In-silico cardiac aging regulatory model including microRNA post-transcriptional regulation

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Abstract

In most developed countries, cardiovascular diseases are among the top causes of death and their development has been shown closely related to aging. In this context, because of their ability to pervasively influence gene networks, miRs have been found as possible key players in the development of cardiac pathologies, suggesting their potential role as therapeutic targets or diagnostic markers. Based on these assumptions, we hereby present a computational study that applies data fusion techniques coupled with network analysis theory to identify a regulatory model able to represent the relationship between key genes and miRs involved in cardiac senescence processes. The proposed model has been validated through an extensive literature analysis, which confirmed that 94% of the identified genes and miRs are related with cardiac senescence. Furthermore, two relevant genes of the model have been also validated by Western blot experiments on heart samples from young and old mice, confirming in vitro their ectopic expression in aged hearts. The pure computationally inferred model presented in the paper is therefore a good candidate to represent the relationship between key genes and miRs involved in cardiac senescence processes, and represents a reliable selection of genes and miRs for further studies, in order to elucidate and better detail their involvement in cardiac aging.

Keywords: Senescence, heart, omic regulation, pathway, microRNA.

1. Introduction

Physiological aging and senescence progressively impair a large set of physiological functions in living organisms leading to a reduced response to stress and increasing the risk of occurrence of a wide set of diseases [1, 2].

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At the cardiovascular level, aging often manifests with reduced cardiac functional reserve, left ventricular hypertrophy, mild fibrosis and reduced endogenous cardiac protection [3, 4]. Cardiovascular diseases are one of the leading causes of death in most developed countries and aging is a dominant risk factor for their development [5]. Therefore, clearing up the regulatory mechanisms contributing to cardiac aging may help the identification of early cardiovascular pathophysiological changes and potentially contributes to the development and improvement of new therapies [6].

Cardiac aging can be favored by both genetic and environmental factors as reported in several published studies. Among the others, the most relevant factors include: decrease in telomerase activity and shortening of the DNA of the telomeres [7, 8], increased oxidative stress [9], loss of mitochondrial function [3] and impaired autophagy [10].

Epigenetic regulation from small non-coding RNAs such as micro RNAs (miRs) also plays a significant role in cell aging processes [11, 12]. miRs are non-coding RNAs that play a key role in the regulation of gene expression. They act at the post-transcriptional level suppressing target protein expression either by promoting messenger RNA (mRNA) degradation or by translational repression [13, 14, 15, 16, 17, 18, 19]. It has been shown that miRs may fine-tune the expression of as much as 30% of all mammalian protein-encoding genes [18], thus influencing a wide spectrum of biological processes such as cell development, stem cell proliferation, division and differentiation, regulation of immunity, apoptosis, cell signaling and metabolism [15, 17, 18, 20, 21]. Moreover, aberrant expression of miRs has been associated with the onset and development of various diseases, such as cancer [18][22] [23], metabolic disorders [24], autoimmune diseases [21], acute cellular rejection following transplantation [25, 26] and cardiac pathologies [27, 28, 29, 30].

In this paper we show the application of a computational workflow that combines data fusion techniques with network analysis theory. Data fusion techniques allow to scrape and integrate a huge amount of information regarding selected biological processes available in public omic databases, and to combine them in the form of complex regulatory networks. Network theory is then used to mine the complexity of the generated networks in order to extract an in-silico model able to represent the relationship between key genes and miRs involved in cardiac senescence processes. This model, that extends the preliminary work presented by the authors in [31], has the potential to support the identification of molecular mechanisms and pathways involving miRs responsible for cardiac aging processes. An extensive literature based validation suggests strong connection between genes of the identified model and aging related processes. This has been further confirmed by Western blot experiments on heart samples from young (2 month-old) and old (18-month old and 26 month-old) mice on two relevant genes of the identified model.

2. Materials and methods

We developed a semi-automated workflow that was able to infer a regulatory network whose nodes showed a key regulatory role in cardiac aging. Fig. 1 shows the overall data flow that takes advantage of two Cytoscape plugins: (i) ReNE [32], and, (ii) NetworkAnalyzer [33]. We started from a list of differentially expressed genes related to cardiac aging, which allowed us to identify a set of KEGG's pathways involving these genes and describing biological processes related cardiac aging. By integrating these pathways with their transcriptional and post-transcriptional regulators we were able to compute gene centrality measures across the networks that eventually allowed us to produce a list of high centrality nodes. Such nodes resulted well interconnected, resembling a highly interconnected sub-network able to dispatch key regulatory signals previously associated with senescence [20].

2.1. Gene selection

A set of genes related to cardiac aging in *Rattus norvegicus* were identified from microarray expression data (Affymetrix Rat Genome U34 Array, annotation table with netaffx build 32) available in the Gene Expression Omnibus (GEO)[34] under accession number GDS399 (`http://www.ncbi.nlm.nih.gov/geoprofiles?term=GDS399[ACCN]`). The selected experiment investigates the effects of reduced adrenergic signaling in the aged heart by comparing young (3-4 month) and aged (20-22 month) male Fischer-344 heart ventricle [35].

Differentially expressed genes were extracted from the dataset using the GEO "Up/down filter". Pseudogenes, expressed sequence tags and duplicates were removed from the obtained list.

To identify *Homo Sapiens* homologs the list of genes was loaded in the ReNE Cytoscape plugins [32] (http://apps.cytoscape.org/apps/rene) and processed using the "Genetic information – fast" option of the plugin. This operation looks for all gene symbols with a valid human unigene id allowing us to remove genes without human homologs, and to generate a list of differentially expressed genes named here as *Differential Expressed Genes Network* (DEGN).

