MICRORNA FUNCTION IN MUSCLE

DEVELOPMENT

Jianfu Chen

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Approved by:

Da-zhi Wang

Mark Majesky

Cam Patterson

Scott Hammond

Yi Zhang

ABSTRACT

MICRORNA FUNCTION IN MUSCLE DEVELOPMENT

(Under the direction of Dr. Da-zhi Wang)

MicroRNAs (miRNAs) are a class of non-coding RNAs of ~22 nucleotides in length that

post-transcriptionally regulate gene expression. While there are more than 600 miRNAs

identified in human genome, the biological functions of miRNAs are largely unknown. Here

we show that microRNA-1 (miR-1) and microRNA-133 (miR-133) are specifically expressed

in cardiac and skeletal muscle. Paradoxically, miR-1 and miR-133 exert opposing effects

during skeletal muscle development *in vitro* and *in vivo*. miR-1 promotes myogenesis by

targeting histone deacetylase 4 (HDAC4), a signal dependent chromatin regulator that

represses MEF2 activity. MEF2, in turn, potently activates miR-1/-133 expression. In

contrast, miR-133 enhances myoblast proliferation by repressing serum response factor

(SRF), an essential regulator for muscle proliferation and differentiaiton. Together, these

findings suggest that miR-1 and miR-133 are integrated into existing genetic circuits that

control skeletal muscle development.

We address the function of miRNAs during heart development using mouse genetic

approaches. Cardiac-specific deletion of Dicer, a RNase III endonuclease responsible for

miRNA maturation, leads to rapidly progressive dilated cardiomyopathy (DCM), heart

failure, and postnatal lethality. Dicer mutant mice show misexpression of cardiac contractile proteins and profound sarcomere disarray. Functional analyses indicate significantly reduced heart rates and decreased fractional shortening of Dicer mutant hearts. Consistent with the role of Dicer in animal hearts, Dicer expression was decreased in end-stage human DCM and failing hearts and, most importantly, a significant increase of Dicer expression was observed in those hearts after left ventricle assist devices were inserted to improve cardiac function. Together, our studies demonstrate essential roles of Dicer in cardiac contraction and indicate that miRNAs play critical functions in normal cardiac development and function

To examine the potential involvement of miRNAs in muscle stem cells, known as satellite cells, we examined miRNA expression profiles during satellite cell differentiation and skeletal muscle regeneration. miR-1 and its isoform, miR-206, are sharply up-regulated during satellite cell differentiation and down-regulated in muscle injury. miR-1 and -206 facilitate satellite cell differentiation by restricting satellite cell proliferative potential. We identify Pax7, an essential stem cell maintenance gene of satellite cells, as one of direct regulatory targets of miR-1 and -206. Knock down *in vivo* using antagomirs specifically against miR-1 and -206 in neonatal mouse skeletal muscle enhances satellite cell proliferation and Pax7 protein levels. Conversely, sustained Pax7 expression due to the loss of miR-1 and -206 repressive elements at Pax7 3' UTR inhibits myogenic progenitor differentiation. Our studies reveal a critical role of miR-1 and -206 in satellite cells and suggest that miRNAs participate in a regulatory circuit that allows rapid gene program transiton from cell proliferation to differentiation by repressing the expression of stem cell maintenance gene.

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ABBREVIATIONS

RISC: RNA-induced silencing complex

RBD: RNA binding domain

EMT: Epithelial-mesenchymal transition

HCM: Hypertrophy cardiomyopathy

DCM: Dilated cardiomyopathy

GPCR: G protein coupled recept

AV canal: Atria ventricle canal

PE: Proepicardium

ECM: Extracellular matrix

EPDCs: Epicardial-derived mesenchymal cells

PTGS: Post transcriptional gene silencing

PAZ domain: piwi-argonaute-zwille domain

TGF-β: The transforming growth factor –beta

DML: Dorsal medial lip

VLL: Ventral lateral lip

MRFs: Myogenic regulatory factors

SMCs: Smooth muscle cells

RA: Retinoic acid

VIGS: Virus-induced gene silencing

RNAi: RNA interference

CHAPTER 1

BACKGROUND TO MICRORNA-MEDIATED GENE REGULATION

A brief history of microRNAs (miRNAs)

