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Original

Role of ncrnas in modulation of liver fibrosis by extracellular vesicles / Chiabotto, G.; Camussi, G.; Bruno, S.. - In: EXRNA. - ISSN 2398-0060. - ELETTRONICO. - 2:AprilMayJune(2020). [10.1186/s41544-020-00050-5]

Availability:

This version is available at: 11583/2996377 since: 2025-01-08T13:19:46Z

Publisher:

BMC Springer

Published

DOI:10.1186/s41544-020-00050-5

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REVIEW

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Role of ncRNAs in modulation of liver fibrosis by extracellular vesicles



Giulia Chiabotto^{1*} , Giovanni Camussi¹ and Stefania Bruno^{1,2}

Abstract

Extracellular vesicles (EVs) are small membrane vesicles carrying bioactive lipids, proteins and nucleic acids of the cell of origin. In particular, EVs carry non-coding RNAs (ncRNAs) and the vesicle membrane may protect them from degradation. Once released within the extracellular space, EVs can transfer their cargo, including ncRNAs, to neighboring or distant cells, thus inducing phenotypical and functional changes that may be relevant in several physio-pathological conditions. This review provides an overview of the role of EV-carried ncRNAs in the modulation of liver fibrosis. In particular, we focused on EV-associated microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) involved into the development of liver fibrosis and on the potential use of EV-associated ncRNAs as diagnostic and prognostic biomarkers of liver fibrosis.

Keywords: miRNA, lncRNA, non coding RNA, extracellular vesicles, exosomes, microvesicles, hepatic stellate cells, liver fibrosis

Introduction

Hepatic fibrosis is a reversible wound-healing response in the liver that occurs in response to a chronic liver damage, such as alcoholic liver disease (ALD), non-alcoholic steatohepatitis (NASH), hepatitis B virus (HBV) and hepatitis C virus (HCV). If uncontrolled, progressive hepatic fibrosis can lead to cirrhosis, hepatocellular cancer (HCC) and end-stage liver disease. Liver fibrosis is caused by a protracted imbalance in extracellular matrix (ECM) production and degradation [1, 2]. The main source of ECM components in the development of liver fibrosis is provided by hepatic stellate cells (HSCs), which reside within an area between hepatocytes and sinusoidal endothelial cells, known as “space of Disse”, and represent approximately 8%-14% of the cells in the normal liver [3, 4]. Following liver injury, HSCs undergo an activation process and transform from quiescent, vitamine A-storing cells into highly proliferative, myofibroblast-like cells, upregulating the synthesis of alpha-1 type I collagen (Col1 α 1) and the expression of alpha

smooth muscle actin (α -SMA), which confers contractility and promotes wound closure [1].

HSC activation is a critical event during liver fibrosis and is controlled by several cytokines and growth factors. A contribution to all stages of chronic liver disease progression, from initial liver injury through inflammation and fibrosis, is provided by transforming growth factor beta (TGF- β) [5]. Liver injury induces the release of active TGF- β by damaged hepatocytes and inflammatory cells, thus upregulating the synthesis and release of several growth factors and cytokines involved in fibrogenesis, including platelet-derived growth factor (PDGF), connective tissue growth factor (CCN2), tumor necrosis factor alpha (TNF- α) and various interleukins (ILs), such as IL-1 β , and IL-6 [6–10]. Moreover, increased levels of TGF- β enhance hepatocyte destruction and mediate HSC activation, resulting in a wound-healing response that implies myofibroblasts generation and ECM deposition [5]. CCN2 is associated with the development of liver injury and its overexpression can lead to fibrosis aggravation [10]. TNF- α is involved in cholestasis-induced liver fibrosis [11]. IL-1 β stimulates the synthesis of ECM [12], while IL-6 induces collagen synthesis and exacerbates hepatic inflammation [13].

* Correspondence: giulia.chiabotto@unito.it

¹Department of Medical Sciences, University of Torino, Corso Dogliotti 14, 10126 Torino, Italy

Full list of author information is available at the end of the article



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A growing body of research has shown that the activation of HSCs is orchestrated by a complex regulatory machinery, which also involves the dysregulation of several non-coding RNAs (ncRNAs) [14]. In the last decade, the pivotal role of microRNAs (miRNAs) and long ncRNAs (lncRNAs) in the activation of HSCs and progression of liver fibrosis has been intensively described [15–17]. Circulating levels of ncRNAs have been recognized as potential biomarker for various diseases, including liver fibrosis [18, 19]. An important example of a biological carrier that can transport abundant quantities of ncRNAs between cells and different tissues is that of the extracellular vesicle (EV). EVs are nanometer-sized, membrane-bound vesicles released by cells in both physiological and pathological conditions, which are mainly involved in cell-to-cell communication.

In this review, we will present the current knowledge about the role of EV-associated ncRNAs in the onset of liver fibrosis and we will discuss their potential use as biomarkers of hepatic fibrosis.

Non-coding RNAs

Recently, advances in RNA microarrays and next-generation sequencing of transcriptomes, have led to identification of several ncRNAs associated with a number of pathological conditions, including liver fibrosis. NcRNAs can be divided into two major classes of molecules, according to their transcript size: lncRNAs, with more than 200 nucleotides, and short ncRNAs which contain less than 200 nucleotides and include miRNAs, small interfering RNA, small nuclear RNA, small nucleolar RNA, tRNA-derived fragments and piwi-interacting RNA (piRNA).

