The admiR-able advances in cardiovascular biology through the zebrafish model system

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REVIEW

The ad*miR*-able advances in cardiovascular biology through the zebrafish model system

Dafne Gays · Massimo Mattia Santoro

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Abstract MicroRNAs are small non-coding RNAs endogenously expressed by all tissues during development and adulthood. They regulate gene expression by controlling the stability of targeted messenger RNA. In cardiovascular tissues microRNAs play a role by modulating essential genes involved in heart and blood vessel development and homeostasis. The zebrafish (*Danio rerio*) system is a recognized vertebrate model system useful to study cardiovascular biology; recently, it has been used to investigate microRNA functions during natural and pathological states. In this review, we will illustrate the advantages of the zebrafish model in the study of microRNAs in heart and vascular cells, providing an update on recent discoveries using the zebrafish to identify new microRNAs and their targeted genes in cardiovascular tissues. Lastly, we will provide evidence that the zebrafish is an optimal model system to undercover new microRNA functions in vertebrates and to improve microRNA-based therapeutic approaches.

Keywords MicroRNA · Zebrafish · Heart · Blood vessel · Development

MiRNAs: function and mechanism

MicroRNAs (e.g., miRs or miRNAs) are evolutionary conserved small non-coding RNAs, usually 21–25 nt long, that regulate gene expression at a post-transcriptional level. MiRNAs act by impairing target mRNA expression through an antisense mechanism. They recognize partial or full complementary sequences that are present mainly in the 3′-UTR (untranslated region) of mRNAs and thereby inhibit protein synthesis [1, 2]. Based on their locations in the genome, microRNAs can be intergenic and so transcribed individually as single transcriptional units or intragenic (intronic or exonic) and then transcribed together with the genes in which they are located. Often, more miRNAs are located close by in the genome and form clusters; in this case they are transcribed in a single polycistronic unit that after cleavage releases single microRNAs [3, 4].

MiRNAs are transcribed by RNA polymerase II as a stem-loop precursor known as pri-miRNA. In the nucleus it is cleaved by a ribonuclease III enzyme called *Drosha* with its cofactor DiGeorge syndrome critical region 8 (DGCR8) into 60–100-nucleotides hairpins called pre-miRNA that can leave the nucleus through exportin 5. In the cytoplasm a second RNase, *Dicer*, removes the loop that generates a duplex containing the mature miRNA sequence and a 'passenger strand' miRNA sequence (called *star*). Subsequently, the miRNA duplex is recognized by the protein complex *Argonaute* to form the RNA-induced silencing complex (RISC). Thus the 'seed' region of mature miRNA (nucleotides 2–8) guides RISC on target mRNA(s). MiRNAs post-transcriptional regulation of gene expression can be achieved through different mechanisms: (1) degradation of the targeted mRNA and/or (2) translation repression. Recently, a role for miRNA in translational simulation has also been proposed [2, 5–7]. While miRNAs commonly repress their mRNA targets, in rare cases it has been observed that miRNA stimulation of mRNA translation occurs [8].

MiRNAs have numerous high- and low-affinity targets and therefore have the potential to modulate multiple pathways. Often many of these targets can themselves

D. Gays · M. M. Santoro (✉)
Department of Biology, Biochemistry and Genetics, Molecular Biotechnology Center, University of Turin, Via Nizza 52, 10126 Turin, Italy
e-mail: massimo.santoro@unito.it

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control the expression and function of miRNAs generating regulatory circuits [9–12]. In positive feedback circuits, miRNAs repress a repressor, leading to activation of transcription factors that activate miRNA expression. The regulatory loop formed between miR-1, HDAC4, and MEF2 exemplifies this form of regulation in skeletal muscle [13]. Here, repression of HDAC4 by miRNA-1 derepresses the activity of the MEF2 transcription factor, which in turn activates expression of miRNA-1 and other target genes. On the other hand, miRNAs can also establish negative feedback circuits by repressing activators of miRNA/mRNA expression. In a negative regulatory loop, miRNAs repress transcription factors that are required for miRNA expression, leading to decreased expression of miRNAs. The reciprocal regulatory interaction among a posterior Hox gene, nob-1, and miRNA-57 has been found to be essential for regulation of posterior cell fate determination in *C. elegans* [14]. On one hand, the Hox gene is required for normal activation of miRNA-57 expression, and on the other, the Hox gene functions as a direct target of and is repressed by the miRNA. Given the conservation of the two genes, a negative feedback loop between Hox and miRNA genes might be broadly used across species to regulate cell fate along the anteroposterior axis [14].

In addition, 3′-UTRs can contain binding sites for multiple miRNAs, allowing redundancy and cooperation between them. Collectively, these mechanisms establish a complex network of gene regulation that fine-tunes the biological processes [9, 15].

MiRNAs are widely expressed in almost all vertebrate tissues, and functional roles for miRNA during development have been extensively described [16, 17]. MiRNA expression has been linked to a wide range of physiologic processes, including development, differentiation, and cell proliferation. Therefore, it is likely that miRNAs participate in nearly every physiological process since miRNA expression is temporally and spatially regulated.

The role of miRNAs in shaping heart and vascular networks in vertebrates

The heart is the first organ to form and function during embryo development, and its continued contraction is essential for life. Processes that lead to heart development are very complex, and their alterations can result in congenital heart disease; functional abnormalities in the adult heart result in a variety of disorders, among which are arrhythmias, cardiomyopathies, heart failure, and sudden death [18, 19]. In vertebrates, the linear heart tube forms from the migration of bilateral populations of myocardial and endocardial cells and their fusion at the midline. Then, morphogenetic movements lead to the formation of a looped, multichambered organ [20]. The heart has the function to pump blood in the vascular system. The vascular system is a highly organized network of arteries, capillaries, and veins that supplies virtually all body tissues, enabling an efficient exchange of oxygen and nutrients and removal of waste products. The vascular system is indispensable for embryonic development and adult life, and its aberrant alterations are associated with numerous diseases, including cancer, atherosclerosis, retinopathy, and stroke [21]. The generation of the vascular system starts with a process called vasculogenesis that give rises to the primary vascular plexus of the embryo from which new vessels sprout in a process called angiogenesis [22]. During this phase, the vascular plexus progressively expands and remodels into a highly organized and stereotyped vascular network. The development and maturation of the cardiovascular system also require the recruitment of smooth muscle cells that stabilize vessels and regulate blood flow [23, 24]. After birth, angiogenesis still contributes to organ growth, but, during adulthood, most blood vessels remain quiescent until they receive physiopathological cues that stimulate endothelial cells (ECs) proliferation.