2.2. Pathway selection

Genes contained in DEGN were used to identify a list of pathways from KEGG [36] involving the identified set of genes and therefore potentially describing biological processes responsible for the aging process. All pathways in KEGG were processed using the KEGG mapper API (`http://www.genome.jp/kegg/tool/map_pathway1.html`) to count how many genes from DEGN they contain, identifying a set of 109 *Homo sapiens* pathways involving genes from DEGN. The analyzed pathways were ranked according to this gene count. Figure 2.2 graphically shows the gene count of the top 50 ranked pathways. Looking at the figure we can see a first area (green section) containing 10 pathways with high gene count and therefore highly relevant for our analysis. After the first 10 pathways the tendency of the curve is to flatten. We used this distribution to define a cut-off threshold in this ranked list of pathways. The green area is

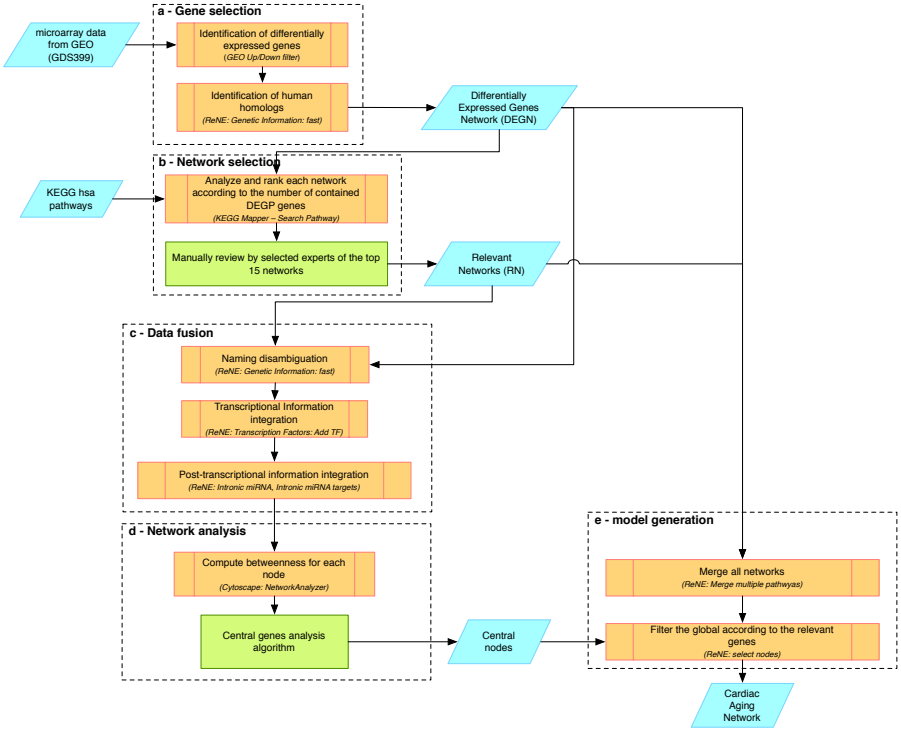

Figure 1: Computational workflow used to infer the cardiac aging model. The workflow starts from a dataset of microarray expression data and (a) identifies a set of differential expressed genes in the dataset, (b) uses these genes to identify a set of relevant KEGG networks, (c) applies data fusion techniques to the selected networks to increase their informative content, (d) applies network analysis theory on the networks to identify central nodes and (e) uses the identified central nodes to build the final in-silico model. Cyan elements identify input/intermediate/output data. Orange blocks are processing tasks resorting to external services, software or database whereas green block correspond to local tasks.

the area of interest for our analysis. Nevertheless, to avoid loosing important information we enlarged this area by adding 5 additional pathways from the rank (orange area) thus selecting a list of 15 top ranked pathways. These top 15 pathways were manually reviewed by a set of experts in order to identify a list of *relevant networks* (RN).

2.3. Data fusion

Both the DEGN and the RN were individually processed resorting to the ReNE Cytoscape plugin [32] to increase their informative content. The following computational tasks were applied:

1. *Naming disambiguation*: since different databases use different ways to

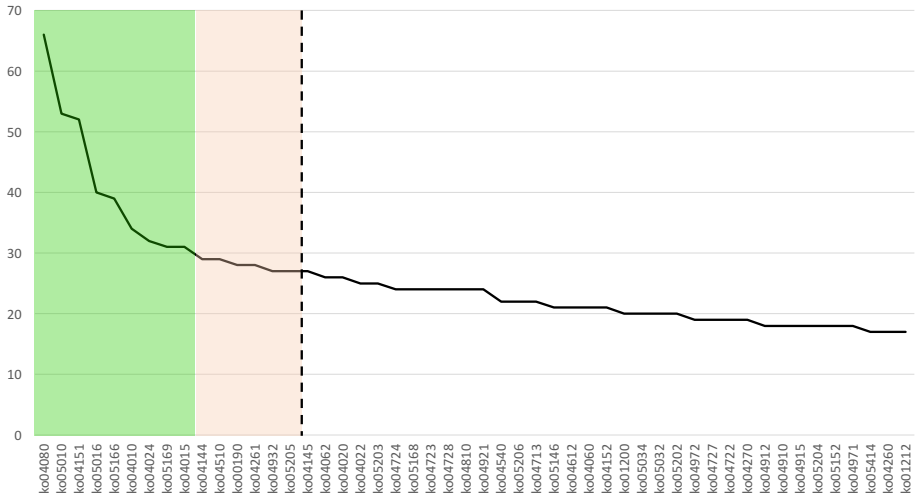

Figure 2: *Homo sapiens* pathways from KEGG containing genes from DEGN. Pathways are ranked based on the number of genes they contain. For clarity only the top 50 pathways out of the 109 identified ones are reported. The full list is available in the supplementary data.

identify omic entities, all symbols contained in the analyzed networks were converted according to their NCBI [37] and Uniprot [38] unique identifiers, thus allowing to navigate across public omic repositories without ambiguities. This operation was performed resorting to the "Genetic information – fast" functionality offered by ReNE.

2. *Transcriptional information integration*: it is well known that miR mediated post-transcriptional regulation of selected genes is not always a direct interaction but is often indirectly mediated by translational repression or mRNA degradation of their transcription factors (TFs) [20, 39, 40, 32]. Considering these indirect interactions is therefore very important. However, pathway stored in KEGG lack systematic information regarding TFs. The TFs of each gene of the analyzed networks were systematically retrieved from TargetMine [41] and integrated in the analyzed networks creating new TF nodes with outgoing edges directed to their target genes. This operation was performed using the ReNE "Transcription Factors – Add TF" functionality.
3. *Post-transcriptional information integration*: information regarding miRs hosted by the network genes, not available in KEGG, was retrieved from the miRIAD database [42] using the ReNE "Intronic miRNA" functionality and added to the analyzed networks. It is well-known that these miRs are co-expressed with their host genes [43] and often act as regulatory hubs that lead to complex post-transcriptional regulatory motifs acting as fine tuning genetic regulatory signaling cascades. For each identified

miR the list of its targets was computed by ReNE using the "Intronic miRNA targets" functionality that resorts to information available in the TargetHUB online database [44], thus connecting miRs with their targets in the pathways.