MicroRNAs (miRNAs)

MiRNAs are approximately 22 nucleotides in length and were first described in 1993 by Ambros and colleagues [20]. They act as negative regulators of gene expression by promoting mRNA degradation or post-transcriptionally repressing mRNA translation [21–25]. Interestingly, miRNAs not only exert their function intracellularly, but are also exported from cells associated with Ago2 protein [26], bound to high-density lipoproteins [27], or incorporated in extracellular vesicles (EVs) such as exosomes [28] or apoptotic bodies [29]. Since miRNAs are involved in the coordination of several biological processes, such as cell proliferation and differentiation, migration, apoptosis and metabolism [22, 30–33], alteration in their expression profiles are frequently related to various pathological processes, including liver diseases [34, 35]. Several studies pointed out the role of circulating miRNAs as biomarkers of hepatic diseases. For instance, increased levels of circulating miR-122, one of the most abundant miRNA expressed in hepatocytes, have been identified in viral

hepatitis [36–40], liver cirrhosis [41], drug-induced liver injury (DILI) [40, 42], HCC [43], ALD [40] and non-alcoholic fatty liver disease (NAFLD) [37, 44, 45]. Various miRNAs are implicated in the development of liver fibrosis. Since HSC activation is considered a critical initial step in hepatic fibrogenesis, interest has grown in the study of miRNAs that coordinate signaling pathways associated with myofibroblast activation, such as phosphatase and tensin homolog (PTEN)/Akt, epithelial-to-mesenchymal transition (EMT)/ERK1, nuclear factor kappa B (NF- κ B) and peroxisome proliferator-activated receptor-gamma (PPAR γ). One of the most studied anti-fibrogenic miRNAs is miR-29, which is downregulated by TGF- β in activated HSCs [46]. When overexpressed, miR-29 leads to decreased HSC proliferation and increased HSC apoptosis, via inhibition of phosphoinositide-3-kinase (PI3K)/AKT pathway [47]. The downregulation of miR-155 has also been observed in activated HSCs from patients with cirrhosis and its enhanced expression results in HSC apoptosis with the inhibition of the EMT process and the ERK1 signaling pathway [48]. One of the predominantly upregulated miRNAs during fibrogenesis is miR-21, which is expressed in HSCs after PDGF stimulation and is associated with decreased expression of PTEN and consequent activation of Akt [49]. Similar to miR-21, miR-181b is involved in PTEN/Akt signaling pathway, being upregulated in HSCs by TGF- β 1 and promoting increased Col1 α 1 synthesis and α -SMA expression [50]. During HSC activation, an inverse correlation has been described between PPAR γ and the expression of miR-33a, miR-34a and miR-34c. In fact, the inhibition of these three miRNAs increased PPAR γ expression, which in turn decreased the expression of α -SMA [51, 52]. Among the miRNAs involved in NF- κ B signaling pathway, miR-126, miR-221 and miR-222 are listed. MiR-126 overexpression in HSCs is associated with increased TGF- β 1 and type I collagen expression and reduced NF- κ B inhibitor alpha (I κ B α) levels, which lead to NF- κ B upregulation [53]. The expression of miR-221 and miR-222 in activated HSCs is inhibited by I κ B and enhanced by pro-fibrotic cytokines TGF- α and TNF- α [54].

Long non-coding RNAs (lncRNAs)

lncRNAs were first described in 2002 by Okazaki and colleagues [55], as a very heterogeneous population of ncRNA molecules with a size that varies from 200 nucleotides to 100 kilobases. As mRNAs, lncRNAs are transcribed by RNA polymerase II and then subjected to polyadenylation and splicing mechanisms [56]. Moreover, they can display a long open reading frame with more than 100 amino acids that usually characterizes protein-coding transcripts [57]. For these reasons, lncRNAs can be easily confused with protein-coding transcripts. However, unlike mRNAs, lncRNAs generally show low cellular expression levels, and exhibit tissue or

cell type-specific expression [58]. Despite the lack of a common method for lncRNA identification, lncRNAs are usually classified into six categories, according to their genomic localization: sense lncRNAs, antisense lncRNAs, bidirectional or divergent lncRNAs, intronic lncRNAs, intergenic lncRNAs and enhancer lncRNAs [59, 60]. Since lncRNAs can be found both in the nuclear and in the cytoplasmic fraction, they can be also distinguished based on their cellular localization into signal, decoy, guide and scaffold lncRNAs [60, 61]. In the nucleus, lncRNAs exert their effects on chromatin architecture by interacting with chromatin remodeling proteins, thus regulating transcriptional activity of neighboring genes in “cis”, or in “trans”, away from their site of synthesis [62–66]. In the cytoplasm, lncRNAs may bind one or multiple miRNAs and act as miRNA precursors, or as miRNA sponges, also known as competing endogenous RNAs (ceRNAs), to either increase or reduce their expression and function [67]. lncRNAs are mainly regulators of development, pluripotency and proliferation, while some of them may function as oncogenes or tumor suppressors [68–71]. Moreover, lncRNA expression may be associated with patho-physiological conditions, including liver diseases and hepatic fibrosis. lncRNA-Hotair contributes to the progression of liver fibrosis through a miR-29b-mediated epigenetically modulation of PTEN expression [72] and by acting as ceRNA of miR-148b, which coordinates the DNA methyltransferase 1 (DNMT1)/MEG3/p53 pathway in HSCs [73]. lncRNA-Hotair also recruits the polycomb repressing complex 2 (PRC2) to lncRNA-MEG3 promoter, thus contributing to MEG3 methylation-dependent downregulation, which has been reported in carbon tetrachloride (CCl₄)-induced mouse liver fibrosis models, human fibrotic livers and in human activated HSCs [73, 74]. The TET3-mediated downregulation of lncRNA-HIF1A-AS1 leads to enhanced HSC proliferation and reduced apoptosis [75]. Both lncRNA-PVT1 and lncRNA-APTR promote the activation of HSCs and the progression of liver fibrosis [76, 77]. lncRNA-PVT1 contributes to EMT process by competitively binding miR-152 and silencing PTCH1 expression [76], while lncRNA-APTR increases cell cycle progression and proliferation of HSCs by inhibiting p21 expression [77]. On the other hand, lncRNA-p21 overexpression prevents liver fibrogenesis by promoting the upregulation of p21 [78] and by sponging miR-17-5p [79], thus suppressing the Wnt/β-catenin pathway-mediated cell-cycle progression and proliferation in HSCs.

