HHS Public Access

Author manuscript

Curr Top Dev Biol. Author manuscript; available in PMC 2016 June 01.

Published in final edited form as:

Curr Top Dev Biol. 2012 ; 100: 279–317. doi:10.1016/B978-0-12-387786-4.00009-9.

MicroRNAs in Heart Development

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Abstract

MicroRNAs (miRNAs) are a class of small noncoding RNAs of ~22 nt in length which are involved in the regulation of gene expression at the posttranscriptional level by degrading their target mRNAs and/or inhibiting their translation. Expressed ubiquitously or in a tissue-specific manner, miRNAs are involved in the regulation of many biological processes such as cell proliferation, differentiation, apoptosis, and the maintenance of normal cellular physiology. Many miRNAs are expressed in embryonic, postnatal, and adult hearts. Aberrant expression or genetic deletion of miRNAs is associated with abnormal cardiac cell differentiation, disruption of heart development, and cardiac dysfunction. This chapter will summarize the history, biogenesis, and processing of miRNAs as well as their function in heart development, remodeling, and disease.

1. Introduction

Gene expression, as a basic biological process, can be characterized as a response to stimuli. However, the level of change in gene expression needs to be carefully controlled to ensure the proper cellular response; similarly, a lack of adjustment may lead to abnormal cellular function. Thus, the regulation of gene expression and its mechanisms of action are fundamental to all organisms and have been thoroughly studied in multiple species and organ systems. Therefore, the recent determination of a new class of small RNAs (microRNAs, miRNAs), as key elements in this regulatory repertoire, has generated enormous interest in this field. It is now known that miRNAs function within the genetic regulatory network at the posttran-scriptional level. By imperfect base pairing with mRNAs in a sequence-dependent manner, these miRNAs repress gene expression by degrading target mRNAs and/or inhibiting their translation. Some miRNAs are expressed broadly, while others are restricted to specific tissues or cell types. Roles for miRNAs have been demonstrated in the regulation of a broad range of biological activities and their aberrant expression has been correlated with embryonic malformations and organ dysfunction. Heart function, in particular, is essential for proper vertebrate embryogenesis, and the early morphogenesis and development of this organ are dependent on tightly regulated genetic networks. This chapter is intended to offer a summarized review of the history, biogenesis, and processing of miRNAs, and their function during heart development.

2. An Abridged Overview of Heart Development

Eukaryotic cell functions, including growth, proliferation, differentiation, and survival, rely on several mechanisms including respiration and energy production. Respiration is the

diffusion of oxygen into cells, where it will be used as a final acceptor of protons, in the respiratory chain, for the production of energy in the form of ATP (Alberts et al., 1994). In "simple" multicellular organisms, such as the worm (*Caenorhabditis elegans*) oxygen can freely diffuse into the cells; however, in higher invertebrates and vertebrates, oxygen has to be brought directly into contact with the internal tissues and cells by the lymph or blood through a circulatory system. Directional flow within a circulatory system allows the exchange of oxygen and nutrients for waste produced by cells and tissues (Harvey, 1999, 2002). The directionality of the lymph or blood flow is produced by the pumping action of the heart, which beats rhythmically in an organized and regular manner to force fluid movement from the caudal to the cranial end of an organism (Challice and Virágh, 1974; Nishii and Shibata, 2006). This cardiac pump takes multiple forms, from a simple single cardiac tube in invertebrates (i.e., the "fruit fly" *Drosophila melanogaster*) to a two-chambered structure in fish (i.e., the "zebrafish" *Danio rerio*), a three-chambered heart in amphibians (i.e., the "frog" *Xenopus laevis*) and reptiles, and finally to a four-chambered heart in birds (i.e., the "chicken" *Gallus gallus*) and mammals (i.e., the "mouse" *Mus musculus*) (Harvey, 1999, 2002; Kirby, 2007).

Heart function is required early during embryogenesis for survival and the subsequent growth of other tissues and organs. The cells that form the heart originate from cardiac progenitors specified during embryonic gastrulation, a dynamic cellular process which results in the formation of the three germ tissue layers: ectoderm, mesoderm, and endoderm. The concerted and simultaneous activity of endodermal and ectodermal signaling permits the formation of the mesoderm and restricts the location of the cardiogenic cells, as well as their specification. Several molecules with inducing or inhibiting properties are involved in each of the different stages of development (Harvey, 1999; Kirby, 2007; Olson, 2006). For cardiac development, these different stages include (1) specification of cardiac progenitors, (2) initial migration of these cells during gastrulation, (3) further cellular migration to form the cardiac fields, (4) formation of the cardiac crescent, (5) formation of the tubular heart, (6) cellular recruitment at the poles of the heart, and (7) cardiac chamber differentiation (Kirby, 2007).

Tracing analyses utilizing vital dyes and cell/tissue explant experiments have shown that early cardiac progenitor cells are located in the anterior region of the primitive streak in the chick and mouse embryo (Buckingham *et al.*, 2005; Harvey, 2002; Srivastava, 2006). At the onset of gastrulation, initial posterior to anterior migration of these cells is regulated by the activity of the FGF family of signaling factors including FGF2, FGF4, and FGF8 which have been proposed to signal through FGFR1 (Ciruna and Rossant, 2001; Harvey, 2002). Similarly, bone morphogenic protein 2 (BMP2), a member of the TGF-β superfamily of growth factors, is initially expressed in the posterior end of the primitive streak by the onset of gastrulation and implicated in early cell migrations (Andrée et al., 1998).

At embryonic day 6.5 (E6.5), as a result of FGF-8-dependent expression of *Mesp-1* and *Mesp-2*, cardiac progenitor cells migrate bilaterally to the anterior and lateral region of the embryo colonizing the splanchnic layer of the lateral plate mesoderm and form two early lateral cardiac fields (Buckingham *et al.*, 2005; Harvey, 1999; Kitajima *et al.*, 2000; Saga *et al.*, 1999). Canonical *Wnt/β-catenin* signaling within the neural ectoderm induces the

Mesp-1 positive mesodermal lineage during early gastrulation (Bondue *et al*., 2008; Saga *et al*., 1999). These data confirm crucial roles for the early action of Bmp2 and FGF signaling in conferring cardiogenic potential (Lough *et al*., 1996), guiding the migration of progenitor cells, and restricting the location of the cardiac fields. It is also aided by the activity of a concentration gradient of the Wnt/β-catenin, which will induce the underlying anterior mesoderm into head tissue (Brade *et al*., 2006; van de Schans *et al*., 2008).

Approximately a day later (E7.5), these early cardiogenic progenitor cells migrate cranially and converge across the midline region of the embryo to form a horseshoe-shaped epithelial fold, the cardiac crescent. The lateral cardiac fields in mouse and chicken embryos were initially identified by the expression of Nkx2-5, a vertebrate homeodomain transcription factor that is the homologue of the *D. melanogaster* tinman gene (Bodmer *et al*., 1990; Kasahara *et al*., 1998; Lyons *et al*., 1995; Tanaka *et al*., 1999). In mouse and chick embryos, *Nkx2-5* is the *Nkx* factor most widely expressed in the early lateral cardiac fields and cardiac crescent during cardiogenesis (Lyons *et al*., 1995; Tanaka *et al*., 1999). However, in contrast to the function of *tinman* in flies, no Nkx gene acts a master regulator of cardiogenesis (Newman and Krieg, 1998). In a dynamic process, the medial region of the cardiac crescent subsequently bulges and moves cranially bringing the lateral regions toward the midline; these two lateral cell populations eventually fuse to form the heart tube (E8.0). It is during the heart tube forming stage that the primitive heart regions can be anatomically and molecularly identified for the first time. From caudal to cranial, these structures are: (1) The inflow tract, where blood is collected to be directed into the heart and which is composed of the right and left horns of the sinus venosus which are the continuation of the common cardinal veins; (2) the primitive atrial chamber; (3) the primitive ventricular chamber; and (4) the outflow tract, which at this stage is known as the conotruncus or bulbus cordis (Buckingham *et al*., 2005; Kirby, 2007; Srivastava, 2006). During the formation of the heart tube, *Wnt/β-catenin* signaling, especially Wnt-3a and Wnt-8, is necessary for the induction and maintenance of the expression of *Isl-1* (Cohen *et al*., 2007) and *Fgf10* (Lizhu *et al*., 2007). These factors are crucial to the correct formation of the second heart field and the expression of *Shh*, *Bmp4*, and *Bmp7* (Tzahor, 2007).

At E9.5, the heart begins a substantial remodeling process with the rightward bending of the heart tube to realign the cardiac chambers (looping); the caudal region of the cardiac tube, including the two sinus venosus horns, is brought cranially to position them dorsally to the outflow tract. By E10.5, the process of cardiac looping is nearly complete as the sinus venosus is shifted to the right side of the heart. The appearance of two outgrowing structures, called the sinus valves, demarcates the junction of the right common cardinal vein and the right atrium. Also, the first signs of septation of the cardiac chambers are evident at E10.5 (Challice and Virágh, 1974; Van Mierop and Gessner, 1970). By E11.5, the caudal region of the heart is finally positioned dorsal to the arterial pole and the subsequent septation within the cardiac chambers, in oxygen breathing vertebrate species, are the final steps in their specification. Initial septation of the outflow tract, by formation of internal cushions, divides it into a pulmonary and arterial arch. The right sinus venosus horn is brought in juxtaposition to the right atrium, and the left horn reduces in size and becomes the coronary artery. The atria and ventricles are separated by the interatrial and

interventricular septa, respectively (Buckingham *et al*., 2005; Harvey, 2002; Kirby, 2007; Van Mierop and Gessner, 1970).