Cardiovascular formation and function are precisely controlled by a network of transcription factors closely intertwined with families of microRNAs that modulate multiple aspects of cardiovascular development, function, and dysfunctions [12]. The importance of microRNAs in the regulation of cardiovascular development and function has been demonstrated through earlier studies on vertebrate mutants of Dicer, the enzyme required for microRNA biogenesis [25–29]. The complete loss of Dicer function leads to severe developmental defects in both zebrafish and mouse. To overcome the early lethality, conditional Dicer mutant mice were created [30]. Cardiac-specific deletion of Dicer leads to cardiomyopathy, heart failure, and postnatal lethality [31]. Again, in mammals specific inactivation of Dicer in endothelial cells shows that endothelial microRNAs are important for postnatal angiogenic responses to a variety of stimuli, including VEGF, tumors, limb ischemia, and wound healing [32]. In mouse the loss of Dicer in vascular smooth muscle cells lead to a dilated, thin-walled blood vessels that also exhibited impaired contractility [33]. Zebrafish maternal-zygotic Dicer mutants (that lack both maternal and zygotic Dicer) display abnormal morphogenesis during gastrulation, brain formation, somitogenesis, and heart development. However, it has been shown that the injection of miRNA-430 rescues early gastrulation defects, enabling the analysis of the loss of microRNAs in tissues that otherwise would not develop [28, 34]. Recent studies on zebrafish *Dicer* mutant embryos demonstrated excessive endocardial cell formation, suggesting a role for miRNA in cardiac development [35]

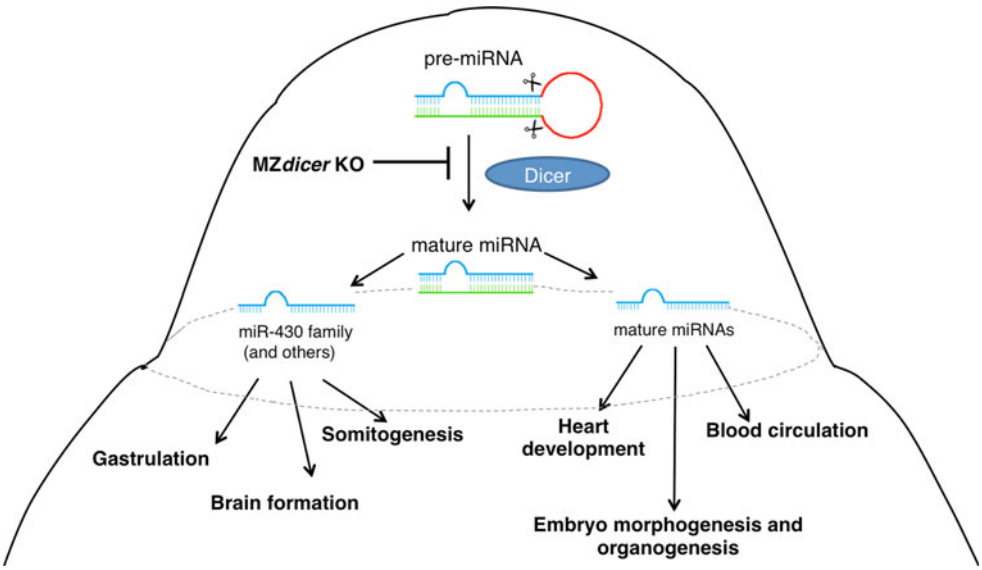

Fig. 1 Molecular and functional consequences of the loss of Dicer activity in maternal-zygotic dicer mutants embryos (MZdicer KO). In zebrafish mutants lacking dicer enzymatic activity, pre-microRNAs are not processed in mature microRNAs, leading to several developmental defects. The lack of miR-430 miRNAs leads mainly to gastrulation, brain formation, and somitogenesis defects. The loss of other microRNAs affects embryo morphogenesis and organogenesis, as well as heart development and blood circulation

(Fig. 1). These studies indicate that the zebrafish system can be a valuable tool to investigate microRNA's function in vivo, especially for studying cardiovascular development and homeostasis where this model presents some unique features among other vertebrate and mammalian models [36].