Circular RNAs (circRNAs)

Circular RNA (circRNA) is one of the latest areas of focus in the field of ncRNA research. It is a unique type of single-stranded lncRNA arranged in a closed loop structure, which is highly stable and resistant from RNA degrading enzymes [80, 81]. In recent years, increased

research has focused on the role of circRNAs as ceRNA or miRNA sponges, revealing their strong association with the development of various diseases and their possible use as diagnostic biomarkers [82–84]. The first report indicating an involvement of circRNAs in liver fibrosis comes from Chen and colleagues [85], who identified differentially expressed circRNAs associated with HSC activation. In a model of radiation-induced HSC activation, they found 179 upregulated and 630 downregulated circRNAs compared with quiescent HSCs. In a CCl₄-induced murine model of hepatic fibrosis, Zhou and colleagues have shown the differential expression of 69 circRNAs, of which 14 were upregulated and 55 downregulated in injured livers in respect to healthy controls [86]. In both experimental settings, bioinformatic analyses indicated that the dysregulated circRNAs may target miRNAs and proteins involved in the activation of HSCs, thus contributing to the development of liver fibrosis [85, 86]. Among the anti-fibrogenic circRNAs, hsa_circ_0007874 (also known as circ-MTO1) has proved to inhibit HSC activation and liver fibrogenesis by regulating miR-17-5p and Smad7 [87]. Instead, a positive correlation with liver fibrosis has been demonstrated for hsa_circ_0071410, since its inhibition attenuated irradiation-induced HSC activation by increasing the expression of miR-9-5p [85], mmu_circ_0000254 (also known as circ-PWWP2A) that contributed to HSC activation and proliferation by sponging miR-203 and miR-223 [88] and hsa_circ_100759 (also known as circ-4099) that increased H₂O₂-induced HSC injury by inhibiting miR-706 [89].

Extracellular vesicles

The term “EVs” refers to a heterogeneous population of lipid bilayer membrane-bound vesicles, originated from different sub-cellular compartments and released by cells into the environment in both physiological and pathological conditions. Due to their considerable heterogeneity and the lack of specific markers that could distinguish one type of vesicle from another, EVs are currently classified based on their size, biogenesis and secretion mechanisms [90, 91].

At present, the most considered EV populations are exosomes, ectosomes and apoptotic bodies. Exosomes are nano size vesicles (30–100 nm) with endosomal origin. They are released into the extracellular space following the inward and reverse budding of multivesicular bodies (MVBs)-membranes and their fusion with the cell surface [92]. This process, called exocytosis, is coordinated by the endosomal sorting complex required for transport (ESCRT) machinery that contributes to exosome formation by controlling ubiquitinated cargo selection (ESCRT-0), MVB intraluminal membrane budding (ESCRT-I and -II) and the scission from the endosomal membrane (ESCRT-III) [92–94]. The ESCRT machinery acts in coordination with auxiliary proteins involved in

exosome biogenesis and secretion, such as hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) [95, 96], tumor susceptibility gene 101 protein (TSG101) [96], apoptosis-linked gene-2 interacting protein X (ALIX) [94, 97] and vacuolar protein sorting-associated protein 4 (VPS4) [98]. However, exosome biogenesis could also occur through an ESCRT-independent mechanism [99]. This process is supported by tetraspanins (e.g. CD63, CD81, CD9), transmembrane proteins enriched in EVs, which are known to participate in cargo sorting and in exosome release [100–104]. Other proteins involved in the transport of MVBs to the plasma membrane for exosome release are small GTPases which mainly belong to the Ras [105] and Rab family [97, 106–109]. Finally, the synthesis of ceramide also plays a crucial role in exosome generation. In fact, the inhibition of specific enzymes involved in ceramide synthesis, such as neutral sphingomyelinase 2, has proved to reduce exosome release [110].

Ectosomes, also defined as shedding vesicles or microvesicles (MVs), include different populations of vesicles, the smallest with a diameter ranging from 50 to 200 nm and the largest with a diameter up to 1,000 nm, some of them derived from pre-apoptotic cells. Unlike exosomes, ectosomes arise directly from plasma membrane budding and contain cytoplasmic constituents [111, 112]. The modifications in the plasma membrane curvature result from changes in lipid and protein interactions, which involve the arrestin domain-containing protein-1 (ARRDC1) and the late endosomal protein tumor susceptibility gene 101 (TSG101) [113]. The process of ectosomes vesiculation and release also relies on cytoskeleton rearrangements, controlled by the signaling cascade of Ras-related GTPase ADP-ribosylation factor 6 (ARF6) [114, 115]. Finally, apoptotic bodies are EVs with a diameter ranging from 1,000 to 5,000 nm that are released by cells undergoing programmed death and may contain nuclear fragments and intact chromosomes [116].

In the last decades, EVs have emerged as important mediators of an evolutionarily well-preserved mechanism of intercellular communication [117]. In fact, following their release into the microenvironment, EVs can efficiently deliver selective patterns of proteins, bioactive lipids and nucleic acids [118, 119]. The transfer of EV cargo into recipient cells can induce epigenetic and functional changes into target cells [120]. Several studies have shown that EVs are particularly enriched in different RNA species that, when transferred to recipient cells remained functional and can modify cellular behavior [28, 121–124]. The EV encapsulation efficiently protects RNA stability from the degrading activity of enzymes, like RNases, which are present in the extracellular space and in biological fluids [121, 125]. Besides the main typical RNA species, like mRNAs, miRNAs and ribosomal RNAs, EVs may shuttle lncRNAs and small ncRNAs,