Differentiation of cells within the heart is regulated by a variety of transcription factors which are again dependent on the expression of first or second heart field factors. The bHLH transcription factors, *Hand1* and *Hand2*, whose expression marks the left and right ventricles, respectively, are key examples. *Hand2* is dependent on the expression of *Isl-1*, while *Hand1* is dependent on the expression of *Nkx2-5* (Buckingham *et al*., 2005; Cai *et al*., 2003). Nkx2-5 interacts with the T-box family of transcription factors and is involved in the activation or repression of gene expression required for the differentiation of numerous cardiac structures. Several members of the T-box family have been identified to be expressed in cardiac tissue including *Tbx-1, -2, -3, -5, -18*, and *Tbx-20* (Plageman and Yutzey, 2005). Interaction of *Nkx2-5* and Tbx5 results in activation of the Atrial Natriuretic Factor (ANF) gene and the differentiation of the cardiac chambers and cardiac trabeculation in the ventricles (Hiroi *et al*., 2001). Nkx2-5 expression is dispensable for the initial formation of the sinoatrial node (SAN) (Blaschke *et al*., 2007; Espinoza-Lewis *et al*., 2009, 2011; Kasahara *et al*., 1998). However, the interaction of Nkx2-5 with Tbx2 or Tbx3 results in the repression of the *ANF* gene and *Connexin-40* (*Cx40*) during differentiation of the cardiac conduction system, including the SAN (Habets *et al*., 2002; Hoogaars *et al*., 2004). Later in development, cardiac outgrowth takes place and by E14.5 the final shape of the heart is achieved; the sinus venosus has regressed and slowly integrated into the dorsal wall of the right atrium, the outflow tract has divided into the pulmonary and arterial arches, septation of the cardiac chambers is complete, and the ventricular chambers have become the major volumetric components of the heart (Buckingham *et al*., 2005; Harvey, 1999, 2002; Kirby, 2007; Srivastava, 2006).

3. The World of the miRNAs

We have briefly summarized some aspects involved in embryonic cardiac morphogenesis and the molecules involved in its regulation. As noted above, cardiac progenitor cells arise from the specification and determination of a special region of the mesoderm. Early determination is driven by the activity of the transcription factors Nkx2-5 and Isl-1 (Cai *et al*., 2003; Kasahara *et al*., 1998; Lyons *et al*., 1995; Tanaka *et al*., 1999). Also, it is implied that the final cellular response in the form of expression of a genetic profile is a result of not only the activity of transcription factors, but also the concerted activity of extracellular molecules, their signal transduction, and fine-tuning regulatory mechanisms. Gene regulation is achieved by the direct binding of transcription factors (activators or repressors) to *cis*-regulatory elements in order to up- or downregulate gene expression. This process can be enhanced or inhibited by the activity of extracellular signals, the activity of intracellular mediators, the status of chromosomal structure, the activity of transcription cofactors, and posttranslational modification of the protein product. In short, gene expression is regulated at the transcriptional, translational, and/or posttranslational level (Alberts *et al*., 1994; Srivastava, 2006). In recent years, a novel mechanism has been described which functions at the posttranscriptional level to modulate and fine-tune gene expression by targeting the mRNA. Such mRNA posttran-scriptional regulation results in either mRNA degradation or

mRNA translation inhibition. This elegant mechanism is carried out by a newly described class of small RNA molecules, the miRNAs.

3.1. miRNAs: A brief history

Embryonic development is a well-organized, highly regulated event with strict spatiotemporal requirements. Stages are categorized by the observation of characteristic anatomical and molecular changes. In the "worm" *C. elegans*, several stages of development can be easily observed by simple visual inspection. From fertilized egg, to embryo, to larva, to adult, the worm's life cycle is completed in about 2 days. The larva stage is the lengthiest and is divided into four stages named L1–L4 (Anderson, 1995). Defects in any of these stages through the disruption of the temporal patterns of cell division and differentiation conducted by regulatory "heterochronic genes" result in larval or adult abnormalities (Chalfie *et al.*, 1981). Mutations in these heterochronic genes result in the induction of cell fate transformations, such as recapitulation of an earlier phenotype at late stages or by adopting late stage phenotypes prematurely (Ambros and Horvitz, 1987; Chalfie *et al.*, 1981).

Initially, heterochronic mutations were related to and identified in four *C. elegans* genes (*lin-4, lin-14, lin-28*, and *lin-29*) (Ambros and Horvitz, 1984). The *lin-4* mutation resulted in cell lineage reiterations which main-tained earlier larval phenotypes. This resulted in supernumerary moults and the continuous production of larval-specific cuticle with the extension of late larval stages (Ambros and Horvitz, 1984; Chalfie *et al.*, 1981). The *lin-14* gain-of-function mutation, which produces a semidominant allele, results in cell lineage retarded development; certain late stage cells adopt fates expressed in cells at earlier stages. In contrast, *lin-14* loss-of-function mutations, which result in null alleles, generate precocious cell lineage development, with certain early stage cells adopting late stage fates prematurely (Ambros and Horvitz, 1987). Interestingly, the *lin-4* and *lin-14* mutant phenotypes were found to occur in the same cell lineages (Ambros and Horvitz, 1987). Additionally, it was found that *lin-14* mutant phenotypes were *lin-4* dependent (Ambros and Horvitz, 1987; Arasu *et al.*, 1991; Ruvkun and Giusto, 1989; Wightman *et al.*, 1993). Those observations suggested a direct regulatory mechanism between these two genes (Ambros and Horvitz, 1987; Arasu *et al.*, 1991; Chalfie *et al.*, 1981). The *lin-14* gene product is a protein that is detected at high levels only in the early larval L1 stage. Surprisingly, however, RNA protection assays revealed that *lin-14* mRNA was expressed and stable in all larval stages (Ruvkun and Giusto, 1989). Extensive screening for lin-14 mutants facilitated a large analysis of the *lin-14* gene structure and determined a regulatory sequence in the *lin-14* 3′UTR as responsible for the negative regulation of gene expression, specifically in inhibiting mRNA translation (Lee *et al.*, 1993; Wightman *et al.*, 1993). Similarly, gene structure analysis, in addition to a highly laborious gene cloning strategy, suggested that the *lin-4* gene product is not a protein. Indeed, RNA protection analysis demonstrated that this locus produced two small RNA products, a 69-nt-long product (*lin-4L*) and a 21-nt-long product (*lin-4S*) (Lee *et al.*, 1993). Computational analysis indicated that the *lin-4L* was a precursor-like molecule which includes the *lin-4S* product in a hairpin or stem-loop secondary structure and that both were complementary to conserved repeated sequences in the *lin-14* negative regulatory 3′UTR in a nonperfect manner. However, the 5′ 6–8nt of

lin-4S displayed an exact match with that of the *lin-14* 3′UTR (Lee *et al*., 1993; Wightman *et al*., 1993). Simultaneously, it was reported that the *lin-14* 3′UTR could mediate the downregulation of the expression of an unrelated protein (Wightman *et al*., 1993). A construct comprising the *LacZ-lin-14*_3′UTR driven by the *Collagen-10* promoter (*Col10-LacZ-lin-14*_3′UTR) was engineered and injected into wild-type *C. elegans* embryos. X-gal staining showed the expected fading coloration of cells after the L2 larval stage. In contrast, injection of the construct into a *lin-4* null mutant line (e912) resulted in the maintenance of the X-gal staining in all four larval stages, thus demonstrating the mechanism of the novel predicted regulatory mechanism (Wightman *et al*., 1993).

Similarly, genetic screening for mutations to suppress the synthetic sterile phenotype in *C. elegans* was performed. Several candidate genes were identified, including *let-7* (Reinhart *et al*., 2000). *let-7* mutations result in supernumerary moults in the L4-adult stage reiterating earlier larval patterns of cell division. This defect was partially suppressed by mutations in the *lin-41, lin-14, lin-28*, and *lin-42* genes. Gene structure, Northern blot, and molecular analyses showed that the *let-7* product was not a protein but a 21-nt-long small RNA molecule. Additionally, computational analysis predicted and determined that the *lin-14, lin-28, lin-41, lin-42*, and *daf-12* heterochronic genes contain nonperfect complementary sequences in their 3′UTR. Transgenic *C. elegans* containing a *Col10-LacZ-lin-41*_3′′UTR construct demonstrated that *let-7* acts in similar fashion to *lin-4* (Reinhart *et al*., 2000). Due to their nature and their specific function in regulating the temporal patterns of cell division in the *C. elegans* larvae, both *lin-4* and *let-7* gene products were termed small temporal RNAs (stRNAs) (Lee *et al*., 1993; Pasquinelli *et al*., 2000).

In order to provide a complete overview of this new regulatory mechanism, we believe it is necessary to briefly describe a few experimental landmarks. These discoveries were reported in between the determination of the *lin-4* and the *let-7* mechanism of action and helped shape the idea that small RNAs were indeed important players in the regulation of gene expression.

It was observed that overexpression of a sense or antisense RNA in plants (Hammond *et al*., 2001b; Jorgensen *et al*., 1996; Que and Jorgensen, 1998) or the injection of antisense RNA in *C. elegans* resulted in down-regulation of gene expression (Fire *et al*., 1991; Guo and Kemphues, 1995). Based on those empirical results, it was hypothesized that the regulation of gene expression by overexpression of a sense RNA was effective due to the quenching of the necessary factors needed for translation of the targeted mRNA molecule (cosuppression). However, a different mechanism was believed to be responsible for the downregulation of expression by over-expression of an antisense RNA; it was thought to induce the formation of a long double-stranded RNA (dsRNA) molecule, thus inhibiting translation (Fire *et al*., 1991; Hammond *et al*., 2001b; Jorgensen *et al*., 1996; Que and Jorgensen, 1998). It was known that dsRNA could affect gene expression by triggering interferon inducible pathways that inhibit translation, known as a “panic response,” through the activation of protein kinases. However, the effects observed when using antisense RNA were gene specific differing from the broad translation inhibition due to kinase activation (Hammond *et al*., 2001b; Williams, 1999). Strikingly, more dramatic effects in down-regulation of gene expression and higher gene specificity were found in *C. elegans* when sense and antisense

RNA molecules were introduced at the same time (Fire *et al*., 1998) leading to the thought that a specific mechanism for RNA silencing must exist. This mechanism was termed RNA interference or RNAi (Fire *et al*., 1998).