2.4. Network analysis

Network topology parameters were computed for each processed network using the Cytoscape NetworkAnalyzer plugin [33]. In detail, centrality measures were computed for each node of the analyzed networks, since they have been demonstrated particularly useful to identify key players in biological processes [45][46]. NetworkAnalyzer provides two centrality measures: (i) closeness centrality, and (ii) betweenness centrality. The closeness centrality uses information about the length of the shortest paths within a network. However, since this distance is only defined for pairwise strongly connected nodes, it cannot be used in our experimental setup. On the other hand, the betweenness centrality quantifies the ability of a node to monitor the communication between other nodes. Every node that is part of a shortest path between two nodes can monitor the communication on the path. Counting how many communications a node can monitor provides an intuitive definition of centrality [45]. Shortest-path betweenness centrality was applied to mammalian transcriptional regulatory networks and it was noted that betweenness appears to be an interesting topological characteristic in regard to the biological significance of distinct elements [47].

Betweenness centrality was therefore exploited to identify nodes whose role is central enough to possibly induce misbehavior when ectopically expressed under aging conditions according to the algorithm reported in Figure 3. The algorithm first analyzes each network filtering the nodes according to their betweenness centrality and retaining only the top T1 nodes (lines $1-4$). It then analyzes all possible pairs of networks searching for common nodes in each pair (lines $5-9$). This information is used to construct a histogram able to sort nodes according to the number of times they concurrently appear in a couple of networks, i.e., those nodes that have the potential to jointly influence more than one network (lines $10-18$). The top T2 nodes of the histogram are selected as list of high centrality nodes. Such nodes are promising regulators with a key role in signaling cascades related to aging.

Selecting the two thresholds T1 and T2 is not an easy task and basically depends on the target size for the final model. However, some basic considerations can be taken into account when selecting these two thresholds. Threshold T1 performs a filtering working locally on each single network. Among the two thresholds, T1 is the most selective one. Setting a low level for T1 means that each network retains only very few nodes with high betweenness. This means that in the second part of the selection algorithm only those nodes that are high in rank in several networks will have a chance to enter the construction of the histogram and will therefore have a role in the construction of the final model. This in turns focuses the model toward the most informative genes, discarding the less connected genes. Threshold T2 instead simply acts on the

final histogram enabling to have a fine control on the size of the model. This is particularly important whenever time consuming validation activities must be performed on the identified genes. In this paper we selected both T1 and T2 equal to 40 genes. Considering that in this paper we worked with networks whose average size is 600 nodes, the selected T1 threshold allowed us to have a very strong filtering working on average with only the top 7% of the genes of each network and therefore focusing on the most informative genes. Threshold T2 was instead selected in order to keep the complexity of the validation analysis under control. It is worth to remark, that the two thresholds affect the size of the model incrementally. Relaxing the constraint would only have the effect of increasing the size of the model adding additional information.

Require: Cut-off thresholds T1,T2
Output: List of central nodes
1: **for** i = 1 **to** count(nets) **do**
2: **sort desc** nodes of nets[i] by betweenness
3: nets[i] = **top** T1 nodes of nets[i]
4: **end for**
5: **for** i = 1 **to** count(nets) **do**
6: **for** j = 1 **to** count(nets) **do**
7: cg[i,j] = nets[i] ∩ nets[j]
8: **end for**
9: **end for**
10: **for each distinct** node n in cg **do**
11: **for** i = 1 **to** count(nets) **do**
12: **for** j = 1 **to** count(nets) **do**
13: **if** $g \in$ cg[i,j] **then**
14: histogram[n]++
15: **end if**
16: **end for**
17: **end for**
18: **end for**
19: **sort desc** histogram;
20: **return** (**top** T2 nodes of histogram[)

Figure 3: Selection of central nodes of a network.

2.5. Model generation

High centrality nodes identified in the previous step were finally mapped to a global network. The "Merge multiple pathways" functionality of the ReNE Cytoscape plugin was used to merge all the analyzed networks (DEGN plus RN) into a single global model. The merging process produced a large network that was finally filtered in order to retain only relations involving the identified central nodes thus reducing its complexity. This filtered subnetwork is a good candidate to highlight the most important inter-pathways regulatory entities, defined accordingly to their high centrality.

3. Results

A set of 191 differentially expressed genes out of 8,799 available microarray data were identified using the GEO "Up/down filter" on the GSE421 Dataset. The list was preprocessed removing non-genes references and duplicates (see Section 2.1) obtaining a list 166 candidate differentially expressed genes (DEGN) in heart aging. By looking for human homologs of the identified genes the following entities have been discarded: *Gstm7*, *LOC687048*, *LOC100365443*, *LOC100363469*, *LOC688869*, *Reg3b*, *LOC100364956*, *LOC100360403*, *Ly6al*, *LOC290595*, *Olr1642*, *RT1-M4*, *LOC100362894*, *Kng1l1*, thus obtaining a list of 152 human homologs with a valid NCBI ID.

KEGG pathways were ranked according to these 152 genes (see Section 2.2) and the 15 top ranked pathways were manually analyzed selecting the following 7 relevant networks (RN):

- *MAPK* (hsa04010): MAP kinases are involved in many different cellular functions like apoptosis, proliferation, survival, growth arrest, differentiation, motility, metabolism and senescence. Alterations in the MAPK signaling pathway have been reported in several tissues during aging [48].

- *Rap1* (hsa04015): promotes integrin and cadherin signaling and is activated by shear stress to regulate NO production in endothelial cells. *Rap1* deficiency in murine endothelium leads to endothelial dysfunction and hypertension, common diseases in the elderly [49].

- *Neuroactive ligand-receptor interaction* (hsa04080): cardiovascular diseases and aging are both associated with changes in the neurohumoral system, mainly adrenergic and renin-angiotensin systems. In particular, it has been reported a decrease in catecholamine-responsiveness in the elderly [50] and its regulatory effect in pathways disturbed in heart failure [51].

- *cAMP* (hsa04024): is a secondary messenger generated by adenylate cyclase, downstream to β-adrenergic signaling. Genetic inhibition of *cAMP* signaling in the mouse myocardium protects against heart failure, attenuates heart aging and prolongs lifespan (interestingly, in the heart of these mice, the $Raf1/MEK/ERK$ and the AKT pathways are activated) [52].

- *PI3K-Akt* (hsa04151): controls a multitude of biological processes in myocardial cells, e.g., cardiomyocyte hypertrophy, survival, energy production, contractility, and response to stress. Some evidences indicate that attenuation of the $PI3K/AKT$ pathway contributes to age related changes in myocardium [53].