In 1993, Victor Ambros and his colleagues discovered that a gene, *lin-4*, does not encode a protein but instead a non-coding RNA (Lee et al., 1993). Genetic studies indicated that *lin-4* encodes a trans-acting negative regulator of *lin-14* through *lin-14* 3'-untranslated region (3'UTR) at post-transcriptional level (Lee et al., 1993; Wightman et al., 1993). The Ambros and Ruvkun labs observed that 22-nt-length *lin-4* RNAs base pairs with multiple complementary sequences within *lin-14* 3' UTR. The Ruvkun lab further demonstrated that these complementary sites are essential in *lin-4*-mediated repression of *lin-14* protein, in which *lin-14* mRNA levels have no noticeable change (Lee et al., 1993; Wightman et al., 1993). These studies suggested that *lin-4* represses *lin-14* mRNA translation as part of the regulatory pathway for the developmental timing controlling in *C.elegans*.

Several years back, Richard Jorgensen overexpressed a pigment synthesis gene in petunias with the goal of deepening the purple color, but instead generated a variegated pigmentation with some lacking pigment altogether. This phenomenon was named "co-suppression" because not only the transgenes were inactive but also the corresponding endogenous genes were silenced (Napoli et al., 1990). In parallel, several groups found that the transgenic plants expressing viral RNAs become resistant to the virus (Angell and

Baulcombe, 1997; Dougherty et al., 1994; Ruiz et al., 1998). Silencing endogenous genes can be triggered by the incorporation of homologous sequences in viral replicon (Kumagai et al., 1995). A fundamental breakthrough in RNA silencings was the discovery that dsRNA is actually the trigger of specific mRNA destruction. Following the observation that sense RNA was as efficient as the antisense RNA in gene suppression in worms (Guo and Kemphues, 1995), Andrew Fire and Craig Mello discovered that dsRNAs was at least ten fold more potent as a silencing trigger than was sense or antisense alone and they coined the term RNA interference (RNAi) (Fire et al., 1998). It then became clear that the VIGS, post-transcriptional silencing phenomena in worms and plants may share the same biological root, that is, dsRNA is the real trigger for RNA silencing. However, it remained unclear how dsRNA-silencing triggers are generated and what mechanisms underlie dsRNA-induced gene silencing.

Our mechanistic understanding of RNAi is mainly based on genetic studies in worms and plants, and biochemical studies in *Drosophila*. In the latter respect, Carthew and colleagues showed that injection of dsRNA into embryos resulted in potent and specific gene interference at the post-transcriptional level (Kennerdell and Carthew, 1998). Sharp's group demonstrated that *Drosophila* embryo extract is competent for RNAi and the incubation of dsRNA in this cell free extract reduces the synthesis of luciferase protein from its mRNA (Tuschl et al., 1999). These studies suggested that dsRNA might trigger the formation of a nuclease complex that targets homologous RNAs for degradation. Hannon's group isolated this effector nuclease, RISC (RNA induced silencing complex), from *Drosophila* S2 cells in which RNA silencing has been triggered by dsRNAs (Hammond et al., 2000). The next question was how RISC identifies its cognate target RNAs? Fire and Mello originally

proposed that a certain derivative from dsRNAs identifies the substrate of RNAi. The first experimental clue for this "guide RNA" came from Hamilton and Baulcome's studies in plants. 25 nt antisense RNAs were detected in the plants in which post-transcriptional gene silencing (PTGS) was induced by transgenes or viruses, therefore indicating that 25-nucletide antisense RNAs represent the specificity determinants of PTGS (Hamilton and Baulcombe, 1999). Similar small RNAs were found in the *Drosophila* embryo extract incubated with dsRNA (Zamore et al., 2000). These studies connected transgene co-suppression in plants with RNAi in animals. This silencing is triggered by dsRNAs which are converted by certain machinery into ~21-25 nt RNAs and join the effector complex RISC to cleave homologous RNA substrates. This model suggests dsRNA is cleaved into ~22 nt RNAs, and this was further supported by the studies from *Drosophila* embryo extract (Zamore et al., 2000). What converts dsRNAs into ~22 nt sequences? Hannon and colleagues identified the enzyme Dicer using biochemical and candidate approaches and demonstrated that Dicer processes dsRNA into 22 nt RNA (Bernstein et al., 2001).