Independently, a similar regulatory system, drawing parallelism to the RNAi mechanism, was previously described in plants, termed posttran-scriptional gene silencing (PTGS). This process, which is guided by small ~22–25nt RNA molecules, was identified after the introduction of a long antisense RNA (Hammond *et al*., 2001b; Jorgensen *et al*., 1996; Que and Jorgensen, 1998). Following the methodology described in PTGS studies, similar results were later obtained in *D. melanogaster* S2-cultured cells (Fire *et al*., 1991) and in the "worm" *C. elegans* (Guo and Kemphues, 1995).

Thus, the description of ~22–25nt small RNA molecules which guide the PTGS/RNAi mechanism resembled the earlier description of the small temporal genes *lin-4* and *let-7*. stRNAs and RNAi molecules share bio-chemical and mechanistic characteristics; they are small in size, double stranded, and possess a 5′-phosphate group and a 3′-hydroxyl group in a 2-nt tail (Bartel, 2004; Bartel and Bartel, 2003). It was inferred, from the latter characteristic, that RNase III must be responsible for such end-product. Indeed, *Dicer*, previously identified to be a component of the PTGS complex, was also found to be involved in the maturation of the stRNA (Grishok *et al*., 2001; Hammond *et al*., 2000). Ironically, it was by using RNAi against the human Dicer gene in mammalian cell cultures that it was demonstrated that *let-7* maturation was DICER dependent (Hutvágner *et al*., 2001). The analysis of DICER-produced biochemical products, along with an elegant cloning strategy and bioinformatic predictions, led almost simultaneously and fortuitously to the discovery of small RNAs resembling stRNAs in several model systems including *D. melanogaster*, *C. elegans*, and in *HeLa*-cultured cells. Although these new small RNAs were not expressed in a timely fashion as the stRNAs, biochemical similarities proved sufficient to classify them into a new and large class of small RNAs which include *lin-4* and *let-7* as founding members. Due to their short length, they were termed miRNAs (Lagos-Quintana *et al*., 2001; Lau *et al*., 2001; Lee and Ambros, 2001).

3.2. miRNA: Biogenesis

The small ~22-nt-long single-strand noncoding miRNA molecules are first expressed as a long transcript known as primary miRNA (pri-RNA) either as a single independent amplicon, as part of a polycistronic RNA molecule, or from intronic sequences within a protein-coding gene host (Fig. 10.1) (Bartel, 2004; Sayed and Abdellatif, 2011). The single or polycistronic molecule is characterized by the adoption of a secondary structure known as the stem-loop or hairpin (Bartel, 2004; Sayed and Abdellatif, 2011). Initial nuclear processing of the long pri-RNA molecule is performed by the microprocessor complex composed of the Class I RNAse type III *Drosha* and the stabilizing protein *Pasha* (Partner of *Drosha*, in *C. elegans*), or *Dgcr8* (*DiGeorge* critical region gene 8, in mice and humans). This process generates a ~70-nt-long RNA molecule in a two-step highly consistent, characteristic, and sequence-independent manner (Denli *et al*., 2004; Han *et al*., 2006; Lee *et al*., 2003; Wu *et al*., 2000). dsRNA is recognized by Dgcr8 and cleaved by Drosha in a staggered fashion, ~11nt from the base (Gregory *et al*., 2004; Han *et al*., 2006). The result of

the staggered cleavage is a hairpin-like molecule, also known as precursor-miRNA (pre-miRNA), with a 5′-phosphate in the forward strand and a 3′-hydroxyl group in the 2 nt-overhang tail on the reverse strand (Han *et al*., 2006). Intronic miRNAs, known as miRtrons, are expressed along with their host gene and the pre-miRNA is a product of splicing. These miRtrons have been shown to bypass *Drosha/Dgcr8* initial processing and have been reported in *D. melanogaster, C. elegans*, and mammals (Ruby *et al*., 2007).

Importins and Exportins are involved in the active nuclear transport of proteins and small RNAs such as pre-rRNA and tRNA (Bohnsack *et al*., 2004). Exportin-5 is a Ras–GTP-dependent active transporter involved in the nuclear export of adenovirus VA1 (a small 160-nt small noncoding RNA) and in tRNA transport (Bohnsack *et al*., 2004; Yi *et al*., 2003). Exportin-5 binds to the 3′ 2nt-overhang in tRNAs (Bohnsack *et al*., 2004; Calado *et al*., 2002), a characteristic also found in Drosha/Dgcr8-processed miRNAs. Indeed, Exportin-5 was identified as the nuclear receptor which mediates miRNA export to the cytoplasm by recognizing and binding to the 3′ 2-nt overhang and dsRNA region of the pre-miRNA in a sequence-independent manner (Bohnsack *et al*., 2004; Lee *et al*., 2011; Lund *et al*., 2004; Yi *et al*., 2003).

In the cytoplasm, the pre-miRNA is further processed by excision of the loop structure in order to convert the hairpin or stem-loop small RNA into a short dsRNA molecule. Dicer was initially identified as an RNase type III involved in the processing of large dsRNA molecules for the production of siRNA in PTGS in plants (Bartel and Bartel, 2003; Hammond *et al*., 2001b; Jorgensen *et al*., 1996; Que and Jorgensen, 1998) and RNAi in *C. elegans* and *D. melanogaster* (Bernstein *et al*., 2001; Fire *et al*., 1998; Grishok *et al*., 2001; Hutvágner *et al*., 2001). DICER, as an RNase type III, recognizes the double-stranded nature of the pre-miRNA and cleaves in a staggered manner at about two helical turns from the base of the hairpin excising the loop from the hairpin and producing a mature imperfectly matched dsRNA molecule (Grishok *et al*., 2001; Hutvágner *et al*., 2001; Ketting and Plasterk, 2000). Dicer homologues have been found in several organisms and are known as *Dicer-like* (*Dcl-1-4*) in *Arabidopsis thaliana*; *Dcr* (*Dcr-1* and *Dcr-2*) in *D. melanogaster*; and *Dicer-1* in mice, rats, and humans (Bartel, 2004).

The mature dsRNA or RNA duplex is composed of the mature miRNA as one of the strands (guide strand), while the other strand is known as miRNA* (passenger strand). Small RNA high-throughput cloning has identified that the majority of the strands screened correspond to miRNAs, while a low number of clones represent miRNA*s, indicating that miRNA:miRNA* duplexes are short lived and that miRNA*s are quickly degraded. However, even though miRNA*s are found in low numbers, a few have been reported to be functional (Bartel, 2004; Sayed and Abdellatif, 2011).

3.3. miRNAs, RISC, and gene silencing mechanism

The two-step RNA processing driven by Drosha and Dicer produces a dsRNA molecule or miRNA:miRNA* duplex which is recognized and bound by a large protein complex known as the RNA-induced silencing complex (RISC). “Loaded” into the RISC complex, the miRNA: miRNA* duplex is unwound and the miRNA guide strand is separated from the

miRNA* passenger strand. The passenger strand is readily degraded while the guide strand directs mRNA targeting.

Several genetic screens in *A. thaliana*, *C. elegans*, and *D. melanogaster* suggested the existence of a conserved protein complex implicated in silencing activity induced by small dsRNA. In *A. thaliana*, mutations in the *AGO-1* gene result in aberrations during leaf development and defects in the floral organs. The leaves are smaller and their outgrowth is retarded and their shape resembles a small squid; thus the mutants were known as Argonautes. Additionally, AGO-1 mutants are ineffective in PTGS (Bohmert *et al*., 1998). A similar phenotype was previously observed in the suppressor of gene silencing (*SGS-2/SDE-1*) mutant plants (Bartel, 2004; Bartel and Bartel, 2003; Hammond *et al*., 2001b). Additionally, RNAi deficiencies were found in *Neurospora crassa QDE-2* mutants (Catalanotto *et al*., 2000), and in *C. elegans rde-1, rde-4*, and *mut-7* mutants (Ketting *et al*., 1999; Tabara *et al*., 1999) among others (Fagard *et al*., 2000; Hammond *et al*., 2001b). Interestingly, these latter genes show shared homology to the *A. thaliana* AGO-1 product (Fagard *et al*., 2000), indicating the presence of a conserved molecular mechanism involved in gene silencing. In *D. melanogaster*, S2-cultured cells transfected with a mixture of a *Luciferase* expressing construct and dsRNA targeting the Luciferase ORF were subjected to lysate fractionation (Hammond *et al*., 2000, 2001a). Certain fractions possessed RNAi processing ability, determined by the presence of small RNAs and absence of the *Luciferase* mRNA, and also coprecipitated a low-abundance protein. Protein sequence homology searches again determined that this protein showed a high homology to AGO-1; thus it was termed AGO-2 and classified as a member of the Argonaute superfamily of proteins (Hammond *et al*., 2000, 2001a). Several other Ago proteins have been identified; *C. elegans* contains 27 members, while only four Ago proteins (AGO-1, AGO-2, AGO-3, AGO-4) are found in mice and humans (Pratt and MacRae, 2009).

The Argonaute superfamily of proteins can be subclassified into three groups: (1) the Ago subfamily, to which siRNA and miRNA bind; (2) the PIWI subfamily, to which piRNAs bind (piRNAs are a subclass of PIWI interacting small RNAs (rv27nt in length) expressed in the germ line regulating gene silencing of retrotransposons particularly during spermatogenesis); and (3) a class of *C. elegans* Ago-like proteins (worm Ago's or WAGO's) (Kapoor *et al*., 2008). Argonaute proteins are ~100kDa in average and are at the core of every RISC (Kawamata and Tomari, 2010). Argonaute proteins are also known as PPD proteins due to the conservation of the PAZ and PIWI domains. The PAZ domain is an independently stable domain with a β-barrel core which weakly binds single-stranded RNA as well as dsRNA (Cerutti *et al*., 2000; Yan *et al*., 2003). The PIWI domain, also involved in single-stranded RNA binding, recognizes the 5′-phosphate of the guide strand (Cerutti *et al*., 2000). Human AGO-2 was found to be sufficient and necessary for processing of small RNAs (Hammond *et al*., 2001a; Meister *et al*., 2004). Interestingly, although all Ago proteins are capable of binding small dsRNA, it is only AGO-2, in humans and mice, which possesses the catalytic activity known as "slicing" (Kawamata and Tomari, 2010).