- *Alzheimer's disease and Huntington's disease* (hsa05010, hsa05016): protein misfolding plays a crucial role in neurodegenerative diseases. Increasing evidences indicate that, in the myocardium, mechanical and oxidative stress together with other pathological conditions lead to protein

misfolding and that misfolded proteins play a relevant role in the onset and progression of cardiomyopathies [54]. Recently, extensive links have been identified between Alzheimer's disease and cardiovascular diseases in large-scale genome-wide association studies [55].

Interestingly after this manual review all selected pathways are from the green area of Figure 2.2.

As a result of the transcriptional and post-transcriptional information integration described in Section 2.3, three new TF nodes and seven miR nodes were globally inserted in the processed networks. Moreover, as a result of this process, the list of genes in the DEGN was connected and transformed into a new network thus obtaining a list of eight processed networks qualitatively depicted in Fig. 3-A. These networks were processed as described in Section 2.4, thus identifying the list of 35 central nodes reported in Table 1.

Table 1: List of 35 identified central nodes.

NAME	**TYPE**	**NAME**	**TYPE**
ADCY1	Gene	*PTOV1*	Gene
BRAF	Gene	*RAF1*	Gene
C00575	Gene	*RAP1A*	Gene
CHUK	Gene	*RAPGEF3*	Gene
CREB3	Gene	*RRAS2*	Gene
CSF1R	Gene	*ST8SIA4*	Gene
EIF4A2	Gene	*TECR*	Gene
ENO1	Gene	*hsa-miR-1248*	miR
FAS	Gene	*hsa-miR-208A*	miR
GNAQ	Gene	*hsa-miR-4691*	miR
MYH6	Gene	*hsa-miR-639*	miR
NDUFS8	Gene	*hsa-miR-661*	miR
MAP2K1	Gene	*hsa-miR-6728*	miR
MAP2K3	Gene	*hsa-miR-7113*	miR
MAPK1	Gene	*MYC*	TF
PIK3R5	Gene	*NFKB1*	TF
PLCB1	Gene	*TP53*	TF
PLEC	Gene		

Finally, the 8 networks were merged obtaining a very large network qualitatively reported in Fig. 3-B. The network complexity was reduced by collapsing protein nodes into their related coding genes (protein information are only required for intermediate processing) obtaining a network composed of 914 nodes (i.e., genes, TFs, and, miRs) and 6,527 edges. By filtering this complex network with the set of identified central nodes we were able to extract a sub-network containing 35 interconnected nodes qualitatively depicted in Fig. 3-C. A detailed view of the network depicting the 35 identified regulators and their interactions is reported in Fig. 5. This network represents the proposed in-silico regulatory

model of cardiac aging.

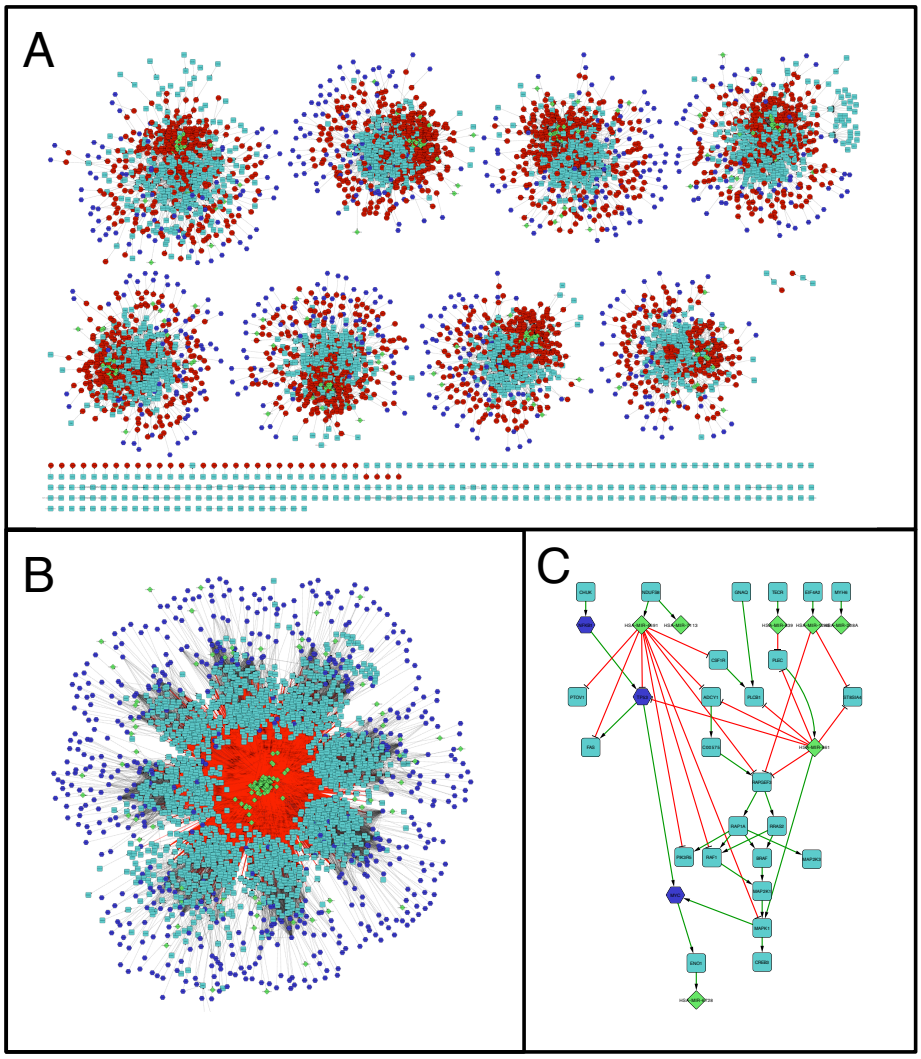

Figure 4: Conceptual processing steps. A – Seven KEGG networks (hsa0401, hsa04015, hsa04024, hsa04080, hsa04151, hsa05010 and hsa05016), and a list of 152 differentially expressed genes (extracted from GEO) were processed using ReNE to add transcriptional and post-transcriptional regulators (green nodes: miRs, dark blue nodes: TFs, red nodes: proteins). B – the processed networks were merged resulting in a large merged network. C – after centrality analysis nodes with highest betweenness centrality were extracted along with their regulatory links, resulting in a small filtered network.

All input data, intermediate and final results, procedures and source code

of the scripts used to process the data are reported in the supp-data.zip file available as supporting file for this paper.

4. Discussion

The proposed model was validated by both a very extensive literature review and by Western blot experiments on heart samples from young (2 month-old) and old (18-month old and 26 month-old) mice on two relevant genes of the model.

4.1. Literature validation

Literature validation confirmed that the nodes of the filtered subnetwork have been reported in different studies on heart aging response.