The zebrafish model system to study cardiovascular miRNA functions

The zebrafish as a model for cardiovascular studies

The zebrafish (*Danio rerio*) system has emerged in the past years as an ideal vertebrate model organism to study a wide variety of biological processes [37–39]. Some of the advantages of the zebrafish animal model system include high fecundity, external fertilization, rapid development, and optical clarity. Last but not least, the zebrafish has low-cost maintenance compared to other vertebrate model systems. The importance of zebrafish for cardiovascular research relies on some unique characteristics such as the early development of a functional cardiovascular system (beating heart, aorta, cardinal vein, and blood) that is already formed by 24 h post-fertilization (hpf) [20, 40]. Since the zebrafish embryo is relatively small and aquatic, oxygen can diffuse passively through tissues, and thus embryos can live more than 3 days without a functional cardiovascular system [41]. This allows genetic manipulation of cardiovascular development for longer than it would be in mammals, where the absence of a functional cardiovascular system is fatal at its early stage of development. Until 5 days post-fertilization (dpf), the embryos are nearly transparent, allowing in vivo visualization during development (even at single-cell resolution) of the heart, blood vessels, and other tissues, without instrumentation or manipulation other than the use of a stereomicroscope. Furthermore, the generation of tissue-specific transgenic lines is relatively easy thanks to the availability of hundreds of fertilized eggs with the injection of DNA and RNA constructs [42]. Such a method usually uses a native tissue-specific promoter to drive expression of a fluorescent reporter protein, such as green fluorescent protein (GFP). The following transgenic lines are of great interest for cardiovascular studies: Fli1:GFP (which expresses GFP in endothelial cells and some neural crest-derived cells) [43], kdrl:GFP (which expresses GFP localized to endothelial cells) [44], gata1:dsRED (which expresses dsRED in erythrocytes) [45], cd41:GFP (which expresses GFP in thrombocytes) [46], cmlc2:GFP (which expresses GFP in cardiomyocytes) [47], and tgnl:Cherry (which expresses cherry in smooth muscle cells) [48]. Coupled with impressive optical clarity, these transgenic lines allow observation of in vivo cellular behavior in a way impossible in other models. Transparent zebrafish embryos are also well suitable for in vivo time-lapse imaging. The fast acquisition speed of a spinning disk and two-photon confocal microscopy reduces the recording time significantly when millimeter-sized embryos need to be imaged at high resolution and at short time intervals. Light sheet fluorescence microscopy could also be very useful in zebrafish [49, 50]. These attributes, including the possibility of carrying out gain- and loss-of-function studies have led to the emergence of the zebrafish as an excellent embryological model that provides a unique opportunity to uncover novel insights into the molecular genetics of development and disease.

More recently, the zebrafish has become an attractive model to study the effects of genetic variations identified in

patients with cardiovascular defects by candidate gene or whole-genome-association studies [51, 52]. Moreover, zebrafish are suitable for forward genetics approaches, which allow the unbiased identification of novel and unanticipated cardiovascular genes. Small and large mutagenesis screens have been conducted in zebrafish and have led to a wealth of heart and endothelial mutants, several of which have already been characterized in detail and positionally cloned [41, 53]. Zebrafish mutants with various cardiovascular phenotypes that closely correlate with human disease, such as congenital heart disease, cardiomyopathies, and arrhythmias, have also been isolated [54]. The pool of zebrafish mutants for which the causal gene mutation has been identified is constantly growing. The human orthologs of several of these zebrafish genes have been shown to be involved in the pathogenesis of human cardiovascular diseases (CVD), known to be the most common cause of worldwide deaths [51, 54]. Cardiovascular zebrafish models also provide the opportunity to develop and test novel therapeutic strategies, using innovative technologies such as high-throughput in vivo small molecule screens.

The zebrafish as a model for miRNA studies

Zebrafish is a helpful model system to study miRNA function and characterize miRNA:mRNA target interactions [55–58]. Important information regarding the conservation of miRNA genes in vertebrates has been derived from the identification and characterization of miRNAs in zebrafish. *Danio rerio* have also proven to be a particularly valuable system to study miRNA functions in developmental processes. In the last decade different analyses have led to the identification of 415 zebrafish miRNAs, and based on seed sequence identity, it has been possible to group these into 44 families [59–61]. However, the exact number of zebrafish miRNAs might be higher, since genome sequencing and annotation has not been fully completed. Thus far, most zebrafish miRNAs exhibit tissue-specific patterns of expression [62].

miRNA expression analyses

To understand the function of specific microRNAs, it is important to determine their spatial and temporal expression pattern. Several methods have been developed to evaluate miRNA expression in zebrafish. Due to their relatively small size and optical transparency, zebrafish are useful for whole-mount in situ hybridization (ISH) analyses. However, compared to gene expression analysis, ISH detection of miRNAs can sometimes be challenging because of the microRNA's relatively small size (usually 21–25 nt), which then lowers the probe signals. Therefore, non-radioactive-labeled synthetic RNA oligonucleotide probes, such as 2-*O*-methyl RNA oligonucleotides, and locked nucleic acids (LNA) are currently used since they can overcome this problem, allowing an overall enhancing of the probe signal [6, 63]. To distinguish between genes that encode the same mature miRNA sequence, Postlethwait and collaborators [64] developed the use of digoxigenin-labeled riboprobes designed to bind to miRNA primary transcripts, allowing the observation of miRNA expression for both intergenic and intronic miRNAs. Alternatively to ISH, zebrafish microRNA expression patterns have also been investigated by qPCR, microarray, RNA-seq, and Northern blot analyses at different developmental stages and in different tissues [55, 59, 65]. As a result, a set of specific miRNAs displays cardiovascular-specific expression patterns, suggesting functional roles in differentiation or homeostasis of these miRNAs during development in zebrafish.

Control of microRNA expression

Modulation of miRNA expression can be achieved in several ways in zebrafish. For gain-of-function studies, the most widely used technique is the injection in one-cell eggs of double-stranded miRNA mimics to promote single miRNA overexpression [66, 67]. It must be taken into consideration that in this way the specific miRNA is overexpressed not only in the tissues where it is endogenously expressed, but also in the entire embryo. However, it could be useful to mimic situations in which a microRNA is spatio-temporally deregulated. To analyze the role of microRNA gain of function in a specific tissue, DNA constructs in which a tissue-specific promoter controls pri-miRNA expression can be used. More sophisticated genetic techniques based on the CreERT2/loxP system have been described in zebrafish, and they can also provide temporal and tissue-specific miRNA expression in zebrafish embryos [68, 69]. These strategies will allow the study of miRNA functions not only in the developing embryos, but also in adult animals and their exclusive tissues of interest.