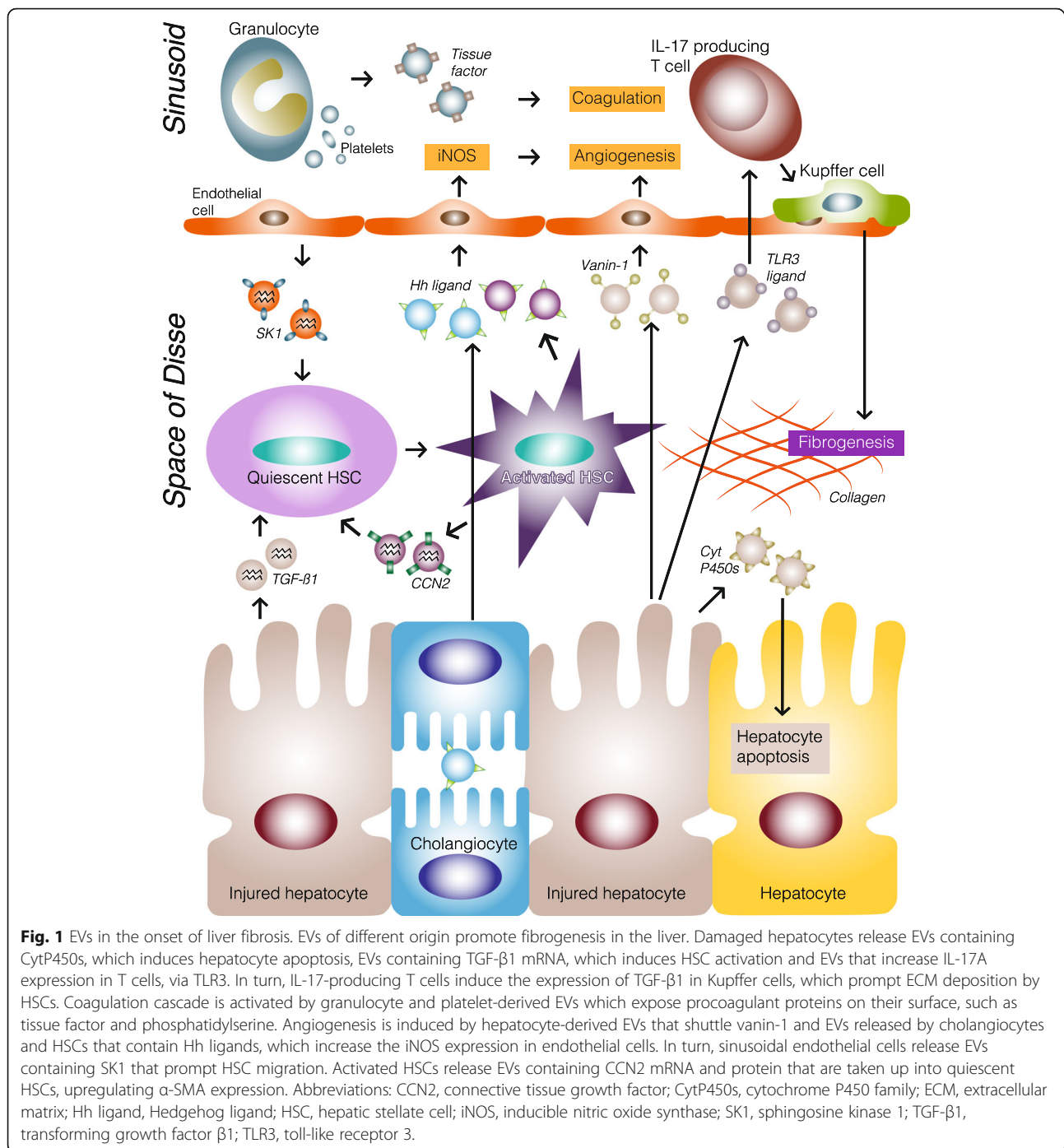
such as tRNA-derived fragments, piRNA, small nucleolar RNA, Y-RNA and vault RNA [126–131]. Interestingly, EVs can also contain circular RNAs [132] and several mRNAs fragments enriched in the 3'-untranslated regions [133], suggesting their role as ceRNA to regulate translational activity of mRNAs in recipient cells.

EVs are abundant in all biological fluids, such as blood, urine, bile, amniotic fluid, synovial fluid, vitreous fluid, cerebrospinal fluid, breast milk, saliva, semen, tears [91]. However, most studies have focused on plasma-derived EVs, because they are easily accessible. Since EVs retain the molecular signature of the cell of origin, they can be exploited as biomarkers for diagnostic and prognostic purpose in patients with hepatic fibrosis.

EVs in the onset of hepatic fibrosis

Consistent with their role in intercellular communication, EVs are involved in different steps of the complex scenario of hepatic fibrosis development [134] (Figure 1). Progressive liver fibrosis usually arises from a recurrent epithelial injury that results in hepatocyte dysfunction and apoptosis, by an intrinsic intracellular stress-mediated pathway, or alternatively, through an extrinsic death receptor-mediated pathway [135, 136]. Damaged hepatocytes can produce exosomes that contain proteins involved in the metabolism of lipoproteins, endogenous compounds, and xenobiotics. Interestingly, exosomes from injured hepatocytes are also enriched in proteins involved in cellular detoxification process, such as the cytochrome P450 family (CytP450s) [137]. Among these enzymes, CytP450 2E1 when overexpressed may promote hepatic apoptosis through a mechanism of oxidative stress [138]. Therefore, injured hepatocyte-derived exosomes containing CytP450s may participate in the development of liver fibrosis by promoting hepatocyte apoptosis. Hepatocytes treatment with CCl₄ induces the release of exosomes able to increase the expression of IL-17A in $\gamma\delta$ T cells, via toll-like receptor 3 [139]. In turn, proinflammatory IL-17-expressing T cells modulate the production of TGF- β 1 in Kupffer cells and can directly activate ECM deposition by HSCs, thus enhancing liver fibrosis [140].