In *D. melanogaster*, the Ago-2-containing lysate fraction was determined to be ~500KDa, much larger than the predicted molecular weight for Ago-2 (Hammond *et al*., 2001a). This discrepancy indicates that Ago-2, albeit being highly important, is only one component of a

larger protein complex. Indeed, several other components of this large protein complex have been discovered and described. TRBP (TAR RNA-binding proteins) has been found and reported to be a Dicer protein partner involved in the "loading" of the RISC (Chendrimada *et al*., 2005). TRBP functions in a similar manner to *D. melanogaster* R2D2 and Loquacious; however, TRBP, R2D2, and Loquacious are not homologous proteins (Czech *et al*., 2008; Liu *et al*., 2003). GW182/Tnrc6 proteins as well as the helicases Rck and MOV10 initially identified to be a component of the RNA processing bodies (P-bodies) recruit "loaded" Ago-2 to P-bodies where final unwinding and RNA processing occurs (Kulkarni *et al*., 2010). Dcp-1 and Dcp-2 (decapping enzymes) are also present in P-bodies and are involved in RNA processing by excising the 5′-GpC-RNA cap to initiate RNA degradation (Kawamata and Tomari, 2010; Kulkarni *et al*., 2010).

As noted above, the miRNA:miRNA* duplex is produced in a two-step process directed by the activity of Drosha/Dgcr8 in the nucleus and Dicer in the cytoplasm, respectively. This miRNA:miRNA* duplex is bound by Ago-2 (this complex is also known as a loaded RISC) and further processed. This involves unwinding of the duplex and selection of the functional miRNA guide strand, followed by degradation of the nonfunctional passenger strand and final assembly of the complete RISC (also known as holoenzymatic-RISC or holo-RISC). This process prepares the miRNA for the recognition of and annealing to its target mRNA (Bartel, 2004; Kawamata and Tomari, 2010; Sayed and Abdellatif, 2011).

Loading of the miRNA:miRNA* duplex into Ago-2 is not a random process. First, it is hypothesized that the transfer of the duplex from Dicer to Ago-2 is guided by direct contact. Both Dicer and Ago-2 contain a conserved PAZ domain, involved in weakly binding RNA molecules, and believed to be involved in Dicer:Ago-2 protein–protein interaction. Indeed, Dicer coimmunoprecipitates with Ago-2 in *D. melanogaster* S2-cultured cells (Hammond *et al*., 2001a). However, Ago proteins are unable to load miRNAs without the involvement of stabilizing factors. In *D. melanogaster*, this Dicer-interacting protein is known as R2D2, while in humans, a factor with a similar function is called the TAR-binding protein 2 (TRBP2); however, these two proteins are not homologous and a true R2D2 homologue has not yet been found in humans (Czech *et al*., 2008; Kawamata and Tomari, 2010; Liu *et al*., 2003).

Additionally, loading of the miRNA:miRNA* duplex is directed by the thermodynamic stability of the duplex itself and is sequence dependent, including center mismatches near or in guide positions. Guide positions vary and are specific for the type of Dicer or Ago proteins involved in the loading process; generally, guide positions involve nucleotides at residues 7–11. Each Ago protein has a preference for the 5′end nucleotide present in the miRNA:miRNA* duplex. This 5′nucleotide, along with sequence mismatches, confers the duplex with asymmetry in the thermodynamic stability of the strands directing the loading of the guide strand. The orientation of the duplex is also sensed by the three-dimensional conformation of the recognizing protein. Interestingly, for the *A. thaliana* AGO-1 and AGO-2 proteins, the positional swapping of the MID and PIWI domains switches their nucleotide preference resulting in a preferential loading of the passenger strand. However, in humans, AGO proteins do not have a preference for the 5′nucleotide and it seems that loading is a simpler process. Nevertheless, human AGO proteins appear to have a preference

for duplexes with center mismatches and disfavor duplexes with mismatches at the ends (Kawamata and Tomari, 2010; Kawamata *et al.*, 2009).

Two processes have been described for the unwinding and separation of the miRNA:miRNA* duplex. First, Ago-2 (in flies as well as humans) has been shown to cleave and nick the passenger strand (Leuschner *et al.*, 2006; Matranga *et al.*, 2005; Miyoshi *et al.*, 2005; Rand *et al.*, 2005). In an Ago-2 slicing activity-dependent manner, separation by degradation of the passenger strand has been reported as a result of the activity of the Mg^{2+}-dependent endonuclease known as C3PO (component 3 promoter of RISC), in *D. melanogaster*, as well as the activity of the exonuclease QIP (QDE-1 interacting protein), in *N. crassa* (Liu *et al.*, 2009; Maiti *et al.*, 2007).

Second, a slower slicer-independent unwinding and separation of the passenger strand has been reported. This process is accelerated by and is the effect of the lower thermodynamic stability of the duplex compared to that of the miRNA:target duplex in a sequence-dependent manner. Additionally, in an ATP-independent manner, sequence pairing and center sequence mismatches have been shown to be essential for the unwinding of the miRNA:miRNA* duplex. Thus, in this case, it could be noted that the passenger strand is simply replaced by the target mRNA due to a “mirror image” effect in which the passenger strand acts as a first “target” sequence replaced by a more stable mRNA target sequence (Kawamata and Tomari, 2010; Kawamata *et al.*, 2009). This hypothesis is reinforced by the enhanced stability of the miRNA–RISC provided by the target mRNA; in other words, abundance of target mRNA increases accumulation of the cognate miRNA within the RISC. Moreover, enhanced accumulation of passenger strands is observed when a synthetic target is introduced in the system (Chatterjee *et al.*, 2011).

miRNA complementary sequences are most commonly located in the 3′- or 5′UTR of the target mRNA molecules. The miRNA pairing to the mRNA target sequence occurs in a nonperfect manner, commonly presenting central sequence mismatches. However, miRNAs are perfectly complementary to their target sequence at nucleotide ~2–8 in the 5′ of the miRNA, known as the “seed” sequence (Lee *et al.*, 1993; Wightman *et al.*, 1993). The presence of the miRNA in the cellular cytoplasm triggers the aggregation of the RISC. The RISC associates with several proteins involved in mRNA decapping to induce recircularization of the mRNA molecule; these include exonucleases to induce deadenylation and/or polyribosomes in order to block the translation of the mRNA target (Bartel, 2009).

3.4. miRNA expression and regulation

Initially, mutations in the *lin-4* gene were rescued by the introduction of a 683bp genomic DNA fragment, indicating that all or mostly all the regulatory elements for transcription and regulation of the *lin-4* gene were located within this fragment. Also, following gene structure analysis and cloning strategies, it was discovered that the *lin-4* gene was transcribed and expressed as a single amplicon (Ambros and Horvitz, 1987). RNA polymerase III drives the transcription of small RNAs including tRNAs, snRNAs, and U6 RNAs, and it was believed to be the miRNA transcription initiator. However, evidence accumulated to suggest that RNA polymerase II was also involved in the transcription of

miRNA genes. Currently, it is believed that RNA polymerase II is the major player responsible for the transcription of miRNAs with few being transcribed by RNA polymerase III (Bartel, 2004; Sayed and Abdellatif, 2011).

Computational prediction and cloning analyses have shown that miR-NAs are highly conserved and can be located in the genome embedded within an intron of a host gene (miRtron) or as a single- or polycistronic unit. The location of the miRNA gene is an important determinant of its expression and regulation. As a miRtron, miRNA expression is dictated by regulatory elements present in the promoter region of the host gene. miR-208a and miR-208b are located within intron-31 of the *α-MHC* and intron-29 of the *β-MHC* gene, respectively (Callis *et al.*, 2009; van Rooij *et al.*, 2009). As a single or a polycistronic unit, miRNA genes have their own *cis*-regulatory sequences and transcription is modified in a tissue- and cell-specific manner drawing similarities to transcription regulation of protein-coding genes. miR-1 and miR-133 contain conserved CArG boxes in their promoter regions and are directly regulated by SRF (Liu *et al.*, 2008; Zhao *et al.*, 2005). Indeed, in an elegant model of interregulation, it has been shown that in skeletal muscle, miR-133-modulated SRF expression represses cell proliferation, while miR-1-modulated HDAC4 expression represses MEF2-activated genes and induces cell differentiation. In a feedback regulatory loop, MyoD (a skeletal muscle transcription master regulator) and SRF regulate the expression of the miR-1/miR-133 polycistron (Fig. 10.2) (Chen *et al.*, 2006). Similarly, SRF, Myocardin, and Nkx2-5 directly regulate the expression of miR145/miR143 in cardiac cells as well as in smooth muscle cells (Cordes and Srivastava, 2009; Liu *et al.*, 2007).

Posttranscriptional regulation of miRNA expression has recently been reported in *C. elegans* and involves the activity of the Terminal Urydil Transferase 4 protein (TUT4). TUT4 is a noncanonical poly (A) polymerase which is recruited to the pre-miRNA by Lin28 protein due to the recognition of a GGAG motif in the terminal loop; it adds a poly (U) tail at the 3′end of the stem-loop, thus inhibiting the processing by the Dicer protein (Heo *et al.*, 2009). Additionally, it has been shown that terminal loop sequences direct the binding of the RNA-binding protein hnRNP A1. Binding of hnRNP A1 to the terminal loop of pri-miRNAs remodels the three-dimensional conformation of the hairpin, thereby modulating miRNA processing. Thus, hnRNP A1 acts as a regulator of Drosha-mediated miRNA processing (Guil and Caceres, 2007; Michlewski and Caceres, 2010; Michlewski *et al.*, 2008, 2010). Similarly, posttranscriptional processing of miRNAs is affected by extracellular signaling. Indeed, pri-miR-21 to pre-miR-21 processing is enhanced by the recruitment of SMAD proteins (the TGF-β and BMP signal transducers) to the Drosha miRNA microprocessor complex by the RNA helicase p68 and the consequent accumulation of pre-miRNA molecules in the complex (Davis *et al.*, 2008).