Inhibition of specific miRNAs can be obtained by the injection of miRNA modulators, among which are cholesterol-modified antagomirs, modifications such as 2′ *O*-methoxyethyl phosphorothioate or 2′ fluoro substitutions, as well as oligonucleotides stabilized by LNA phosphorothioate chemistries. Although these techniques, especially antagomirs, are successfully used in mammals, they do not work quite efficiently in zebrafish embryos, mainly because their chemistry is not compatible with the lipid-enriched zebrafish yolk sac [70]. Conversely, antisense morpholino phosphorodiamidate oligonucleotides (MOs) have been extensively used to knock down gene functions in zebrafish embryos and larvae [71–73]. To inhibit specific miRNAs,

MOs must be designed to block the processing of pre-miRNA or the activity of the mature miRNA [74, 75]. A recent alternative approach to inhibit miRNA function in vivo is to use miRNA 'sponges' or 'decoys.' They contain several miRNA-binding sites, and, when present in a cell as RNA, act as competitive inhibitors for miRNA binding [76, 77]. Placing a miRNA sponge in the 3′-UTR of a reporter gene (dGFP in most cases), it is also possible to obtain information about the miRNA expression pattern and to down-modulate it at the same time. Interestingly, miRNA sponges have also been used to generate miRNA tissue-specific and stable knockdown [78, 79]. The use of sponges can be useful to overcome morpholinos off-target effects and to obtain a stable or prolonged knockdown not achievable with morpholinos because of their relative short half-life. Recently, targeted gene inactivation via zinc finger nucleases (ZFN) has been established for zebrafish genome [80, 81]. This technique, allowing the introduction of targeted lesions in the zebrafish genome, could, in principle, be used to selective targeted pre-miRNA sequences. The TALEN technology could also be used to induce somatic mutagenesis in zebrafish microRNA genes [82, 83].

Finally, compared to mammalian models the zebrafish system also has the unique feature of being able to perform "transient" knockdown (by morpholino) and rescuing (by mimics) of microRNAs in whole embryos. Such genetic techniques cannot be achieved so easily in rodents in a short period of time [36].

Target prediction and validation tools

Several open-access bioinformatic databases have been developed to facilitate the analysis of miRNAs and their target prediction. However, prediction of miRNA–mRNA interactions still remains challenging, owing to the short length of miRNAs, requirement of only partial homology for binding, redundancy among members of an miRNA family, and the existence of multiple putative miRNA recognition sites [84, 85]. The majority of the computational target prediction programs are based on several features, such as complementarity between the 5′-seed of the miRNA and the 3′-UTR of the target mRNA, thermodynamic stability of the miRNA-mRNA duplex, conservation among species, and the presence of several miRNA target sites. However, in zebrafish different in silico target prediction programs exist. They use prediction algorithms based on mammalian counterparts [86]. Such prediction programs must be used carefully in zebrafish studies since (1) the zebrafish genome is not fully sequenced, (2) only a limited number of miRNA targets have been experimentally validated, and (3) there is low conservation among species regarding mature microRNA sequences as well as the target prediction. Experimental high-throughput studies (such as poly(A)-site mapping followed by RNA-Seq) are needed to allow for generation of zebrafish precise prediction algorithms [27]. To validate predicted miRNA–mRNA interactions, several in vitro experimental approaches can be used, such as biochemical methods (luciferase assays, qRT-PCR, Western blot, RNA seq), "omics" approaches (SILAC, LAMP), and RISCome analysis (quantification of mRNAs in the RNA-induced sequencing complex; RISC RNA sequencing, RIP-chip) [87]. The zebrafish system allows the validation of target mRNA in vivo. Novel techniques have been recently implemented in the zebrafish model such as fluorescent miRNA sensor and MO target protector. In the first case, validation of the target prediction is tested using a reporter assay based on monitoring GFP/cherry fluorescence in zebrafish embryos microinjected with mRNA encoding the fluorescent reporter fused to the 3′-UTR of the target gene in the presence or absence of a specific miRNA duplex [67, 88]. In this assay, a decrease in fluorescence in the presence of miRNA duplex indicated miRNA-mediated repression and then the confirmation of the target in vivo. In the second case, MOs are designed to be complementary to specific miRNA-binding sites in target mRNAs. In this way, MOs have been shown to efficiently protect the target mRNA from translational inhibition or degradation [89, 90]. To study the regulation of a particular target, it is important to first establish that the 3′-UTR is regulated by a particular miRNA. miRNAs may speed degradation or slow translation of their targets, but repression caused by either mechanism can be assessed by measuring the protein output of a reporter. The efficiency of "target protectors" to block the interaction of miRNAs with a particular binding site in a target mRNA can also be investigated by co-injections with reporter mRNAs containing a GFP/cherry coding sequence and a 3′-UTR region with the miRNA-binding site [65, 91]. Subsequently, a tested target protector can be used to study whether protection of a specific target mRNA from silencing has any biological effect also in a tissue-specific manner (by generation of a cell autonomous miRNA sensor) [65, 67].

MicroRNAs involved in heart and vascular development and homeostasis in zebrafish

The functions of individual miRNAs in normal and pathological angiogenesis are just starting to be unveiled. Single miRNAs may regulate cardiovascular functions either cell-autonomously or non-cell autonomously. Recent progress toward understanding the functions of specific miRNAs in cardiovascular development and homeostasis has been done. Here, we described those miRNAs whose