A relevant fibrogenic role in HSC activation is played by CCN2, an ECM-expressed cystein-rich protein that is produced in fibrotic liver tissue. CCN2 is involved in adhesion, migration, proliferation, survival and differentiation of HSCs and exhibits strong pro-fibrogenic properties [141]. CCN2 overexpression promotes the deposition of ECM, the development of fibrotic lesions and mediates TGF- β 1-dependent fibrotic pathways in HSCs [142, 143]. Furthermore, exosomes derived from injured epithelial cells contain TGF- β 1 mRNA that can rapidly initiate myofibroblasts activation [144]. Recently, it has been demonstrated that CCN2 mRNA and protein are packaged into activated HSC-derived EVs and efficiently transferred into



quiescent HSCs, resulting in the upregulation of CCN2 downstream targets, such as α -SMA, and the activation of HSCs [145].

Another important aspect of HSC activation is the enhanced migration potential. During hepatic fibrosis, endothelial cells can release sphingosine kinase 1 (SK1)-containing EVs that can prompt HSC migration [146]. The EV-induced HSC migration depends on EV adhesion, which consists in the binding of the EV-associated

fibronectin with α V β 1 integrin on HSCs, followed by the dynamin-dependent internalization of EVs. As intrahepatic angiogenesis and sinusoidal remodeling promote fibrogenesis in the liver [147], HSC-derived EVs may contribute to fibrogenesis by modifying endothelial cell fate. It has been suggested that EVs derived from hepatocytes exposed to saturated, free fatty acid display a proangiogenic effect both *in vitro* and *in vivo* [148]. The release of EVs is regulated by the ectoenzyme

pantetheinase, also called vanin-1, which is found primarily in liver, kidney and intestine, and has been associated with cell adherence and migration. High levels of hepatocyte-derived EVs associated with angiogenesis and early liver fibrosis were also detected in an *in vivo* model of dietary-induced steatohepatitis. The genetic inhibition of caspase3 effectively reduced circulating levels of hepatocytederived EVs and protected mice from angiogenesis and fibrosis [148]. Another important observation is that PDGF-stimulated HSCs and cholangiocytes release EVs enriched in hedgehog ligands that can alter the phenotype of hepatic sinusoidal endothelial cells [149]. *In vivo* experiments on rats subjected to bile duct ligation (BDL) have also confirmed that EVs isolated from plasma or bile contain hedgehog ligands, that can induce the expression of activation markers, such as inducible nitric oxide synthase and CD31, in hepatic sinusoidal endothelial cells [149]. All these findings highlight the role of EVs in the paracrine crosstalk between endothelium and HSCs in liver fibrosis.

Since hypercoagulation has proved to enhance liver fibrosis in chronic liver disease [150–152], another important EV contribution to hepatic fibrosis may be the activation of the coagulation cascade. It has been demonstrated that EVs derived from platelets or granulocytes in patients with sepsis have procoagulant properties and support thrombin generation, in proportion to the severity of the disseminated intravascular coagulopathy [153, 154]. Therefore, EVs contribute to liver fibrosis not only by promoting HSC migration and activation, but also by exhibiting proangiogenic and procoagulant effects that strengthen their pro-fibrogenic potential.

The contribution of EVs containing ncRNAs to liver fibrosis

To date, a very large number of ncRNAs involved in the development of liver fibrosis have been described [15, 155, 156]. In this chapter we will focus only on EV-associated miRNAs and lncRNAs and discriminate in particular pro-fibrotic ncRNAs, which are upregulated in fibrogenesis, from anti-fibrotic ncRNAs, which are downregulated during liver fibrosis (Table 1).

Pro-fibrotic ncRNAs shuttled by EVs

Several studies have shown that EVs produced by hepatocytes exposed to lipotoxic stress can induce fibrotic HSC activation [161, 165, 180]. In particular, Feldstein and colleagues have demonstrated that hepatocyte-derived EVs contain several miRNAs that target PPAR- γ , and identified miR-128-3p as a pro-fibrogenic miRNA that efficiently mediates EV effects on PPAR- γ inhibition and HSC activation [161].

Exposure to alcohol and its metabolites, or hepatitis viral infection can also lead to hepatic injury and fibrogenesis. Recently, miR-19b and miR-92 expression has been found

increased in exosomes isolated from activated HSCs and from the plasma of ALD cirrhotic patients [162]. These two miRNAs belong to the miR-17-92 cluster (miR-17a, -18a, -19a, -19b, -20a, and -92), mostly involved in the regulation of the TGF- β pathway [181]. In the liver, miR-17-92 members coordinate different cellular pathways by targeting genes involved in lipid metabolism [182], inflammatory responses [183], EMT [184], cell proliferation [181, 185], apoptosis and necrosis [183]. Interestingly, miR-19 is present into EVs derived from HCV-infected hepatocytes and is transported into HSCs, where it targets SOCS3 and activates the STAT3-mediated TGF- β signaling pathway, leading to HSC activation and enhancing liver fibrosis [163]. Other miRNAs that have been detected in EVs released by HCV-infected hepatocytes are miR-122 [166] and miR-192 [172], two miRNAs expressed in large quantities in healthy hepatocytes [186]. In particular, the exosomal transfer of miR-192 into HSCs induces their fibrotic activation, while treatment of HSCs with miR-192 inhibitor can effectively revert the myofibroblast transdifferentiation of HSCs [172].