Additionally, chromatin structure and three-dimensional configuration have also been shown to contribute to the regulation of miRNA expression. Gene compartmentalization in various genomic domains is achieved by the periodic looping of chromatin also known as scaffold/matrix-attachment regions (S/MARs). S/MARs expose or hide specific *cis*-regulatory sequences in a cell- and tissue-specific manner where exposed elements are bound by the transcriptional machinery to up- or downregulate gene expression. Disruption in the expression of S/MAR-binding proteins, such as SATB1 and SMAR1, among others,

included within the transcriptional machinery, leads to chromatin modification and gene regulation alteration. Accumulation of S/MAR-binding proteins has been shown in upstream regions of the miR-17-92 cluster, as well as in the individual miRNAs let-7b, miR-17, miR-93, and miR-221 (Chavali *et al*., 2011).

4. miRNAs in Cardiac Development, Function, and Disease

Retrospectively, the first indication that miRNAs play an important role in embryonic development came to light with the genetic evidence that the small RNAs *lin-4* and *let-7* played a role in the posttranscriptional regulation of the expression of other heterochronic genes (Lee *et al*., 1993; Reinhart *et al*., 2000; Wightman *et al*., 1993). Subsequently, the discovery of miRNAs in other organisms and the description of the molecular machinery responsible for miRNA processing and function consolidated the notion that miRNAs indeed play an important role in several cellular processes. To date, cloning of small RNA molecules, quantitative RT-PCR screening, as well as microarray expression profiling have identified a large number of miRNAs in many tissues and organisms (Glazov *et al*., 2008; Lagos-Quintana *et al*., 2001, 2002, 2003; Lau *et al*., 2001; Lee and Ambros, 2001). Detailed information about miRNAs is accumulated in the miRNA database known as “miRBase.” According to its most recent release (Release 18, November 2011), there are 18, 226 entries expressing 21, 643 mature miRNA products in 168 species. In the mouse, there are 741 entries expressing 1283 mature miRNA sequences; in the human database, there are 1527 entries expressing 2108 mature miRNA sequences (miRBase: http://www.mirbase.org).

Expression and functional dissection of the mechanism of miRNA fine-tuning of gene expression has revealed considerable intersection and cross talk with well-described signaling and transcriptional networks for the regulation of development, morphogenesis, cell fate determination, and other cellular processes. In this section, we will discuss the function of miRNAs in heart development and summarize their role during cardiac remodeling and in disease.

4.1. miRNA function during embryonic stem cell differentiation into cardiomyocytes

Processing of mature miRNAs is a multistep biological process driven by the activity of the small RNA microprocessor (*Drosha/Dgcr8*) in the nucleus and Dicer in the cytoplasm. In flies, depletion of the Dicer homologue *Dcr-1* results in the derepression of target genes with noticeable effects during development and patterning (Lee *et al*., 2004). Similarly, targeted mutation of the mouse *Dicer* gene resulted in early embryonic lethality as a result of the depletion of the embryonic stem cell (ESC) pool (Bernstein *et al*., 2003). In tissue culture, mouse ES cells carrying a conditional mutation of the Dicer gene display severe defects in differentiation, aberrant expression of stem cell markers, reduction in epigenetic silencing, and absence of all small dsRNA molecules (Kanellopoulou *et al*., 2005). Although these cells maintain a slow rate of proliferation, they do not contribute to the formation of embryonic chimeras when injected into blastocysts (Kanellopoulou *et al*., 2005). Similarly, a *Dgcr8* null mutation in mouse ES cells results in the accumulation of pri-miRNAs and the absence of precursors and mature miRNA. However, *Dgcr8* null ES cells differ from *Dicer*

null ES cells in that the former express stem cell molecular markers normally (Wang *et al.*, 2007).

miRNA and mRNA expression profiles have documented that mouse and human ES-derived cardiomyocytes, compared to fetal and adult heart tissue, exhibit a correlation between miRNA expression and the up- or downregulation of mRNA expression. Although it has been demonstrated that miRNAs target mostly 3′UTR mRNA sequences, such an inverse correlation in miRNA and mRNA expression indicates that miRNAs are surely involved in the cardiac differentiation regulatory network (Synnergren *et al.*, 2011). Indeed, cardiac differentiation of human fetal cardiomyocyte progenitor cells, or mouse ES cells, is accompanied by an upregulation of several miRNAs including miR-1, miR-133, and miR-499. Correspondently, adenoviral overexpression of miR-1 and miR-499 reduced progenitor cell proliferation by 25% and 15%, respectively, while enhancing differentiation (Sluijter *et al.*, 2011). Simultaneously, lower protein levels of several progenitor cell or stem cell molecular markers were observed (Ivey *et al.*, 2008; Sluijter *et al.*, 2011). Downregulation of the Notch signaling pathway has been demonstrated to be necessary for normal muscle differentiation, while its overexpression inhibits such lineage fate. During cardiac differentiation, Delta, a Notch ligand, is targeted by miR-1. miR-1 lowers the protein levels of Delta as hESC cardiac differentiation progresses (Ivey *et al.*, 2008). Similarly, miR-1 targets and induces the translational repression of HDAC4, a transcriptional repressor of Mef2-dependent activation of muscle-specific genes. Meanwhile, miR-499 has been shown to repress the translation of Sox6 (Sluijter *et al.*, 2011), the sex-determining region-Y box 6 transcription factor which is involved in the maintenance of cardiac development (Cohen-Barak *et al.*, 2003). Moreover, knockdown experiments in ESCs demonstrate that miR-1 is required for normal smooth muscle and cardiac muscle differentiation, at least in part, by modulating the expression of the Kruppel-like factor 4 (Klf4) (Xie *et al.*, 2011). In addition, miR-1 overexpression in ESC attenuates apoptosis by indirectly elevating the levels of p-Akt (which, in turn, diminishes PTEN and Caspase-3 levels) and also by modulating the expression of Hsp60 (Glass and Singla, 2011; Shan *et al.*, 2010). It is predicted that still more miRNAs remain to be identified with significant roles in the regulation of cardiomyocyte cell fate.

4.2. miRNAs during cardiac development

As noted above, miRNAs have been revealed to have an enormous impact during embryonic development, as observed in Dicer null mice (Bernstein *et al.*, 2003), and Dgcr8 null mice (Wang *et al.*, 2007). In the heart, conditional mutation of Dicer by employing cardiac tissue-specific Cre lines has revealed that miRNAs are not only necessary for cardiac embryonic development but also essential for postnatal cardiac maintenance and function (Chen *et al.*, 2008; da Costa Martins *et al.*, 2008; Huang *et al.*, 2010b; Saxena and Tabin, 2011). Surprisingly, it has been shown that the developmental program is unaffected when individual miRNAs are mutated. Nevertheless, such miRNAs appear to be powerful mediators in the modulation of gene expression under stress conditions (Cordes and Srivastava, 2009; Huang *et al.*, 2010a; Tatsuguchi *et al.*, 2007; van Rooij *et al.*, 2007).

Myo-miRs or muscle miRNAs is a term used to describe those miR-NAs specifically expressed or enriched in smooth, skeletal, and/or cardiac muscle (van Rooij *et al*., 2009). In particular, miR-1, miR-133, miR-208, and miR-499 have been reported to be highly expressed in cardiac tissue from the early stages of heart development to the adult. These specific myo-miRs have been shown to be involved in cardiac development, regulating cell proliferation, differentiation, and apoptosis, and also playing an important role in several cardiac diseases, such as cardiac hypertrophy, myocardial infarction (MI), cardiac arrhythmia, and heart failure (Sayed and Abdellatif, 2011; van Rooij *et al*., 2009). In addition, many non-myo-miRs, those expressed in multiple tissues as well as heart muscle, are also involved in the development and differentiation of this organ (Sayed and Abdellatif, 2011).

miR-1 and the closely related miR-206 possess an identical seed sequence and are related in function and expression; however, they are present in different genomic loci and their expression and transcriptional regulation differs. miR-1 is predominantly enriched in cardiac and skeletal muscle tissues, while miR-206 is a skeletal muscle-specific miRNA (Chen *et al*., 2010). Both miR-1 and miR-206 are expressed along with miR-133 as a bicistronic unit from three different genomic loci: in mice, miR-1-1/ miR-133a-2 (chromosome 2), miR-1-2/ miR-133a1 (chromosome 18), and miR-206/miR-133b (chromosome 1) (Liu *et al*., 2007). Cloning screening determined that miR-1 expression accounts for ~45% of the miRNAs found in the adult mouse heart and ~24% in the adult human heart, suggesting its participation in cardiac formation and function (Lagos-Quintana *et al*., 2002). In chick embryos, *in situ* hybridization shows that miR-1 is expressed in early stages of cardiac development, at the onset of cardiomyocyte differentiation and during the formation of the heart tube, as well as in the developing somites where skeletal muscle progenitors are located (Darnell *et al*., 2006). Similar results were obtained by X-gal staining in transgenic mice in which the *LacZ* cassette is driven by miR-1 upstream fragments (Zhao *et al*., 2005). Sequence analysis of this upstream fragment revealed conserved consensus response elements corresponding to the early cardiac determination transcription factor Nkx2-5. Accordingly, the fly transcription factor Tinman and its homologue Nkx2-5 in mice have been shown to regulate the expression of miR-1 by directly binding upstream-specific *cis*-regulatory response elements (Qian *et al*., 2011). The Rho-GTPase CDC42 interacts with Tinman/Nkx2-5 and cooperates to regulate miR-1 expression; more interestingly, CDC42 itself is a miR-1 target. This feedback regulatory network is involved in cardiac output and the formation of a normal myofibrillar architecture (Qian *et al*., 2011). Similarly, SRF, Mef2c, MyoD, and Myogenin have been shown to directly bind consensus-conserved response elements in order to regulate miR-1 expression in cardiac and skeletal muscle tissues (Liu *et al*., 2007; Rao *et al*., 2006). Interestingly, myocardin, a well-known transcription cofactor of SRF, has been recently reported to regulate the expression of miR-1 in vascular smooth muscle cells (Wang *et al*., 2001). Inducible overexpression of myocardin results in an upregulation of the expression of miR-1 which, in turn, inhibits cell proliferation due to the downregulation of the serine/ threonine kinase Pim-1 (Chen *et al*., 2011; Jiang *et al*., 2010).