For the sake of clarity, the role of the identified genes, miRs and TFs was mapped to six main functional classes:

1. inflammatory responses,
2. senescence regulation,
3. beta-adrenergic signaling,
4. *MAP2K* and *ERK1/2* signaling pathways,
5. miRs,
6. Transcription factors.

Five out of the seven identified miRs and all three identified TFs were reported as involved in senescence or inflammatory processes. Their role is of particular interest because they can finely tune the network behavior shading new light into the complex regulatory mechanisms underlying senescence. Overall, 32 out of the 35 network nodes (94%) have been previously reported in studies on aging related processes. The two missing nodes, i.e., miR-4691and miR-7113, represent two interesting candidates for future investigations. In particular, miR-4691 with his role of regulatory hub with 9 regulated nodes is a particularly interesting node to be investigated in laboratory.

4.1.1. Inflammatory Response related genes

NDUFS8. mitochondria are necessary for the pro-inflammatory and pro-oxidant features of senescent phenotype. Their biogenesis stabilizes senescence via a crosstalk with the *ATM*, *Akt*, and *mTOR* signaling pathways. Since the increased mitochondrial mass is invariably reduced by rapamycin the *mTOR* pathway has a central role in mitochondrial regulation. *mTOR* inhibition results in a down expression of *mTOR* complex 1 (*mTORC1*), which integrates stress signals into the regulation of protein and lipid synthesis and autophagy, all of which are involved in the complex pathways mediating mitochondrial homeostasis. In this context, *NDUFS8* is a *mTORC1* downstream regulated gene and its presence may be an hallmark of mitochondrial regulation [56].

EIF4A2. is a member of the helicase family. Helicases are known as guardians of the genome given their role in various DNA metabolic pathways that include DNA recombination, replication, repair, transcription and telomere maintenance. *EIF4A2* seems related to aging by influencing protein translation and mitochondrial translocation efficiency [57].

ENO1. studies reported that *ENO1* is an auto-antigen that has been recognized as a common marker of systemic autoimmune diseases and inflammatory, degenerative and other pathological disorders. It is induced by cellular stress and promote cell growth, glycolysis, migration, and invasion [58].

CSF1R. the colony-stimulating-factor receptor-1 (*CSF1R*) is a type III receptor tyrosine kinase primarily reported as responsible for the proliferation, differentiation and survival of the monocyte–macrophage cell lineage, as well as their recruitment. Two ligands (*CSF1* and *IL34*) function through non-competitive binding to *CSF1R* by activating both phosphatidylinositol 3-kinase *PI3K*-dependent and *RAS*-activated protein kinase-dependent pathways. Senescent cells also revealed that *AKT* inhibition in *CSFR*-expressing cells is accompanied by the ectopic regulation of *CDKN1A*, *SOX2*, *OCT4* and *c-MYC* proteins. This establishes an interesting link between the expression of *CSF1R*, *AKT* activation and physical crosstalk between the *PI3K*, *AKT*, *STAT3*, and *NFKB* pathways [59].

PLCB1. acts as catalyst for the formation of inositol 1,4,5-trisphosphate and diacylglycerol from phosphatidylinositol 4,5-bisphosphate. This reaction plays an important role in the intracellular transduction of many extracellular signals. *PLCB1* has been identified as a pro-inflammatory regulator involved in several cardiovascular diseases as acute, inflammatory, and self-limited vasculitis, Kawasaki Syndrome, endothelial cell inflammation with varying degrees of pro-inflammatory cytokine expression, and coronary artery aneurysm. [60]

4.1.2. Senescence Regulation

PLEC. laminar shear stress (LSS) caused by blood flow is known to regulate endothelial function and to contribute to vascular health. However endothelial cell senescence seems to increase the incidence of cardio vascular disorders. *PLEC1* is an actin binding protein induced in endothelial cells exposed to LSS. Studies demonstrated that several actin binding proteins including *PLEC1* may play a critical regulatory role in the endothelial wound healing under LSS conditions [61].

BRAF. constitutive activation of *BRAF* may drive the proliferation and maturation/senescence of melanoblasts. In mature human skin, descendant melanoblasts reside in the epidermis but they colonize all the tissues of the body, such as the valves of the heart and the meninges [62].

GNAQ. its mutations have been associated with defects in platelet activation and aggregation, and port-wine stains. It is involved in estradiol-responsive pathway and produces age-related differences in functional pathways related to oxidative phosphorylation, synaptic plasticity, and estrogen responsive signaling cascades. *GNAQ* has also been recently reported as possibly responsible (with *NRAS*/*BRAF*-mutations) for transcriptional modifications in uveal melanoma and blue nevi that lead to melanoblast proliferation and melanocyte differentiation [62].

CREB3. is a regulator known to physically associate with the endomembrane system. It has been found ectopically expressed in abnormal lysosomes with impaired function. Abnormalities of lysosomal functions are characteristic of cellular senescence, organismal ageing, atherosclerosis, Alzheimer's and other diseases. Ectopic secretion of lysosomal proteases can, in fact, degradate extracellular matrix, contributing to atherosclerosis, arthritis, aneurysms formation, and other diseases [63].

MYH6

myosin-6 has been demonstrated ectopically regulated in senescent rats against young rats [64].

FAS. is a *TNF* receptor, shown to activate *NFKB*, *MAPK3/ERK1*, and *MAPK8/JNK* pathways. It is involved in transducing the proliferating signals in normal diploid fibroblast and T-cells. *FAS* appears impaired in senescence-associated secretory phenotype (SASP) proteases, i.e., cleave *FAS* ligand and other cell surface proteins required for effective immune function. The macrophage responses result declining with aging, potentially contributing to senescent cell accumulation in old age, which eventually results in a reduced immune function [65].

ADCY1. encodes a member of the of adenylate cyclase gene family. *ADCY1* has been identified as functionally responsible for enhanced forskolin-stimulated association with *ERK* that eventually contributes to *cAMP* elevation in vascular smooth muscle cells. *cAMP* over expression in the plasma membrane intensely strengthen the endothelial barrier function, resulting in cardiac rejuvenation, and suggests possibly regenerative potential in aged tissues. *ADCY1* ectopic activity is also proven sufficient to overwhelm the barrier protective effects of plasma membrane activity to promote endothelial gap formation [66].

CHUK. encodes a member of the serine/threonine protein kinase family. The encoded protein is an inhibitor of the transcription factor *NFKB* complex. In aging, reduced inhibition of NFKB is associated with the pro-inflammatory phenotype in vascular endothelium [67].