The increase in lncRNA-MALAT1 expression is associated with inflammation and fibrosis in NASH patients [187] and is negatively correlated with miR-101b expression levels in fibrotic livers and in activated HSCs [188]. Recently, it has been observed that MALAT1 is contained in exosomes released by arsenite-treated hepatic cells and is transported into HSCs, where it promotes their activation through miR-26b regulation of Col1 α 2 expression [160]. Another lncRNA associated with the progression of liver fibrosis is the lncRNA-H19, which acts as ceRNA of let-7 family upregulating its target high-mobility group AT-hook 2 (HMGA2) and causing cholangiocyte proliferation [157]. The release of exosomal lncRNA-H19 by cholangiocytes is associated with HSC activation and cholestatic liver injury (CLI) [158, 159]. Under cholestatic conditions, the taurocholate acid and estrogen-induced activation of ERK1/2 signaling pathway upregulates lncRNA-H19 in cholangiocytes and in exosomes released by these cells [159, 189]. Exosomal lncRNA-H19 is delivered to hepatocytes, where it downregulates the small heterodimer partner (SHP), which is involved in the regulation of bile acid homeostasis [190]. Moreover, lncRNA-H19-enriched exosomes can be taken up by HSCs both *in vitro* and *in vivo*, resulting in HSC proliferation and activation with the upregulation of pro-fibrotic gene expression [158].

Anti-fibrotic ncRNAs shuttled by EVs

Increasing evidence has pointed out the key role of miR-30a in myocardial [191] and peritoneal fibrosis [192] and its contribution to the suppression of EMT process during HSC activation [193]. It has been observed that miR-30a is downregulated both in activated HSCs and in exosomes derived from activated HSCs [178]. On the

Table 1 EV-associated ncRNAs in liver fibrosis

	ncRNAs	EV expression in hepatic fibrosis	Fibrosis model	Target genes in fibrosis	Functions	Citations
PRO-FIBROTIC	lncRNA-H19	↑ in EVs from damaged cholangiocytes	CLI	HMGA2, SHP	HSC activation, proliferation, differentiation, Biomarker of hepatic fibrosis	[157–159]
	lncRNA-MALAT1	↑ in EVs from damaged hepatocytes	DILI	Col1a2, via miR-26b	HSC activation, Biomarker of hepatic fibrosis	[160]
	miR-128-3p	↑ in EVs from damaged hepatocytes	NAFLD	PPAR-γ	HSC activation	[161]
	miR-17-92 cluster	↑ in EVs from activated HSCs, damaged hepatocytes and plasma	ALD, HCV hepatitis	SOCS3, TGF-β signaling pathway	HSC activation, EMT, lipid metabolism, inflammatory responses, proliferation, apoptosis, necrosis	[162, 163]
	let-7s	↓ in EVs from activated HSCs and plasma	HCV hepatitis	TGF-β signaling pathway	Biomarker of hepatic fibrosis	[164]
	miR-122	↑ in EVs from damaged hepatocytes ↑ in plasma EVs ↑ in EVs from engineered ADSCs	<i>In vitro</i> HSC activation, NASH, NAFLD, ALD, DILI, HCV hepatitis	CCNG1, IGF1R, P4HA1	Survival, proliferation, EMT, fibrosis suppression biomarker of hepatic fibrosis	[161, 165–170]
	miR-181-5p	↑ in EVs from engineered ADSCs	<i>In vitro</i> HSC activation, NASH	STAT3, Bcl-2, Beclin1	Fibrosis suppression	[171]
ANTI-FIBROTIC	miR-192	↑ in plasma EVs	NAFLD, HCV hepatitis		HSC activation, biomarker of hepatic fibrosis	[161, 165, 172]
	miR-199a-5p	↑ in EVs from quiescent HSCs	<i>In vitro</i> HSC activation	CCN2	Adhesion, migration, proliferation survival and differentiation of HSCs	[173]
	miR-214	↑ in EVs from quiescent HSCs, ↓ in EVs from activated HSCs and plasma	<i>In vitro</i> HSC activation	CCN2	Adhesion, migration, proliferation survival and differentiation of HSCs, Biomarker of hepatic fibrosis	[174, 175]
	miR-22-3p, -26a-5p, -27b-3p, -302-3p, -486-5p, -92a-3p, -92b-3p	↑ in EVs from iPSCs,	<i>In vitro</i> HSC activation, NASH, BDL	TGF-β signaling pathway	Fibrosis suppression	[176]
	miR-223	↑ in EVs from engineered BM-MSCs	AIH	NLRP3, caspase-1	Fibrosis suppression	[177]
	miR-30a	↓ in EVs from activated HSCs	BDL	Beclin1	EMT, autophagy	[178]
	miR-34c, -151-3p, -483-5p, -532-5p	↓ in serum EVs	NASH	PDGF, TIMP2, SMAD3 (predicted)	fibrosis suppression, biomarkers of hepatic fibrosis	[179]

other hand, miR-30a overexpression ameliorates hepatic fibrosis by suppressing Beclin1-mediated autophagy and increasing lipid accumulation in HSCs and in murine fibrotic liver tissues after BDL [178].

The association between the downregulation of miR-214 and miR-199a-5p and the increase of CCN2 expression in fibrotic livers and in cultured activated primary mouse HSCs has been described by Brigstock and colleagues [173, 174]. They observed that quiescent HSCs release EVs that inhibit the activation of HSCs and attenuate fibrosis. Next, they demonstrated that exosomes derived from quiescent HSCs were enriched in miR-214 and miR-199a-5p, which both specifically bind to the 3'-untranslated region of CCN2, thus reducing the

expression levels of CCN2 and of its downstream targets Col1α1 and α-SMA [173, 174]. A Twist1-miR-214-CCN2 axis has also been identified in HSCs. EVs secreted by quiescent HSCs contained high levels of Twist1, which can induce miR-214 expression in activated HSCs, thus suppressing CCN2 expression levels and reverting HSCs to a more quiescent phenotype [175].