The early embryonic expression, together with its regulation by early cardiac differentiation determinants, suggests that miR-1 may play an important role in cardiac development and

function. Indeed, miR-1 loss-of-function in *D. melanogaster* is embryonic lethal due to a disruption in the patterning of the dorsal vessel as a result of a defect in muscle differentiation. This results in a failure in cardiac progenitor cell determination, leading to an increase in undifferentiated progenitor cells (Kwon *et al.*, 2005; Sokol and Ambros, 2005). This phenotype is strongly related to the miR-1-mediated downregulation of Delta, a Notch ligand involved in cell–cell signaling required for the differentiation and maintenance of skeletal and cardiac muscle (Kwon *et al.*, 2005). Similarly, in mice, null mutation of miR-1-2 results in noticeable embryonic lethality from E15.5 up to 50% lethality at weaning time. miR-1-2 null mutant embryos show pericardial edema and cardiac dysfunction. Juvenile null mice present cardiac hyper-plasia due to cell proliferation up to three times higher than normal, ventricular hypertrophy, or dilated cardiomyopathy, in addition to defects in heart function related to defects in the cardiac conduction system. In the surviving mice, higher levels of Irx5 (a miR-1 target which is involved in the regulation of the cardiac repolarization gradient through Kcnd2/Kv4.2) and higher protein levels of Hand2 are observed (Costantini *et al.*, 2005; Zhao *et al.*, 2007). In contrast, miR-1 overexpression driven by the β-MHC promoter in transgenic mice during early cardiac formation results in stunted growth and cardiac development arrest at embryonic day E13.5 (E13.5). The hearts show thin-walled ventricles and heart failure due to lower levels of proliferation compared to wild-type controls (Zhao *et al.*, 2005). miR-1 was shown to target the 3′UTR of Hand2, a bHLH tran-scription factor expressed early in development beginning at E7.75–E8.0 in the linear heart tube and further regulating differentiation of the right ventricle (Srivastava *et al.*, 1997).

miR-133 is a component of the bicistronic unit along with miR-1, yet miR-133 expression is not as abundant (Lagos-Quintana *et al.*, 2002). However, miR-133 has been shown as an important player during cardiac development. In an effort to uncover miR-133 functions in the heart, miR-133a-1 and miR-133a-2 were targeted for null mutation. Analyses of single miR-133 gene mutation resulted in no differences when compared to wild-type controls. When analyzing double null mutation of miR-133a-1 and miR-133a-2, it was observed that the double mutation resulted in partial embryonic or postnatal lethality. This lethality is likely related to cardiovascular defects including ventricular septal defects (VSDs) and dilation in the atria and ventricles as a result of increased proliferation and, intriguingly, increased apoptosis near the interventricular septum (Liu *et al.*, 2008; Sayed and Abdellatif, 2011). Mutant animals that escape the early lethality develop dilated cardiomyopathy and present thinner ventricular walls and cardiac failure. However, cardiac hypertrophy and VSD are not observed in mutant mice (Liu *et al.*, 2008). These latter results are in contradiction to previous observations where downregulation of miR-133 by specific antagomirs induces cardiac hypertrophy (Carè *et al.*, 2007). These phenotype discrepancies might be attributed to the imperfect downregulation of miR-133 by the antagomir and/or residual expression of miR-133b since it is not targeted in the miR-133a-1/miR-133a-2 null mice. On the other hand, overexpression of miR-133 during mouse embryonic stages driven by the β-MHC promoter results in a phenotype similar to that found in β-MHC-miR-1 overexpressing mice (including embryonic lethality at mid-gestational stages (rvE15.5), reduced proliferation inducing thinning of the ventricular walls, VSD, and heart failure) (Liu *et al.*, 2008).

As a member of the bicistronic unit with miR-1, miR-133 expression is also regulated by several transcription factors including SRF. Interestingly, the miR-133a-1/miR-133a-2 double knockout mice show a twofold increase in SRF expression and the activation of a smooth muscle genetic network in cardiac tissue (Liu *et al.*, 2008). SRF overexpression in postnatal hearts driven by the α-MHC promoter induces cardiac dilation, in addition to increased heart weight due to hypertrophy and the reactivation of a fetal genetic program. Moreover, the 3′UTRs of SRF, as well as CyclinD2 and CDC42, are targeted by miR-133 indicating that miR-133 is involved in the control of cell proliferation and cell cycle (Carè; *et al.*, 2007; Liu *et al.*, 2008).

Although miR-1 and miR-133 are transcribed as a single molecule, their maturation and function differ. As noted, miR-1 is highly expressed in cardiac and skeletal muscle tissues, while miR-133 appears less abundant. Both miRNAs play important roles in the development of the cardiac tissue, albeit with opposite functions; miR-1 promotes and regulates cell differentiation, while miR-133 induces cell proliferation and the maintenance of the progenitor cell pool (Chen *et al.*, 2006). A similar result is observed when miR-1 is overexpressed in Xenopus embryos where myoblast and cardiac precursor proliferation is reduced while differentiation is enhanced. However, injection of miR-133 results in augmented cell proliferation with a reduction in cell differentiation, more so in myoblast than in cardiac progenitor cells (Chen *et al.*, 2006). This apparent contradiction in the effects of miR-1 and miR-133 in cell proliferation and cell differentiation might be explained by the differences in the nature of their targets and their distinct and possible independent transcription regulation, even though both belong to a bicistronic unit. As noted, miR-1 targets HDAC4, which represses myoblast differentiation, while miR-133 has been shown to target SRF, which is involved in transcriptional regulation of proliferation and differentiation of muscle cells (Chen *et al.*, 2006). Interestingly, SRF directly binds to upstream regions of the miR-1/miR-133 bicistronic unit, which contain consensus CArG boxes, to activate its transcription (Zhao *et al.*, 2005). Moreover, MEF2 and MyoD also directly regulate the transcription of miR-1/miR-133 by binding to an intergenic enhancer containing MEF2 response elements and E-box binding sequences. These intergenic enhancers, present in the miR-1-1/miR-133a-2 and miR-1-2/miR-133a-1 bicistrons, are hypothesized to drive miR-1/miR-133 differential expression (Fig. 10.2). Taken together, these results indicate that regulatory feedback networks are in place in order to regulate cell proliferation versus cell differentiation, in part mediated by miR-1 and miR-133 (Liu *et al.*, 2007).

During cardiac development, not only miR-1/miR-133 but also other miRNAs have been shown to exert an effect. Recently, miR-130a was reported to be involved in cardiac development through targeting the transcription factor Friend-of-GATA 2 (Fog-2). miR-130a is broadly expressed in kidney, liver, testis, and brain but highly enriched in lung and heart. Embryonic overexpression of miR-130a, driven by the β-MHC promoter, results in ventricular hypoplasia and embryonic lethality at mid-gestation stages (E13.5–E14.5), a phenotype resembling that described for Fog-2 null mutant mice (Kim *et al.*, 2009). Similarly in mice, miR-195, a member of the miR-15 family, was shown to induce ventricular hypoplasia and VSD when overexpressed in embryonic stages under the β-MHC promoter, in part by affecting the expression of the Check point 1 protein (Check1).

Knockdown experiments using locked nucleic acid oligomers against miR-15 derepresses Check1 and induces postnatal myocytes to reenter the cell cycle (Porrello *et al*., 2011).

In zebrafish, cardiac expression of miR-138 is found to be restricted to the ventricle. Knockdown experiments targeting miR-138 result in pericardial edema at late cardiac stages (48hpf) reflecting cardiac dysfunction. However, initial cardiac development and formation appears undisturbed. Further, miR-138 knockdown resulted in the expansion of the expression fields of aldh1a2 (retinoic acid dehydrogenase 2-Raldh2), a putative miR-138 target that is involved in retinoic acid synthesis. Derepression of Raldh2 and versican (another putative miR-138 target that encodes a versatile extracellular matrix proteoglycan) in the heart induces a defect in cardiac patterning characterized by the aberrant formation of the atrio-ventricular canal (AVC) (Morton *et al*., 2008). Knockdown of miR-143, a miRNA highly expressed in the cardiovascular system, derepresses the RA signaling pathway which profoundly influences cardiac patterning (Miyasaka *et al*., 2011). Similarly, miR-143 regulates ventricular cardiomyocyte F-actin remodeling. Downregulation of miR-143 results in ventricular collapse by targeting adducin3 (add3), an F-capping protein, affecting F-actin dynamics, cell morphology and contractility (Deacon *et al*., 2010). Further, in an interesting twist, the mechanical input of the heartbeat itself induces the expression of miR-143 although the molecular pathway is not fully known (Miyasaka *et al*., 2011). Additionally, in a screen performed in Dicer mutant zebrafish hearts, Has2, a molecular marker of AVC formation, was found to be upregulated. In turn, overexpression of miR-23, a putative targeting miRNA, was found to repress Has2 expression and to reduce extracellular matrix remodeling and endocardial jelly formation, resulting in impaired cardiac valve formation. Also, miR-23 blocks the EMT (endothelial-to-mesenchymal transformation) induced by the TGF-β signaling pathway possibly due to targeting Has2, icat, and *tmem2* 3′UTR sequences (Lagendijk *et al*., 2011). These studies indicate that miRNAs are novel regulators of EMT, cellular migration driven by signaling cues, and are essential for the normal formation of the cardiac tube. The *Slit/Robo* signaling pathway has been shown to be one of the most important migration/repression signals during development (Kidd *et al*., 1999). miR-218 is expressed from intronic sequences in *SLIT2* and *SLIT3* and targets the *ROBO1* and *ROBO2* mRNA to repress their translation. Overexpression of miR-218 and the consequent repression of the ROBO proteins reduced the activation of the MAPK pathway and disrupted the VEGF-induced migration of endocardial and myocardial cells, leading to an aberrant heart (Fish *et al*., 2011)

The polycistronic miR-17-92 cluster, also called Oncomir-1, comprises seven miRNAs and related miRNA clusters are ubiquitously expressed. The miR-17-92 cluster has been shown to be related to the enhancement of cell proliferation in several cancers, reduction of apoptosis, and increasing tumor angiogenesis (He *et al*., 2005). During cardiac development, null mutation of the related miR-106arv363 and miR-106brv25 clusters resulted in no observable phenotype and mice are viable and seemingly normal; however, null mutation of the miR-17-92 cluster has been reported to result in lung hypoplasia and induce the appearance of VSD at late developmental stages leading to immediate postnatal lethality (Ventura *et al*., 2008).