4.2. Beta-Adrenergic signaling

Chronic activation of beta-adrenergic signaling is deleterious to cardiac function resulting in increased heart rate, contractility, peripheral vasoconstriction and wall stress. Stimulation of beta-adrenergic receptors decreases anti-apoptotic and anti-oxidative stress signaling contributing to senescence and functional deterioration in a cascading effect with downstream induction of *cAMP* and *PKA* that act as inhibitors of the cardioprotective *Raf/MEK/Erk* pathways. In aging human heart, the functional responsiveness of the human cardiac beta-adrenoceptor system decreases along with all the effects mediated by *cAMP*.

PTOV1. androgens have been considered to reduce the incidence of ischemic myocardial disease in men, but they have also been reported to exert atherogenic effects on the human cardiovascular system through promoting plaque formation and enhancing monocyte adhesion to endothelial cells. In this context, *PTOV1* is considered to be one of the testosterone-induced genes involved in androgen-mediated stimulation of *VSMC* proliferation. *PTOV1* may thus play an important role in androgen-related atherogenesis in the male human aorta [68].

TECR. is a poorly characterized testosterone reduction enzyme member of steroid 5-alpha reductase family, which also contains *SRD5A2L2*, *SRD5A1*, *SRD5A2*, *SRD5A3*. *SRD5A1* and *SRD5A2* have been already identified as encoders for proteins targeted in treatments against prostate cancer and male pattern hair loss (male aging related diseases). However the role of *TECR* is still not clear. It has been shown to be involved in the elongation of very long chain fatty acids and reduction of a non-steroid lipid. *NbECR*, a *TECR* homolog in *Nicotiana Benthamiana*, has been identified as responsible for disorganized membrane structures and epidermal cell ablation. Those assumptions make *TECR* a candidate as processor for adrenergic and hormonal signals, capable to possibly lead to vascular membranes impairment [69, 70].

C00575 (cAMP). *cAMP* is a second messenger used for intracellular signal transduction such as transferring into cells the effects of hormones like glucagon and adrenaline, which cannot pass through the plasma membrane. *cAMP* also regulates the function of ion channels and a few other cyclic nucleotide-binding proteins such as *RAPGEF3*. Exchange proteins activated by *cAMP* (*RAPGEFx*), when *cAMP* binds, expose the activated *GEF* domain, allowing them to activate small *Ras*-like *GTPase*, such as *RAP1* [71].

RAP1. a *GTPase* superfamily member highly involved in cell motility and junction formation. It acts as a substrate for protein kinase A and interacts with *NADPH* oxidase (a group of multimeric enzymes whose activity results in the production of O2). In non-phagocytic cells, homologues of the *NADPH* oxidase were found in vascular endothelial cells and smooth muscle cells or transformed cells such as melanoma. Activation of *RAP1* strengthens cell junction

integrity and reduces cell migration by affecting actin cytoskeleton remodeling [72]. *RAP1* also acts as a protective protein for telomeres by relocalizing to the upstream promoter regions of hundreds of new target genes when critical telomere shortening happens. *RAP1* role in delaying senescence and concurring in DNA damage responses makes it (and its surrounding regulators) a good candidate for actively managing senescence [73].

4.3. MAP2K and ERK1/2 signaling pathways

Activation of β-adrenergic receptors results in the production of cyclic *AMP* which in turn phosphorylates many transcriptional factors via the *MAPK* pathway and thus, stimulates protein synthesis and cell growth. In the filtered network, we identified a central cluster of genes that recurs in several high relevance pathways: *Ras*, *Raf*, *MAP2K*, and, *ERK1/2* signaling pathways. Such pathways are also highly interconnected to each other showing a concurrent role in crucial cell-survival regulations. At the cellular level, the *ERK1/2* pathway regulates cell cycle progression, proliferation, cytokinesis, transcription, differentiation, senescence, cell death, migration, *GAP* junctions formation, actin and microtubule networks, and cell adhesion. *MAPK* pathway plays a role in many biological functions like cell cycle regulation, apoptosis, cell survival, senescence, differentiation, and cell growth and migration.

RRAS2. encodes a small *GTPase* involved in diverse processes including angiogenesis, vascular homeostasis and regeneration, and cell adhesion. *RAS* proteins interact with multiple effectors, including *RAF* kinases, *PI-3* kinases, and activate multiple downstream signaling cascades. Of particular interest the RAS/mitogen-activated protein kinase *RAS/MAPK* signaling pathway, which plays a central role in cellular proliferation and differentiation. *RRAS2* also plays a key role in Noonan syndrome, an autosomal-dominant disorder characterized by congenital heart defects. [74]

ST8SIA4. is predominantly expressed in immune cells. It plays an important role in substrate recognition that modulates cell adhesion and signaling. Furthermore, *ST8SIA4* mediates the activity of *PI3K/Akt* signal pathway in a competitive way. Inhibition of *PI3K/Akt* pathway is also able to attenuate the effects caused by the overexpression of *ST8SIA4* on *MDR* [75].

RAPGEF3. is necessary for antioxidant and antiapoptotic effects of exendin, which has an essential role in cardiomyocytes and is involved in the development of heart failure. *RAPGEF3* also drives the inhibitions of oxidative stress and apoptosis in cardiomyocytes thanks to *GLP-1R* which helps in cardioprotection, and also inactivates myosin light chain kinase [76]. Elevated levels of cAMP activate *RAPGEF3* which, in turn, leads to *GTP* binding to *Rap1*. This causes changes to the cortical cytoskeleton and organization of vascular endothelial cadherin in the endothelial junctions, leading to reduced endothelial permeability. Ectopic expression of *RAPGEF3* may thus result in cell aging effect [77].

RAF1. its activation engages the phosphorylation cascade starting from *RAF1* (*MKKK*) to *MAP2K*, and then to the *MAPK* family. *RAF1* was found to be an activator of *MAP2K* in mammalian cells and to form a stable complex with *MAP2K*. Prototypically, growth factors activate *RAS* which recruits and activates *RAF* at the plasma membrane. Once activated, *RAF* phosphorylates and activates *MAP2K*, which in turn activates *ERK1/2*. Eventually activated *ERK1/2* can phosphorylate downstream proteins in the cytoplasm or nucleus, including many transcription factors. Cardiac specific deletion of *RAF1* leads to heart failure without hypertrophy in the absence of external stress, and also increases apoptosis [78].