Recently, it has been shown that stem cell-derived ncRNA-containing EVs may exert an anti-fibrogenic effect. In particular, mesenchymal stromal cells (MSCs) efficiently support the repair and regeneration of injured tissues through the release of paracrine factors, such as EVs [194]. Several papers have explained the therapeutic effects of MSC-EVs in preclinical models of hepatic

fibrosis [195, 196]. Besides the proteins and transcripts shuttled by stem cell-derived EVs, their ncRNA cargo has proved to modulate HSC biology, thus reverting liver fibrosis. Feldstein and colleagues have demonstrated that human induced pluripotent stem cell (iPSC)-derived EVs can effectively deliver anti-fibrogenic miRNAs into HSCs, thus reducing their activation and attenuating liver fibrosis [176]. By performing genomic analysis of iPSC-EV miRNA cargo, they identified 22 highly expressed miRNAs, among which miR-22-3p, miR-26a-5p, miR-27b-3p, miR-302-3p, miR-486-5p, miR-92a-3p and miR-92b-3p all display potential anti-fibrotic properties [176].

Recent advances in nanomedicine, allowed the generation of engineered EVs that may be more effective than naïve EVs against liver fibrosis [167, 171, 177]. Lou and colleagues have demonstrated that lentiviral-induced miR-122 expression in adipose tissue-derived MSCs (ADSCs) increased their therapeutic effect in hepatic fibrosis [167]. The EV-mediated transfer of miR-122 from ADSCs resulted in the cell cycle arrest of HSCs and the downregulation of miR-122-target genes involved in HSC proliferation and collagen maturation, such as prolyl-4-hydroxylase 1 (P4HA1), insulin-like growth factor receptor 1 (IGF1R) and cyclin G1 (CCNG1) [167]. The transient overexpression of miR-181-5p in ADSCs represents another therapeutic strategy against liver fibrosis [171]. As for miR-122, miR-181-5p can be delivered by ADSC-EVs to HSCs, where it modulates the fibrosis-associated STAT3/Bcl-2/Beclin1 pathway, thus reducing TGF- β 1 expression and inducing autophagy in HSCs. Moreover, the *in vivo* administration of miR-181-5p-overexpressing ADSC-EVs attenuated liver injury and reduced liver expression of pro-fibrotic genes, such as type I collagen, α -SMA, vimentin and fibronectin [171]. In a model of autoimmune hepatitis (AIH), the lentivirus-mediated up-regulation of miR-223 in bone marrow (BM)-derived MSCs improved the cytoprotective effect of BM-MSC-EVs, both *in vitro* and *in vivo* [177]. The engineered EVs prompted the increase of miR-223 levels in the liver, which results in the improvement of liver function and the reduction of inflammation and hepatocyte apoptosis, through the downregulation of miR-223-target genes NLRP3 and caspase-1. However, the specific removal of miR-223 from BM-MSCs completely abrogated the therapeutic effects of BM-MSC-EVs [177].

Our group has demonstrated that EVs released by human liver stem cells (HLSCs) can reduce fibrosis in a murine model of NASH [197]. Proteomic analyses performed on HLSC-EV cargo revealed several anti-inflammatory proteins that may contribute to the attenuation of fibrosis. However, the role of HLSC-EVs in the reversal of hepatic fibrosis may be also related to the EV-mediated transfer of specific mRNAs and ncRNAs from HLSCs to injured hepatocytes and activated HSCs. Our previous results on two different *in vivo* models of chronic kidney diseases

have shown that the administration of HLSC-EVs reduced the expression of pro-fibrotic genes α -SMA, Col1 α 1, and TGF- β 1 [198, 199]. Moreover, we have pointed out a correlation between the anti-fibrotic effect of HLSC-EVs and their ncRNA cargo, which includes specific anti-fibrotic miRNAs, such as miR-29a, miR-30a and the let-7 family, that are known to contribute to the reversal of hepatic fibrosis [199].

EVs-shuttled ncRNAs as biomarkers of liver fibrosis

In addition to information exchange, interest has grown in the role of ncRNA associated with EVs as biomarkers in liver diseases and hepatic fibrosis [18, 200, 201].

Research by the group of Brigstock and colleagues has demonstrated that reduced circulating levels of exosomal miR-214 in mice with liver fibrosis reflect the fibrosis-induced modifications in the liver [175]. Moreover, the same group identified decreased levels of miR-34c, miR-151-3p, miR-483-5p and miR-532-5p in serum EVs derived from fibrotic mice treated with CCl₄ and from human patients with liver fibrosis [179]. All of these miRNAs were shown to cooperatively suppress HSC activation, but predictive computational analysis did not pinpoint classical HSC activation markers CCN2, α -SMA or Col1 α 1 as direct targets of these miRNAs, suggesting that they are likely secondary downstream targets. In particular, the overexpression of miR-483-5p counteracts liver fibrosis by suppressing tissue inhibitor of metalloprotease-2 (TIMP2) and PDGF- β [202], while miR-532-5p is predicted to target SMAD3, which modulates CCN2 transcription in HSCs, thus regulating liver fibrogenesis [203].

In the context of chronic hepatitis, the progression of hepatic fibrosis has been related to EVs produced by HCV-infected hepatocytes [163, 164]. In a cohort of patients with chronic hepatitis C, Matsuura and colleagues have observed a negative correlation between levels of circulating or EV-shuttled let-7 family and the severity of hepatic fibrosis. Furthermore, pathway analysis of target genes of let-7 suggested that low levels of let-7 in EVs may be related to hepatic fibrogenesis through the activation of TGF- β signaling in HSCs [164].

The characterization of EVs into the bloodstream of an experimental NAFLD model identified both exosomes and MVs enriched in miR-122 and miR-192 [45]. Diet-induced liver damage led to the downregulation of miR-122 in activated HSCs [204, 205] and in injured hepatocytes [168]. Simultaneously, high miR-122 circulating levels have been detected both in the EV and in the protein-rich (EV-free) serum fractions [168]. Interestingly, increased circulating levels of EV-associated miR-122 were also detected in the plasma of subjects with alcoholic hepatitis [169, 170]. Moreover, during NAFLD progression, levels of miR-122 and miR-192 increased in circulating EVs while decreased in liver cells [165]. The release of these two miRNAs from

damaged hepatocytes in EVs during NAFLD progression may be a possible explanation of miR-122 and miR-192 increased expression in circulating EVs from advanced stage NAFLD patients compared to those from early stage NAFLD patients. These results suggest the role of EV-associated miR-122 and miR-192 as biomarkers of hepatic fibrosis that can be used to monitor and discriminate the stage of the disease in patients with chronic liver injury.