4.3. miRNAs in cardiac remodeling and pathological cardiac processes

Pathological processes are the cause or the effect of a disruption of the normal physiological status of a cell, tissue, organ, and ultimately an organism. These changes are also seen at the molecular level as a dynamic change in gene expression and manifested by altered genetic interactions. In the heart, cardiac pathologies often refer to stress-related disorders as well as congenital diseases which ultimately lead to cardiac tissue remodeling. These pathological defects include cardiac hypertrophy, cardiac dilation, and MI. Genome-wide analyses have uncovered significant changes in genetic expression signatures when the cardiac tissue is under stress conditions, showing a change in mRNA expression along with up- or down-regulation of miRNAs. In particular, under stress conditions, the 3′UTRs of mRNAs were shortened and the repressive function of miRNAs was reduced. Also, there is an activation of a fetal genetic program, which is indicative of a return to a developmental program in order to mitigate the effects of such a stress (Fig. 10.3) (Park *et al.*, 2011; Thum *et al.*, 2007).

miR-208 is expressed specifically in cardiac tissue and is detected as two isoforms, miR-208a and miR-208b. They are transcribed from intronic sequences in the myosin heavy chain (MHC) genes *Myh6* (*α-MHC*) and *Myh7* (*β-MHC*), respectively. These two miRNAs differ only in 3nt but they share an identical “seed” sequence, indicating that both miRNAs might target the same genes. During cardiac development, the MHC genes are expressed at different developmental stages; the slow contractile β-MHC is predominantly expressed in adult skeletal muscle and in the heart during embryonic stages, whereas the fast contractile α-MHC is expressed at late embryonic stages and in the adult heart, becoming the predominant cardiac MHC shortly after birth. The switch from β-MHC to α-MHC is dependent on the activity of thyroid hormone (TH) signaling at birth which directly regulates *α-MHC* and *β-MHC cis*-response elements, repressing *β-MHC* and activating α-MHC expression (Weiss and Leinwand, 1996). Due to their location within introns, miR-208b and miR-208a are coexpressed with their host genes. Therefore, the TH signaling also regulates a miR-208b to miR-208a switch, which was previously unrecognized when investigating the MHC switch, at birth (Callis *et al.*, 2009; van Rooij *et al.*, 2007). In stress models which induce cardiac hypertrophy, such as thoracic aortic banding or transgenic overexpression of calcineurin in the heart (Molkentin *et al.*, 1998), the β-MHC to α-MHC switch at birth is not observed and the β-MHC embryonic levels persist postnatally and in the adult heart (Callis *et al.*, 2009).

In mice, miR-208 null mutations do not show any severe developmental phenotypes. The mice are viable although they exhibit some abnormalities in the cardiac conduction system as adults, including atrial conduction deficiencies and atrial fibrillation. This is consistent with the observation that miR-208a targets GATA4, an early cardiac differentiation marker and a direct regulator of Cx40 expression in the conduction system (Callis *et al.*, 2009). Although miR-208a null mice show normal α-MHC and β-MHC expression levels, under stress conditions, the signature persistence of β-MHC in postnatal stages is not observed (van Rooij *et al.*, 2007). Nevertheless, other cardiac hypertrophy markers such as ANF are upregulated, indicating that the effect of miR-208a mutation is specific in the modulation of β-MHC levels. Indeed, overexpression of miR-208a driven by the α-MHC promoter induces cardiac hypertrophy and the induction of β-MHC but not ANF (Callis *et al.*, 2009). Through

bioinformatic screens, it was predicted and later experimentally demonstrated that the thyroid receptor associated protein 1 (THRAP1) was a miR-208a target. THRAP-1 is necessary for TH signaling by recruiting RNA pol II to *cis*-regulatory elements of target genes, in this case, to downregulate β-MHC and upregulate α-MHC at birth when TH levels increase (Canepari *et al*., 1998). Thus, THRAP-1 expression modulation is determined by miR-208a, providing a direct mechanism for regulation of β-MHC expression. Consequently, the expression of miR-208b is also disturbed in miR-208a null mutant mice under stress conditions. Indeed, miR-208a overexpression represses the expression of miR-208b, and in hypertrophied miR-208a null mice hearts, miR-208b upregulation is stunted (Callis *et al*., 2009). A third member of the miR-208 family, miR-499, is encoded by intron 19 of the mouse Myh7b gene. Similar to β-MHC, Myh7b and miR-499 are highly enriched in skeletal muscle as well as in the embryonic heart and their expression is absent in miR-208a overexpressing mice. Interestingly, over-expression of miR-499 is sufficient to counteract the effects of loss of miR-208a in mice, including the restoration of β-MHC and miR-208b expression to normal levels, and the normal response to TH inhibition (Fig. 10.3) (van Rooij *et al*., 2009).

The expression of miR-1 was reduced in hypertrophic hearts induced by transgenic overexpression of calcineurin as well as in the hearts of human patients presenting with cardiac hypertrophy or aortic stenosis (Sayed and Abdellatif, 2011). On the other hand, knockdown of miR-1 in mice proves efficient in induction of cardiac hypertrophy. Intriguingly, therapeutic delivery of miR-1 has been shown to reverse hypertrophy and preserve cardiac function and result in the upregulation of SERCA2a, ANF, and β-MHC (Karakikes *et al*., 2010). However, results obtained from human patients complicated this issue. It was shown that the expression of miR-1 is up- or downregulated depending on the type of cardiac stress (i.e., cardiac hypertrophy vs. ischemia) indicating that a variety of factors are in play during such events. Indeed, several genes that play an important role in these processes have been shown to be targeted by miR-1, indicating that miR-1 regulates several genetic networks independently in the heart. These genes include Cdk9, involved in the cell cycle; Mef2a, involved in myoblast differentiation; calmodulin, a Ca^{2+}-binding protein involved in several cellular processes; insulin-like growth factor-1, involved in cell growth; and the heat shock proteins Hsp60 and Hsp70, which are involved in apoptosis (Fig. 10.4) (Elia *et al*., 2009; Sayed *et al*., 2007; Shan *et al*., 2010.)

In addition to miR-1, miR-133 has also been shown to be downregulated during the onset of cardiac hypertrophy. Indeed, knockdown of miR-133 was sufficient to induce cardiac hypertrophy; however, as described previously, miR-133a-1/miR-133a-2 double ablation results in aberrant myocyte proliferation and apoptosis, VSDs, and partial embryonic lethality without induction of hypertrophy (Liu *et al*., 2008). The difference in the results might be explained by the residual miR-133b expression in the double knockout model and the difference in the timing of onset of hypertrophy (Abdellatif, 2010; Carè *et al*., 2007). Alternatively, it is formally possible that the oligonucleotide-based miR-133 knockdown could result in “off-target” effects. Additionally, overexpression of miR-133 under the *α-MHC* promoter shows that miR-133 transgenic mice are protected from stress-induced cardiac fibrosis, although the transgenic mice also present with some cardiac conduction deficiencies (Matkovich *et al*., 2010). However, transgenic overexpression of miR-133 under

the *β-MHC* promoter results in early embryonic lethality. Similar to miR-1, the effects of miR-133 during cardiac development and cardiac hypertrophy are results of the interplay between a wide variety of mRNA targets, including Caspase-9 and Hsp70 in apoptosis, SRF in myocyte differentiation, Cdc42 in the regulation of the cell cycle, among others (Abdellatif, 2010; Carè *et al*., 2007).

miR-21, another miRNA which is dramatically upregulated during cardiac hypertrophy, has been shown to repress the expression of the sprouty gene 1 (Spry-1), a known inhibitor of the ERK–MAP kinase pathway, thus enhancing cell survival (Thum *et al*., 2008). Knockdown of endogenous miR- 21 in mice under pressure overload conditions results in attenuation of the hypertrophic response measured in terms of collagen fiber deposition and cardiac function (Thum *et al*., 2008). Conversely, overexpression of miR-21 in mice shows that it exerts a protective effect as determined by the reduction of the myocardial infarct size and reduced apoptosis in the infarct border zone under ischemia conditions. miR-21 was proposed to repress the expression of PDCD24 in this setting (Dong *et al*., 2009). However, these observations do not correlate with results reported for miR-21 null mutant mice. Deletion of miR-21 does not result in any phenotype and mice do not show any differences compared to wild-type controls, when under stress conditions (Patrick *et al*., 2010). Certain differences in experimental details might account for the observed contradictory results, highlighting the critical impact of genetic approaches in deciphering the function of individual miRNAs.

A molecular signature of cardiac hypertrophy is the upregulation of myocardin expression. Indeed, myocardin overexpression in cultured cardiomyocytes is sufficient to induce cardiomyocyte hypertrophy (Xing *et al*., 2006). During hypertrophy, the nuclear factor activator of T cells (NFAT3c and NFATc4) directly regulates myocardin expression by binding to the upstream regions of the myocardin promoter. In turn, miR-9 suppresses myocardin expression by targeting the myocardin 3′UTR, attenuating the effects of hypertrophy. Similar effects are observed upon miR-133a repression of NFAT4c and lowering of its protein levels (Li *et al*., 2010; Wang *et al*., 2010).

During cardiac remodeling, fibrosis or fibrotic deposition is enhanced, resulting in an increase of muscular thickening and the loss of flexibility. Fibrotic deposition involves the enhanced proliferation of cardiac fibroblasts and the enhanced production of extracellular matrix components. Several molecular pathways, including the TGF-β signaling pathway, are involved in these processes. Generation of mice with a null mutation in *Smad-4* results in animals with hypertrophic hearts and is correlated with an upregulation of miR-27b. Silencing of miR-27b is sufficient to mitigate the effects of *Smad-4* null mutation, or the hypertrophy induced in a pressure overload model (Wang *et al*., 2011). Conversely, TGF-β1, an inhibitor of myoblast differentiation, downregulates miR-24 expression by direct binding of Smad-3 and Smad-4 to upstream promoter sequences. In Smad-4 null hearts, miR-24 is upregulated which then induces endothelial cell apoptosis under cardiac ischemic conditions by targeting the endothelium-rich transcription factor GATA2 and the p21-activated kinase PAK4 (Sun *et al*., 2008). Overexpression of miR-24 in zebrafish impairs normal cardiac angiogenesis, and knockdown experiments result in a reduced infarct size by vascularization enhancement (Fiedler *et al*., 2011).