PIK3R5. activates the *Ras-Raf-MAP2K-ERK* signalling cascade. *PI3K* also represents a downstream target of active *RAS*. Suppression of *PI3K* prevents lipofuscin accumulation in aged heart tissue. Lipofuscin is thought to be generated from damaged proteins and post mitotic cells (such as cardiac myocytes) accumulate a large amount of lipofuscin in old age. Inhibition of *PI3K*, in fact, prevents the expression of cellular senescence markers (by decreasing the accumulation of lipofuscin in heart tissue) and most of the age-related changes of gene expression [79, 80].

MAPK1/MAP2K3/MAP2K1. the MAPK cascade is composed of *RAS*, *RAF1*, *MAP2K*, *MAPK1* and *RSK*. It spans from the plasma membrane to the nucleus and trasduces the mitogenic signals downstream from the tyrosine kinase membrane receptor. The first subgroup of *MAPK* (*ERK1/2*) plays an important role in the signaling pathway responsible for the *G0* to *G1* transition in the cell cycle. The MAPK1 pathway is coupled by a GTP-binding protein (Gαq-protein isoform in the heart) that activates the *ERK1/2* pathway. The activation of the *ERK1/2* pathway in heart is lead by a GTP-binding protein (Gαq-protein isoform) that produces diacylglycerol (*DAG*) and inositol-3,4,5-triphosphate (*InsP3*). *DAG* then activates the *MAPK1* signaling cascade, while *InsP3* activates the *MAPK1* pathway via both *RAS*-dependent and -independent mechanisms. The activity of *MAPK1* is stimulated by multiple extracellular stimuli and oncogenes. Its activation regulates several cellular protein kinases, and catalyzes the phosphorylation of some nuclear transcription factors [80]. Finally, mutations in *HRAS*, *KRAS*, *BRAF*, and *MAP2K* have also been discovered to be involved in other genetic disorders with cardiac developmental defects, such as LEOPARD syndrome, cardio-facio-cutaneous syndrome, and Costello syndrome [78].

4.3.1. miRs

MiRs are key regulators of gene expression. By binding to multiple genes at once, they create a complex network of post-transcriptional fine co-regulations.

miR-6728. has been demonstrated to be able to regulate several genes associated with cardiometabolic phenotypes [81].

miR-639. recently emerged as potential novel biomarker of Human bone marrow mesenchymal stem cells aging [82].

miR-661. has been demonstrated to have a regulatory role in small GTPase signalling. It also has an active role in phosphorylation of myosin II and up regulates the cell invasion, indicating a possible oncogenic miR [83]. *miR-661* is also highly associated with nano-sized vesicles released by activated *CD4+* T lymphocytes release [84] showing a correlation with inflammatory response, and with the modulation of the endogenous levels of *iNOS* and nitrite production via *MTA1* mediated control. [85]

miR-1248. studies investigating the relationships between miR profiles and aging reported that serum expression levels of *miR-1248* were significantly lower in old participants (mean age, 64 years) than in young participants (mean age, 30 years). *miR-1248* was found to regulate the expression of mRNAs related to several cytokines (age-associated cytokines *IL6* and *IL8*) and inflammatory-associated pathways (including NF-kB), thus suggesting a relevant role in the aging process. *miR-1248* also influences the impaired DNA repair capacity, then decreased levels of *miR-1248* due to aging directly affect DNA repair pathways [86].

miR-208a. targets thyroid hormone receptor associated protein 1 (*THRAP1*) and myostatin, which are negative regulators of muscle growth and hypertrophy. It is particularly relevant in cardiac myocytes, since its transgenic overexpression in the heart is sufficient to induce hypertrophic growth and arrhythmias in mice. *miR-208a* also has a key role in the expression of cardiac transcription factors, like homeodomain-only protein (*HOP*) and *GATA4*, and for the gap junction protein connexin. Upregulation of *miR-208a* was observed in samples of infarcted heart tissue from patients with myocardial infarction compared to healthy adult hearts. Given its high sensitivity in early setting of acute myocardial infarction (AMI), *miR-208a* seems a reliable biomarker for early AMI diagnosis in humans [29]

4.3.2. Transcription factors

TP53. TP53 encodes a tumor suppressor protein, whose expression is stabilized by DNA damage. In order to suppress tumor development, *TP53* has a key role in leading to senescence as mechanism to prevent tumor formation and to have a trade-off relation with cancer. Overexpression of *TP53*, in fact, induces cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism [58].

NFKB1. is a binding subunit of the *NFKB* protein complex, a transcription regulator that acts as hub by aggregating multiple intra- and extra-cellular stimuli (i.e., cytokines, oxidant-free radicals, ultraviolet irradiation, pathogens). Ectopic activation of NFKB has been associated with inflammatory diseases and inappropriate immune cell development or delayed cell growth. In particular, the *NFKB1* subunit is known to be responsible for proinflammatory arterial

phenotype developed with aging, and is associated to vascular dysfunctions due to impaired nitric oxide processing [87].

MYC. is a multi-functional, nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation. *MYC* is necessary and sufficient for the regulation of Nucleostemin (*NS*) (a nucleolar protein regulating stem cell proliferation and pluripotency). *NS* regulation is essential to preserve the regenerative potential of aging stem cells by antagonizing senescence and by enhancing myocardial regeneration. Levels of *MYC*, positive transcriptional regulators of the cell cycle, are decreased in myocardial senescence along with elevated levels of *TP53*, which eventually result in enlarged myocytes, reduced number of cardiomyocytes, disfigured mitochondrial morphology, telomere attrition, up-regulation of molecular markers of senescence, and decline in cardiac function resembling a heart failure phenotype [88].

4.4. Wet Lab assessment

The expression levels of two proteins belonging to the *MAPK* cascade (*RAF1* – coded by *RAF1* gene and *MEK1* – coded by the *MAP2K1* gene) and identified in the in-silico analysis were tested by Western blot on heart samples from young (2 month-old) and old (18-month old and 26 month-old) mice.

Western blot was performed as described in [89]. Briefly: hearts were lysed in RIPA buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl,1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing Roche complete protease inhibitor cocktail, 10 mM NaF, 1 mM PMSF and 1 mM Na3VO4. Protein extracts were clarified with three sequential centrifugations for 20 minutes at 20,000 g, at 4°C. The following antibodies were used: *MEK1/2* (Cell Signaling), *RAF1* (Santa Cruz) and *Vinculin* (Sigma).

Both proteins showed a decreased expression in old mice (see Fig. 6), supporting in-silico data. This is particularly relevant since the *MAPK* pathway plays a crucial role in adaptation to stress conditions and it has been involved in both adaptive or maladaptive cardiac remodeling [90]. Activation of the *MAPK* cascade has been described as a protective signal in a number of stressful conditions as myocardial infarction [91, 92, 93] and pressure overload [94, 95]. In this view, underexpression of components of the pathway may cause insensitivity to external stimuli and an impairment in heart adaptation.