Table 1 Summary of vertebrate miRNAs involved in cardiovascular development and homeostasis in zebrafish

microRNA	Targets	Functions	Technique	Reference
Cardio(vascular)				
23	Has2	Cardiac valve formation	Knockdown (morpholino); overexpression (mimic)	Lagendijk et al. [35]
	Icat Tmem2			
133	Mps1	Cardiac regeneration	Conditional knockdown (transgene); overexpression (transgene)	Yin et al. [79]
	Cx43			
138	aldh1a2	Cardiac patterning	Knockdown (morpholino)	Morton et al. [70]
	cspg2		Overexpression (RNA injection)	
143	aldh1a2 rxrab	Cardiogenesis	Knockdown (morpholino)	Miyasaka et al. [100]
	add3		Overexpression (mimic)	Deacon et al. [101]
218	Robo1	Migration of heart precursors	Knockdown (morpholino)	Fish et al. [102]
(Cardio)vascular				
24	Pak4	Cardiac and patterning angiogenesis	Overexpression (pre-miRNA injection)	Fiedler et al. [110]
	Gata2			
31	PROX1	Vascular lineage-specific differentiation and sprouting	Knockdown (morpholino)	Pedrioli et al. [112]
			Overexpression (pre-miRNA injection)	
92a	ITGA5	Patterning angiogenesis	Overexpression (pre-miRNA injection)	Bonauer et al. [114]
126	spred1	Vascular integrity and maturation	Knockdown (morpholino)	Fish et al., [119]
	pi3kr2		Conditional overexpression (transgene)	Nicoli et al. [67]
221	cdkn1b	Endothelial tip cell behaviour	Knockdown (morpholino)	Nicoli et al. [65]
	pi3kr1		Overexpression (miRNA duplex)	

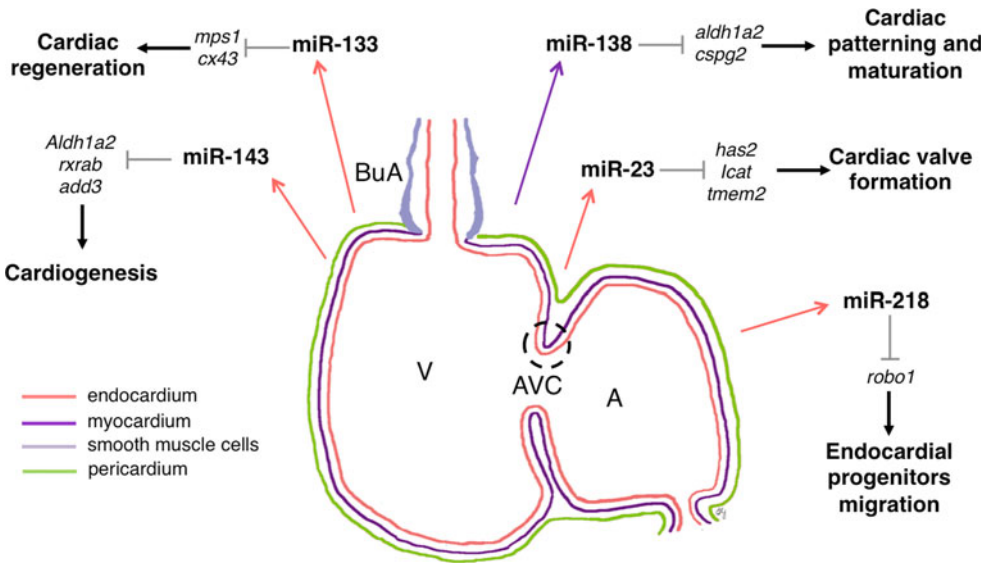

Fig. 2 Schematic representation of zebrafish heart at 72 hpf, which shows the bulbus arteriosus (BuA), atrium (A), ventricle (V), and the atrioventricular valve (AVC, *dashed circle*). *Green* indicates the pericardium, *purple* the myocardium, *pink* the endocardium, and *blue* smooth muscle cells. Each of these tissues expresses specific microRNAs that are shown along with their targets. Inhibition of the specific target expression allows for a specific functional role of the different microRNAs in cardiogenesis (see text for details)

functions in the zebrafish cardiovascular system have been addressed (Table 1).

Heart-specific miRNAs

Heart formation and function are precisely controlled by networks of transcription factors closely intertwined with families of miRNAs that modulate multiple aspects of cardiac development, function, and dysfunctions. Here, we report some miRNAs whose function has also been characterized through the use of zebrafish (Fig. 2).

The miRNA 23

Defects in cardiac valves are the most common subtype of cardiovascular malformations, and in adults they remain a major cause of morbidity and mortality [92]. Increasing the molecular understanding of processes that control heart valve development and remodeling could be useful to improve or develop new therapeutic approaches. In this sense it could be relevant that the discovery of one gene involved in heart valve development is miRNA-23 [35]. It has been showed that zebrafish MZDicer mutants have endocardial defects, including excessive endocardial cushion formation, and Lagendijk et al. demonstrated that they are caused by the loss of miRNA-23 activity. In this process, miRNA-23 acts to downregulate *has2* (hyaluronic acid synthase 2), an extracellular remodeling enzyme known to be required for endocardial cushion and valve formation [93] and whose upregulation in MZDicer mutants authors showed to be responsible for the observed valve defects. Furthermore, they demonstrated that in mouse endothelial cells, miRNA-23 is able to inhibit a transforming growth factor-beta (TGF-β)-induced endothelial-to-mesenchymal transition (EMT), a process that normally occurs during heart valve development. So the authors proposed a model in which miRNA-23, *has2*, and its product hyaluronic acid (HA) create a feedback regulatory loop that could respond to the AVC signal, such as TGF-β, and restrict endocardial cushion formation to the AVC region. Data shown in zebrafish and mouse endothelial cells suggest a conserved role of miRNA-23 whose function in mammalian valve development is still unknown but which could be interesting to investigate.

The miRNA 138

A precise regulation in time and space of gene expression and protein activity is fundamental for a proper development of a chambered heart. Morton et al. [70] demonstrated that miRNA-138 is necessary to establish appropriate chamber-specific gene expression patterns during embryonic development. This microRNA is expressed in specific domains of the heart, and its downregulation leads to ectopic expression in the ventricle of genes normally expressed in the atrioventricular valve region (AVC). As a consequence, ventricular cardiomyocyte morphology and cardiac function are affected. The microRNA function is to partially repress *aldh1a2* in the ventricle, a gene that encodes retinoic acid (RA) dehydrogenase, an enzyme involved in RA synthesis. miRNA-138 also mediates ventricular repression of *cspg2*, a gene positively regulated by RA signaling. This study demonstrates that, targeting multiple members of a common pathway, miRNA-138 establishes discrete temporal and spatial domains of gene expression during cardiac morphogenesis, which is necessary for the correct development of the heart. This microRNA is highly conserved across species as well as

transcriptional networks that control heart patterning [94], and so it could be interesting to analyze miRNA-138 functions in mammalian heart in which RA signaling plays a key role during development [95].