Higher levels of cellular apoptosis have been observed following MI and ischemia/reperfusion (I/R) injury and several miRNAs have been shown to regulate such cellular process. In mice, miR-320 expression is decreased following I/R injury. miR-320 overexpression increases apoptosis and increases the extent of the infarct size after I/R, while miR-320 knockdown shows cell-protective effects through the derepression of Hsp20 (Dong *et al*., 2009; Ren *et al*., 2009). Similarly, miR-1/206 has been shown to function as a cardioprotective factor through the repression of Hsp60 and Hsp70 (Fig. 10.4). Additionally, miR-30, miR-133, and miR-199a were shown to function as apoptotic factors through targeting p53, Caspase-9, and Hif-1a and Sirt-1, respectively (Li, 2010).

5. Final Conclusions

In the past decade, miRNAs have clearly established themselves as important manipulators of gene expression and key regulators of cell fate determination, proliferation, differentiation, and cell death during embryogenesis as well as in postnatal life. Yet, only a fraction of the total number of miRNAs have been studied in detail and the true magnitude of their regulatory ability remains to be determined. Indeed, the miRNA's characteristic "seed" sequence along with its imperfect base pairing allows for one miRNA to target multiple mRNAs; as a corollary, a single mRNA could be targeted by many miRNAs, increasing the level of complexity in the regulation of these intertwined genetic networks. We are confident that we will see more and more reports on the roles of miRNAs in the regulation of a variety of essential biological processes.

Human disease, in general, and cardiac diseases, specifically, are commonly associated with genetic haploinsufficiencies and/or point mutations. Such mutations often lead to the reduction of gene expression and/or function. Therefore, miRNAs, which function as reagents for fine-tuning levels of mRNAs and their protein products, are potential pharmacological targets that can be exploited for therapeutic purposes. With further characterization and the elucidation of context-dependent functions, miRNAs will provide new avenues for the diagnosis, prognosis, and treatment of cardiovascular diseases.

ACKNOWLEDGMENTS

We thank members of the Wang laboratory for discussion and support. We thank Dr. John Mably for careful reading of the chapter and stimulating discussion. Research in the Wang lab was supported by the March of Dimes Birth Defect Foundation and the National Institutes of Health. R. A. E. L. is a postdoctoral fellow grantee of the American Heart Association. D. Z. W. is an established investigator of the American Heart Association.

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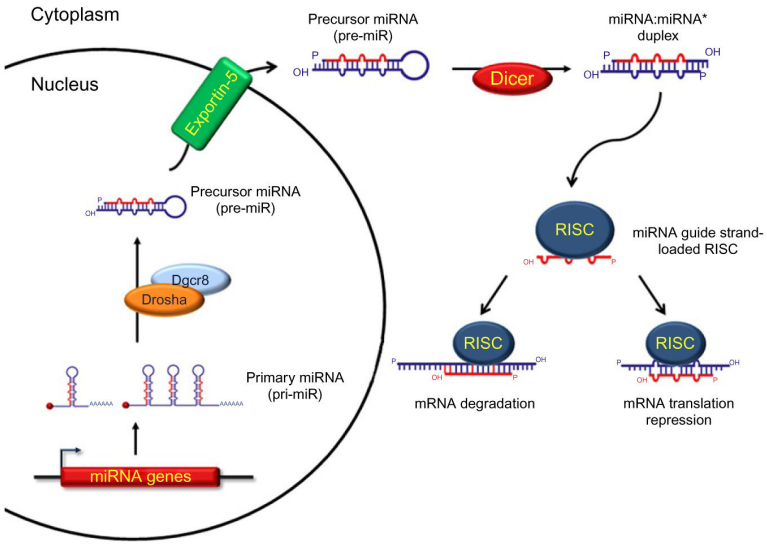

Figure 10.1.

MicroRNA Biogenesis

In the nucleus, a miRNA gene is transcribed into a long RNA molecule containing a single miRNA sequence or several miRNA sequences (polycistron or cluster), known as primary miRNA (pri-miRNA). Pri-miR-NAs are protected by the addition of a 5′-m^7G cap (red ball) and a 3′ poly-A tail. Pre-miRNAs are processed by the microprocessor machinery composed of Drosha and the stabilizing protein Dgcr8, thus producing a precursor-miRNA molecule (pre-miRNA). Pre-miRNAs are transported out of the nucleus to the cytoplasm by the active trans-porter Exportin-5 and further processed by Dicer into a miRNA:miRNA* duplex. The RNA-induced silencing complex (RISC) is assembled and loaded with the miRNA guide strand due to the unwinding of the miRNA:miRNA* duplex. A loaded RISC will facilitate the recognition of mRNA targets leading to the mRNA degradation or the inhibition of translation.

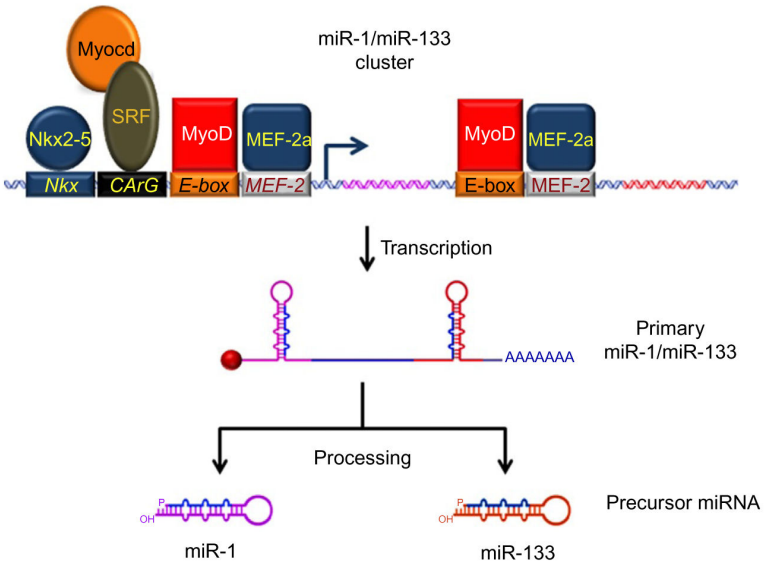

Figure 10.2. Regulation and processing of the miR-1/miR-133 cluster

The miR-1/miR-133 clusters are present in different chromosomes (see text). Several transcription factor response elements are shown in the 5′upstream promoter region as well as in the "intergeneic" region. As shown, *Nkx, CArG, E-box*, and *MEF-2* boxes represent recognition sites for Nkx2-5, SRF, Myogenin/MyoD, and MEF-2a binding, respectively. Further processing of the cluster results in the formation of two hairpin precursor miRNAs, miR-1 (in purple) and miR-133 (in red). Mature miRNA is represented in blue in both hairpins. Primary miRNAs are protected from early degradation by the addition of a 5′-m^7G cap (red ball) and a 3′ poly-A tail.

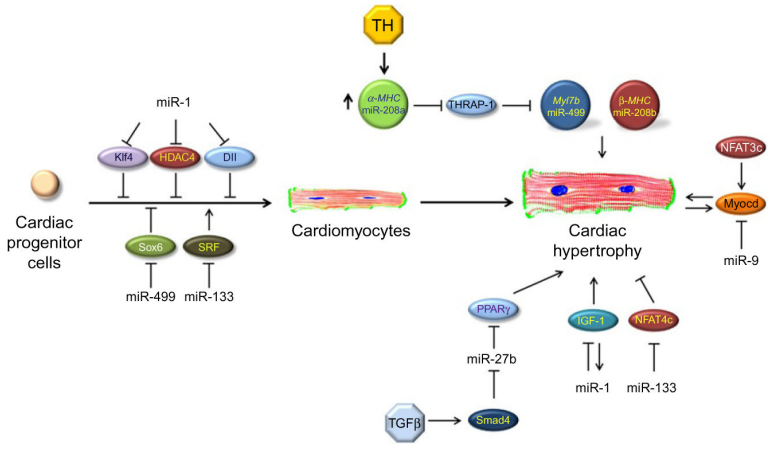

Figure 10.3. miRNA function and integration in the regulatory networks to regulate cardiomyocyte differentiation and disease

Graphic representation of several regulatory networks targeted by miRNAs in cardiomyocyte differentiation from cardiac progenitor cells, cardiomyocytes, and their function in the regulation of cardiac hypertrophy. Also, the integration of miRNAs in such regulatory networks by targeting transcription factors, signaling molecules, and/or structural proteins is shown.

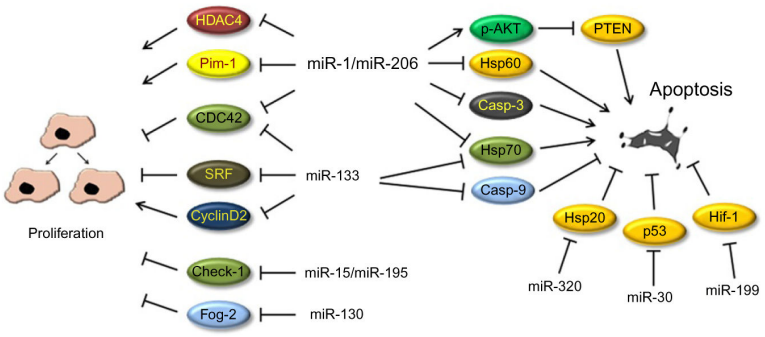

Figure 10.4. miRNA regulatory effects on cellular processes

Graphic representation of several miRNAs' regulatory effects on two opposing cellular processes such as proliferation and apoptosis. It is worthy to note that one miRNA might be involved in both processes by targeting different molecules which, in turn, induce or inhibit such process.