: Chronic Toxicity Studies, OECD Guidelines for the Testing of Chemicals, Section 4. Paris: OECD Publishing (2018).
- 35. Selby MJ, Engelhardt JJ, Johnston RJ, Lu L-S, Han M, Thudium K, et al. Preclinical development of ipilimumab and nivolumab combination immunotherapy: mouse tumor models, *in vitro* functional studies, and cynomolgus macaque toxicology. *PLoS ONE* (2016) 11:e0161779. doi: 10.1371/journal.pone.0161779
- 36. Hartley C, Elliott S, Begley CG, McElroy P, Sutherland W, Khaja R, et al. Kinetics of haematopoietic recovery after dose-intensive chemo/radiotherapy in mice: optimized erythroid support with darbepoetin alpha. Br J Haematol. (2003) 122:623–36. doi: 10.1046/j.1365-2141.2003. 04467.x
- Leder K, Pitter K, LaPlant Q, Hambardzumyan D, Ross BD, Chan TA, et al.. Mathematical modeling of PDGF-driven glioblastoma reveals optimized radiation dosing schedules. *Cell* (2014) 156:603–16. doi: 10.1016/j.cell.2013.12.029
- Roychowdhury S, Chinnaiyan AM. Translating cancer genomes and transcriptomes for precision oncology. CA Cancer J Clin. (2016) 66:75–88. doi: 10.3322/caac.21329
- Nardella C, Lunardi A, Patnaik A, Cantley LC, Pandolfi PP. The APL paradigm and the "co-clinical trial" project. *Cancer Discov.* (2011) 1:108–16. doi: 10.1158/2159-8290.CD-11-0061
- Bester AC, Lee JD, Chavez A, Lee Y-R, Nachmani D, Vora S, et al. An Integrated genome-wide CRISPRa approach to functionalize lncRNAs in drug resistance. *Cell* (2018) 173:649–664.e20. doi: 10.1016/j.cell.2018. 03.052
- Pauli C, Hopkins BD, Prandi D, Shaw R, Fedrizzi T, Sboner A, et al. Personalized *in vitro* and *in vivo* cancer models to guide precision medicine. *Cancer Discov.* (2017) 7:462–77. doi: 10.1158/2159-8290.CD-16-1154

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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