The miRNA 143/145 cluster

In mammals, miRNA-143 and miRNA-145 are two smooth muscle cell-specific microRNAs playing a role in vascular SMC phenotypic switch [96–99]. These microRNAs are expressed in early cardiac progenitors and throughout the embryonic heart before becoming restricted to vascular and visceral smooth muscle cells. Despite being expressed at high levels in the developing heart, no cardiac function for the miRNA-143/145 cluster in developing mammalian heart has been found [98, 99]. Although the downregulation of miRNA-145 affects cardiac development [66], its role in this organ has not yet been fully investigated in zebrafish. On the contrary, a role of miRNA-143 in cardiogenesis in zebrafish has been reported. In particular, it has been demonstrated that miRNA-143 is involved in the conversion of mechanical stimuli into biological output (mechano-transduction pathway) in the heart [100], a process that is important during the later phases of cardiac development when hemodynamic forces play an essential role in the morphogenesis and adaptation of the heart to the circulatory demands. The authors showed that miRNA-143 expression in the myocardium and endocardium of outflow tract and ventricle is heartbeat-dependent. Blocking the heartbeat with the injection of *tnnt2* (cardiac troponin T2) morpholino or with BDM (2,3-butanedione monoxime) treatment, miRNA-143 cardiac expression is reduced or abolished. They showed that the loss of miRNA-143 leads to pericardial edema and heart defects: the atrium of miRNA-143 morphants is swollen, and the ventricle doesn't inflate, maintaining a tube-like structure; the ventricle doesn't beat, and there are blood flow defects. Downregulation of miRNA-143 disrupts ventricular myocardial and endocardial architectures, probably through the repression of *aldh1a2* and *rxrab*, which encode the retinoid X receptor alpha b, and then through an aberrant activation of RA signaling. This study demonstrates that mechanical forces regulate the expression of miRNA-143 to set up a gradient of RA signaling in the developing heart. Understanding how mechanical stimuli are converted in biological responses in the developing heart can be useful for giving insight into the mechanism of both congenital and acquired heart disease and then how to prevent or combat them.

Another study showed that miRNA-143 is required for heart chamber morphogenesis through a direct repression of *add3* (adducin 3), which encodes an F-actin capping protein, in myocardial cells [101]. Deacon et al. observed that downregulation of miRNA-143 inhibits ventricular cardiomyocyte F-actin remodeling. As a consequence, cellular growth and elongation are blocked and cause ventricular collapse and decreased contractility.

We would speculate that the fact that a phenotype is evident in zebrafish but not in mouse would reside in a second copy of miRNA-143, which is present in the zebrafish genome but not in the mouse genome. The second copy of the miRNA-143 is identical to the one located in the miRNA-143/145 cluster, but it is in a different genomic region, and its expression could possibly be controlled by a different transcriptional unit (data not shown). Alternatively, it is conceivable that the cardiac phenotype of the miRNA-143 KO mouse model has not been characterized under ischemic conditions, such as I/R [98].

The miRNA 218

MiRNA-218 is a conserved microRNA situated intronically in *Slit2 Slit3*, genes that encode ligands of Robo receptors. Fish et al. [102] demonstrated that the *Slit/*miRNA-218*/robo* signaling pathway regulates migration of heart precursors in the midline, in part modulating Vegf signaling in the endocardium. The authors observed that downregulation of miRNA-218 causes a delayed heart field migration that results in pericardial edema and abnormal heart looping. The same phenotypes are caused by the overexpression of *robo1*. Demonstrating that *robo1* is a direct target of this microRNA, they found that a ligand-encoded microRNA regulates the expression of its own receptor. They subsequently also observed that Vegf is a mediator of heart field migration in zebrafish: its temporal inhibition leads to delayed heart field fusion. In vivo it interacts with *robo1*, which in turn could facilitate the phosphorylation of Vegfr2 as demonstrated in vitro. This study provides evidence of a *Slit/*miRNA-218*/Robo/Vegf* feedback regulatory loop that precisely regulates the migration of heart fields to form the linear heart tube. It has also been demonstrated in mouse that miRNA-218 regulates vascular patterning by modulation of Slit-Robo signaling [103], highlighting a conserved role of miRNA-218 in this pathway.

The miRNA 133

The zebrafish heart is similar to the human heart in many respects. But unlike the human heart, after injury the zebrafish heart closes wounds rapidly and regenerates cardiac tissue to fully restored cardiac muscle function [104]. Specific molecular networks have been associated to these regeneration events [105, 106]. These findings have suggested a potential role of microRNA in the regulation of heart regeneration and offer hope that this event could also