5. Conclusions

This paper presented a pure computationally inferred model that is able to represent the relationship between key genes and miRs involved in cardiac senescence processes. Interestingly, all the newly introduced regulators, except for two miRs (i.e., miR-4691 and miR-7133), appear strongly related to previous studies on aging related mechanisms. Giving the results obtained on the other regulators, these two miRs represent good candidate for further studies to elucidate their possible involvement in cardiac aging.

6. Human and animal rights

The use of animals was in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health, and was approved by the Animal Care and Use Committee of the University of Torino.

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8. Supporting Files

supp-data.zip: This file is organized into a set of folders containing all information required to reproduce the full computational analysis presented in this paper:

- Step 0 - Input files
- Step 1 - Pathway processing
- Step 2 - Network analysis script
- Step 3 - Model construction

Each folder contains a README file describing in details its content.

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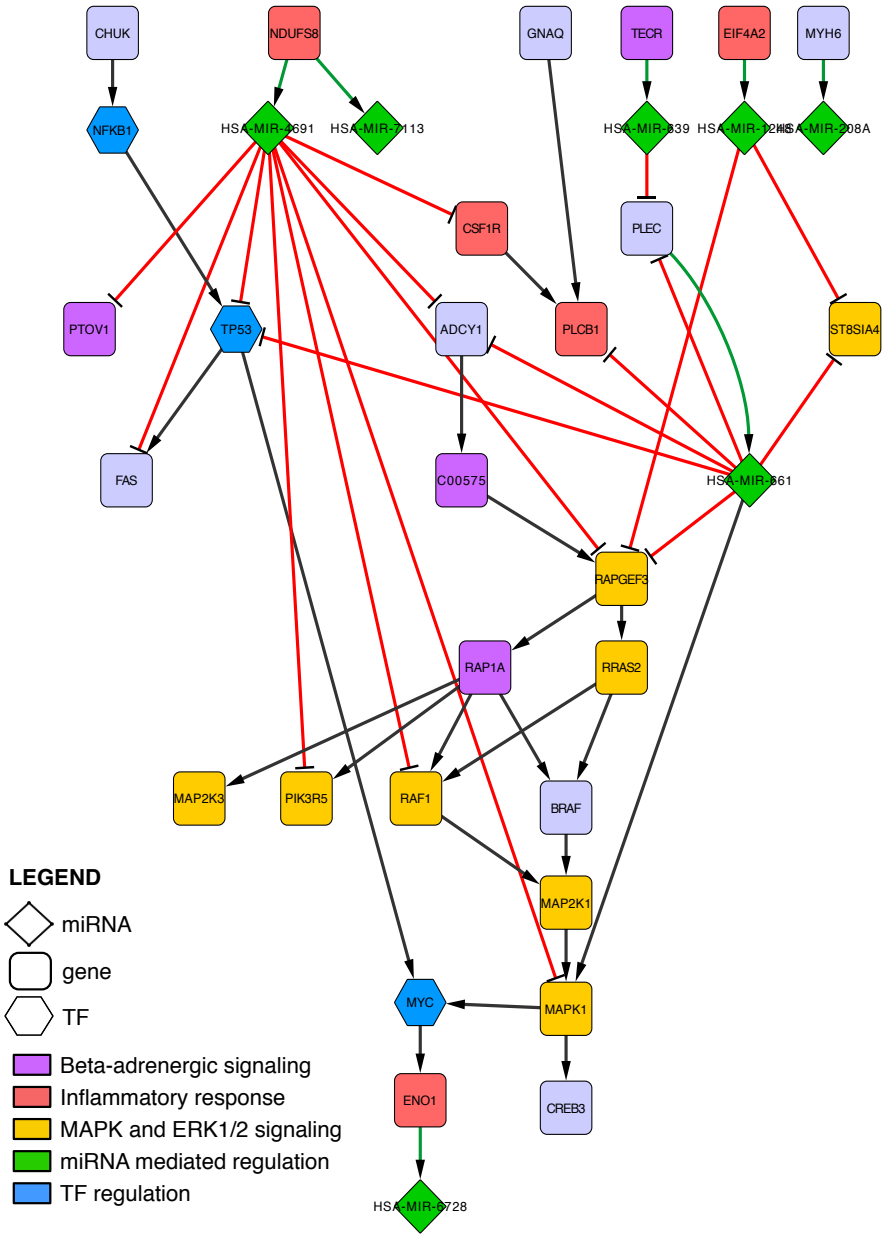

Figure 5: The resulting cardiac aging model. Nodes are colored to highlight their different roles: in purple Beta-Adrenergic signaling related nodes, inflammatory response in red, MAPK-Erk1/2 in yellow, senescence regulation in pale violet, microRNAs in green, and, transcription factors in blue. Edges are colored according to the following criteria: red for miR post-transcriptional repression, green for miR co-transcription with their host gene, and black for regulations inherited from KEGG's pathways.

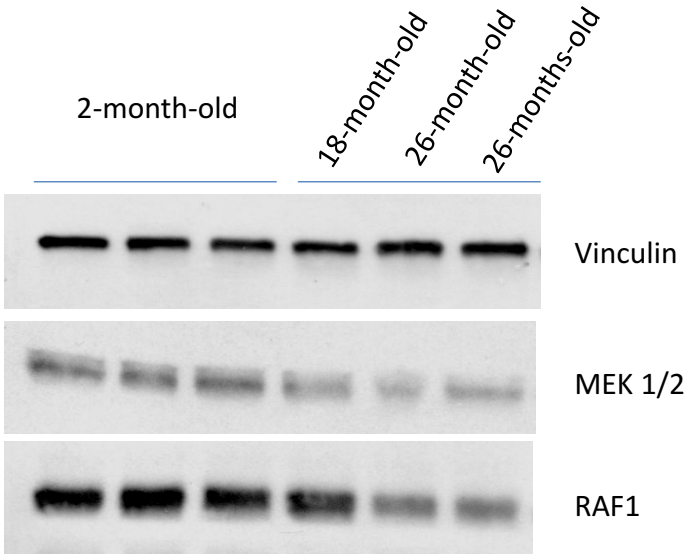

Figure 6: Western blot analysis of heart protein extracts from young (2 month-old) and old (18-month old and 26 month-old) mice for MEK1/2 and RAF1 expression. Vinculin was used as loading control.