The analysis of circulating EVs from subjects exposed to arsenite has pointed out a correlation between the progression of liver fibrosis and the increase of lncRNA-MALAT1 levels in circulating exosomes [160]. This observation encourages the use of EV-associated lncRNA-MALAT1 as a possible biomarker of hepatic fibrosis caused by arsenicosis. Finally, a correlation between serum levels of exosome-associated lncRNA-H19 and the severity of cholestatic liver fibrosis has been observed in both mouse CLI models and in human patients with different types of cholestatic liver disease [157–159]. In particular, the co-expression of lncRNA-H19 and CK19 indicates that these exosomes are mainly released by damaged cholangiocytes during cirrhosis and explain their increase during the progression of hepatic fibrosis.

Conclusions

NcRNAs were proved to have a pathogenic involvement in the development of liver fibrosis, as well as a diagnostic potential. Several ncRNAs are carried by EVs and are protected by their lipid membrane bilayer from degradation. Moreover, EVs may deliver ncRNAs at distance and local sites, thus influencing several physiological and pathological processes. EVs have proved to be active mediators of intercellular communication in a number of pathological conditions, including liver fibrosis. The damaged hepatocytes produce EVs that act in a paracrine manner to regulate key processes of hepatic fibrosis, such as HSC activation, angiogenesis and coagulation. In turn, EVs secreted by circulating cells and sinusoidal epithelial cells modify liver microenvironment, thus influencing the progression of liver fibrosis. Analysis of the EVs cargo, in particular ncRNAs, may provide further insight in the epigenetic modulation of genes and pathways related to hepatic fibrogenesis. Moreover, several studies have shown that EVs are promising biomarkers for diagnostic and prognostic purposes in patients with chronic liver diseases, as they are implicated in many stages of fibrosis development and progression.

In the future, one may envisage the use of EVs to deliver therapeutic molecules to enhance liver regeneration. Preliminary studies have also shown that native EVs carrying anti-fibrotic ncRNAs may have a therapeutic potential. In particular, MSC-derived EVs have proved to be good candidates in the delivery of anti-fibrogenic miRNAs to HSCs, thus ameliorating hepatic

fibrosis. Moreover, engineering native EVs may be a powerful way to improve the efficacy of native EVs. Also the EV-mediated delivery of lncRNAs, especially circRNAs, may represent a valid resource in the reversion of liver fibrosis and for this reason needs further investigation. Finally, determining which kind of cells in the liver are target for ncRNAs shuttled by EVs may help to better understand the patho-physiology of liver fibrosis and may open new therapeutic strategies.

Abbreviations

ADSC: Adipose tissue-derived mesenchymal stromal cell; AIH: Autoimmune hepatitis; ALIX: Apoptosis-linked gene-2 interacting protein X; ALD: Alcoholic liver disease; α -SMA: Alpha smooth muscle actin; BDL: Bile duct ligation; BM-MSC: Bone marrow-derived mesenchymal stromal cell; CCl₄: Carbon tetrachloride; CCN2: Connective tissue growth factor; CCNG1: Cyclin G1; ceRNA: Competing endogenous RNA; circRNA: Circular RNA; CLI: Cholestatic liver injury; Col1 α 1: Alpha-1 type I collagen; CytP450s: Cytochrome P450 family; DILI: Drug-induced liver injury; ECM: Extracellular matrix; EMT: Epithelial-to-mesenchymal transition; ESCRT: Endosomal sorting complex required for transport; EV: Extracellular vesicle; HBC: Hepatitis B virus; HCC: Hepatocellular cancer; HCV: Hepatitis C virus; HLSC: Human liver stem cell; HMGA2: High-mobility group AT-hook 2; HRS: Hepatocyte growth factor-regulated tyrosine kinase substrate; HSC: Hepatic stellate cell; IGF1R: Insulin-like growth factor receptor 1; I κ B α : NF- κ B inhibitor alpha; IL: Interleukin; iPSC: Human induced pluripotent stem cell; lncRNA: Long non-coding RNA; miRNA: MicroRNA; MSC: Mesenchymal stromal cell; MV: Microvesicle; MVB: Multivesicular body; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; ncRNA: Non-coding RNA; NF- κ B: Nuclear factor kappa B; piRNA: Piwi-interacting RNA; P4HA1: Prolyl-4-hydroxylase a1; PDGF: Platelet-derived growth factor; PPAR γ : Peroxisome proliferator-activated receptor-gamma; PTEN: Phosphatase and tensin homolog; SHP: Small heterodimer partner; SK1: Sphingosine kinase 1; TGF- β : Transforming growth factor beta; TIMP2: Tissue inhibitor of metalloprotease-2; TNF- α : Tumor necrosis factor alpha; TSG101: Tumor susceptibility gene 101 protein; VPS4: Vacuolar protein sorting-associated protein 4

Acknowledgements

The authors would like to thank Valeria Chiabotto for figure realization.

Authors' contributions

G.Ch. performed the research of the pertinent literature. G.Ch. and S.B. designed and drafted the manuscript. G.Ca. contributed to revising and editing the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by a grant from "Terapie avanzate per processi fibrotici cronici" (Progetto EV-ER, codice domanda 320-40, Regione Piemonte).

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

G.Ca. is a component of Scientific Advisory Board of Unicyte AG. The authors declare no conflict of interest.

Author details

¹Department of Medical Sciences, University of Torino, Corso Dogliotti 14, 10126 Torino, Italy. ²Molecular Biotechnology Centre, University of Torino, Torino, Italy.

Received: 23 January 2020 Accepted: 29 April 2020

Published online: 18 May 2020

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