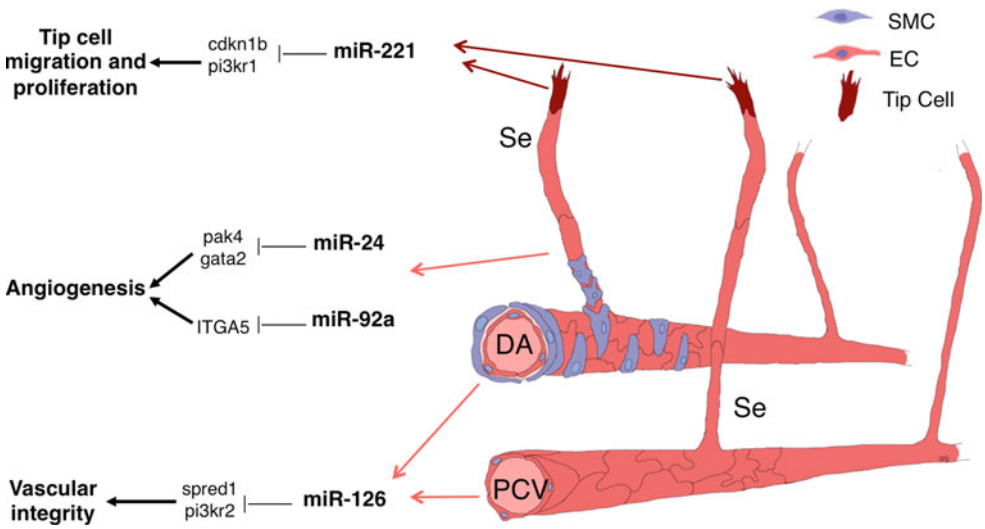

Fig. 3 Schematic representation of zebrafish trunk vessels showing endothelial cells (EC, *pink*), tip cells (*red*), and smooth muscle cells (SMC, *blue*) of the dorsal aorta (DA), posterior cardinal vein (PCV), and intersegmental vessels (Se). The expression of select miRNAs is shown, along with their observed target and functional roles (see text for details)

be induced in mammals. Yin et al. [79], through a microarray analysis of microRNA differentially expressed after the resection of the ventricle apex of the adult zebrafish heart, found that miRNA-133 expression correlates with the regeneration process. In zebrafish, miRNA-133 is downregulated after apex amputation, but progressively returns to be expressed to baseline when regeneration is completed. To study miRNA-133 function in adult heart regeneration, these authors generated two zebrafish transgenic lines through which they modulate miRNA levels in adult animals during regeneration. For overexpression studies, they generated a transgenic line containing 250 bp of genomic sequences flanking the sides of the miRNA-133a1 precursor sequence downstream of the *hsp70* promoter. Adult transgenic animals exposed to a single heat treatment showed a 1.8 increase of mature miR-133. For downregulating miRNA-133, the authors created another transgenic line using an miRNA-133 sponge construct (encoding EGFP cDNA followed by triplicate perfect binding sites for miRNA-133) placed downstream of the *hsp70* promoter. They showed that, remarkably, a single heat shock was sufficient to transiently but efficiently knock down adult cardiac miRNA-133 expression. Using these transgenic lines it was possible to discover that miRNA-133 inhibits myocardium regeneration reducing cardiomyocyte proliferation. Microarray analyses on miR-133-overexpressing/downregulated adult zebrafish identified cell cycle regulators such as monopolar spindle 1 (*mps1*), an essential factor for heart regeneration [107], and cytoskeletal/structural component proteins such as connexin-43 (*cx43*), a gene implicated in human cardiomyopathies [108], as direct targets of miRNA-133. The results of Yin et al. are also consistent with the elevated cardiomyocyte proliferation displayed by miR-133a1/a2 double knockout mice [109]. This work highlights the utility of the zebrafish model for studying the role of microRNAs also in the adult cardiovascular system in physiopathological contexts. Although we are still far from the possibility of inducing heart regeneration mammals, dissecting this process in zebrafish can provide context for understanding and possibly enhancing the cardiac regenerative capacity in mammals.

In summary, miRNA-133, a conserved miRNA known to have a role in cardiac development and disease in mammals [12], regulates zebrafish heart regeneration [79]. This work further demonstrates the versatility of the zebrafish model for miRNA studies not only during embryonic development, but also in the adult animal.

Vascular-specific miRNAs

Formation of a functional vasculature during vertebrate development is essential for embryonic survival. Physiological aspects of the vascular system have been extensively studied, resulting in its clinical importance. Thus far studies have shown a high degree of conservation of the molecular mechanisms that govern zebrafish vascular development as well as in the anatomical structures that comprise the vascular system in vertebrates. In zebrafish embryos both endothelial cell functions and angiogenesis are critically regulated by microRNAs (Fig. 3). Here are some examples of studies conducted by knockdown or ectopic overexpression of specific microRNAs and their effects on angiogenesis and endothelial cell behavior.

The miRNA 24

The miRNA-23/27/24 gene cluster is enriched in human endothelial cells and in highly vascularized tissues such as the heart. Recently, several reports have demonstrated that the miRNA-23/24/27 cluster is involved in heart and

vascular development and homeostasis in zebrafish [35, 110]. Overexpression of miRNA-24 in zebrafish embryos resulted in pericardial edema as well as blood accumulation. Studies in transgenic zebrafish that express GFP in the vasculature showed that miRNA-24 overexpression impaired intersegmental vessel formation and resulted in impaired blood transportation [110]. Morpholino-mediated silencing of the miRNA-24 targets *gata2* and *pak4* in zebrafish mimicked the effects of miRNA-24 overexpression, showing an involvement and potential therapeutic importance of those targets in angiogenesis [110]. These recent data also propose miRNA-24 as a suitable target for therapeutic intervention in cardiovascular diseases [111].

The miRNA 31

Using a TaqMan-based qRT-PCR profiling platform for signature miRNAs, miRNA-31 showed up as an important endothelial miRNA [112]. In vivo gain-of-function studies with zebrafish embryos established that overexpression of miRNA-31 impaired lymphatic development and reduced endothelial venous sprouting. Taken together, these findings indicate that miRNA-31 plays a pivotal role in regulating lineage-specific differentiation within the developing vasculature of vertebrates.

The miRNA 92

Many microRNAs involved in pathological angiogenesis, such as tumor angiogenesis, modulate tumor growth by controlling the production of angiogenic factors and then neovascularization. The miRNA 17–92 cluster is transcribed as a polycistronic unit and selectively targets anti-angiogenic proteins [113]. Inside this cluster, miRNA-92a seems to be important in the control of cardiovascular system homeostasis in vertebrates. By using antagomir approaches, Dimmeler's group determined that miRNA-92a is highly expressed in endothelial cells and regulates angiogenic functions of endothelial cells as evidenced by gain of function and loss of function experiments in vitro [114]. To demonstrate the conservative role of this microRNA, overexpression of miRNA-92a in zebrafish not only impairs in vitro angiogenesis but also blocks intersomitic vessel growth [114]. One of the mechanisms through which miRNA-92 controls endothelial cell functions seems to be the direct regulation of integrin subunit $\alpha 5$ (*itga5*), a crucial regulator of vessel growth and angiogenesis [115]. MiRNA-92a is expressed also in mammal cardiac fibroblasts and cardiomyocytes, and antagomir-92a treatment significantly reduced apoptosis of cardiomyocytes in vivo, but did not directly affect survival of cardiomyocytes in cell culture assays in vitro, indicating that antagomir-92a may influence cardiomyocyte apoptosis via a paracrine mechanism. Interestingly, it has been observed that atheroprotective flow patterns decrease the level of miRNA-92a, which in turn increases KLF2 expression to maintain endothelial homeostasis [116].

The miRNA 126

MiRNA-126 is one of the most evolutionary conserved and enriched microRNAs in human and mouse endothelial cells [117, 118] and is also expressed in the zebrafish vascular tree throughout development [67, 119]. In 2008, different groups analyzed and reported vascular defects in miRNA-126-deficient cells, ranging from zebrafish to human and mice. MiRNA-126 has been clearly shown to be a master non coding RNA in vascular functions acting as a regulator of angiogenic integrity and signaling in vitro and in vivo [119–121] and recently also in heart valve development [122]. In zebrafish, the downregulation of miRNA-126 induced collapsed blood vessels and cranial hemorrhages in the developing organism, suggesting its primary role for maintaining vascular structure during development [119]. Sprouty-related EVH1 domain-containing protein 1 (*Spred1*) and a regulatory subunit of PI3K, PIK3R2 (also known as p85β), two direct targets of miRNA-126, seem to explain these angiogenic defects. MiRNA-126 regulates *Spred1* and *PIK3R2*, which are negative regulators of MAPK [123] and PI3K [124] signaling pathways, respectively. As a consequence, miRNA-126 promotes VEGF and other growth factor signaling in ECs in vivo. As a matter of fact, miRNA-126 may fine-tune angiogenic responses by targeting multiple signaling pathways [119, 125]. Remarkably, deletion of miRNA-126 in mice resulted in the same vascular developmental defects observed in zebrafish, such as delayed angiogenic sprouting, widespread hemorrhaging, and partial embryonic lethality [120, 121], supporting the idea that the zebrafish model can be a simple and easy tool to discovery miRNA functions in vertebrates. Recent studies in zebrafish demonstrate for the first time that miRNA-126 expression and the egfl7 gene are both under the control of blood flow. Blood flow-dependent Kruppel-like factor 2a (*klf2a*) induces expression of miRNA-126 to modulate Vegf signaling [67].

The miRNA 221

Formation of new vessels in normal and pathological conditions requires coordination of distinct cell behaviors between tip and stalk cells [126]. The Vegf and Notch signaling pathways govern this process. Recently, through deep sequencing and functional screening in zebrafish, a new microRNA has been associated to this process [65]. MiRNA-221 has been found to be essential for angiogenesis since it is required for tip cell proliferation and

migration. miRNA-221 promoted tip cell behavior through repression of two targets: cyclin-dependent kinase inhibitor 1b (*cdkn1b*) and phosphoinositide-3-kinase regulatory subunit 1 (*pik3r1*). Importantly, these results in zebrafish indicate the central role of miRNA-221 as a regulatory node through which tip cell migration and proliferation are controlled during angiogenesis [65, 127]. A main role for miRNA-221/222 in endothelial therapeutic applications in humans has also been proposed [128]. In fact, recent work demonstrate that miRNA-221 is regulated by hyperglycemia in HUVEC cells, and lipid-lowering therapies (LLT) with atorvastatin increases EPC numbers and decreases miRNA-221/222 levels in patients with coronary artery disease [129, 130].

Conclusion

In this review, we pointed out the important role of the vertebrate *Danio rerio*, commonly called "zebrafish," in helping to understand microRNA functions in heart and vascular cells not only during development but also in pathological conditions.

Recent discoveries have demonstrated that the zebrafish system can be used to study tissue regeneration since adult zebrafish can effectively regenerate injured hearts submitted to partial surgical amputation of the ventricle area in vivo. Limited information is currently available about the molecular mechanisms that regulate this process [131]. In particular, which microRNAs can be involved in this process remains unclear. Identification of microRNAs active during the regenerative potential of vertebrate/zebrafish tissue may implicate the discovery of new therapeutic approaches to supplement or replace conventional pharmacotherapeutic interventions in humans.

Recently, the possibility has also been suggested that microRNAs can be transported between cardiovascular tissues and regulate athero-vascular protection by an extracellular-vesicle-mediated mechanism [132, 133]. Demonstration of a role for circulating microRNAs could be validated in the zebrafish model system by microinjecting exogenous microRNA-containing liposomes in the blood flow of developing embryos to detect a functional role for mature microRNAs in cardiovascular tissues.

Interestingly, the zebrafish model can be used to perform pharmaceutical high-throughput modifier screens. The use of small RNA molecules such as microRNA products through such screens will serve as ideal entry points for novel drug development in the treatment of many vascular dysfunctions. Also, for miRNA-based therapeutic strategies the zebrafish model will come in handy. Antisense oligonucleotide approaches for inhibiting miRNA function and gain-of-function technologies for replacement of miRNAs are currently being explored as tools for uncovering miRNA biology and as potential therapeutic agents. We think that in the near future significant progress in understanding miRNA biology will be derived from the zebrafish system, and this model will help to advance the technology for therapeutic modulation of miRNA activity in cardiovascular diseases.

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