In the several years after the discovery of *lin-4* RNA, there were no similar RNAs detected in the species other than in the *C. elegans* and no other non-coding RNAs were found in the worms. The situation really changed upon the discovery of the second miRNA, *let-7*, which promotes the transition from late-larva to the adult cell fate in worms (Reinhart et al., 2000; Slack et al., 2000). The *let-7* homologues were quickly identified from *Drosophila*, humans and even other bilateral animals (Pasquinelli et al., 2000). Less than one year later, three labs reported over one hundred additional small non-coding RNAs, including approximately 60 new genes in worms, 20 in *Drosophila* and 30 in humans (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). These small RNAs resemble *lin-4* and

let-7 in that they are ~22 nt in length and endogenously expressed, and are evolutionarily conserved. Extensive cloning efforts identified numerous additional miRNAs in multiple organisms including worms, fishs, *Drosophila* and mammals (Ambros et al., 2003; Aravin et al., 2003; Dostie et al., 2003; Houbaviy et al., 2003; Kim et al., 2004; Lagos-Quintana et al., 2002; Lim et al., 2003a; Lim et al., 2003b). A microRNA registry was set up to catalogue these small RNAs and facilitate naming the new identified ones (Griffiths-Jones, 2004). The first miRNA, *lin-4*, was discovered in 1993 (Lee et al., 1993; Wightman et al., 1993), two years before the first hint of RNAi in worms (Guo and Kemphues, 1995). However, no formal link was made between miRNAs and siRNA until the discovery of the ribonuclease enzyme Dicer. Dicer, the enzyme converting long double-strand RNAs into siRNAs (Bernstein et al., 2001), was found to also process miRNA precursors into mature miRNAs (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001).

The biogenesis of microRNAs

Most miRNAs are located in intergenic regions (> 1kb away from annotated/predicted genes), indicating they are derived from independent transcriptional units. Nevertheless, a sizable minority of miRNAs are located in the introns of known genes with the same orientation as the host mRNAs, implying most of those miRNAs share the same promoters with their host genes (Lagos-Quintana et al., 2001; Lau et al., 2001). ~50% miRNAs are in close proximity with other miRNAs, indicating these miRNAs are derived from polycistronic transcriptional units (Lagos-Quintana et al., 2001; Lau et al., 2001).

Several lines of evidences suggest that miRNA genes are transcribed by RNA polymerase II (Pol II). First, primary transcript microRNAs (or pri-miRNAs) are sometimes

several kilobase in length, which are longer than regular pol III transcripts. pri-miRNAs also

contain internal runs of uridine residues that would prematurly terminate pol III transcripts.

Second, pri-miRNAs have Cap structure and Poly(A) tails. They are sensitive to α- amantin

at the concentration that specifically inhibits pol II but not pol I and pol III (Cai et al., 2004;

Lee et al., 2004b). Last, many miRNAs are differentially expressed during development.

Under the control by pol II associated regulatory factors, a subset of miRNAs can be

regulated temporally and spatially during development and under specific conditions. *lin-4*

and *let-7* have stage-specific expression pattern during worm development (Pasquinelli et al.,

2000) and the *miR-35-miR-42* cluster is preferentially expressed in the *C. elegan* embryos

(Lau et al., 2001). Other interesting examples include miR-1 and miR-133 clusters, which are

cardiac and skeletal muscle specific expressed (Chen et al., 2006; Lagos-Quintana et al.,

2002); miR-122, which is primarily expressed in the liver (Lagos-Quintana et al., 2002), and

the miR-290-miR-295 cluster, which is expressed in embryonic stem cells but not

differentiated cells (Houbaviy et al., 2003). Thus, any given cell type at a specific stage has a

distinct miRNA expression profile, providing enormous potential for the regulation of the

transcriptome. Some miRNA are remarkably abundant. It has been reported that there are

more than 50,000 molecules of each miR-2, miR-52, and miR-58 in one worm cell, which is

more abundant than the U6 snRNA of the spliceosome (Lim et al., 2003b).

Although transcriptional regulation is the main regulatory mechanism for miRNAs,

emerging evidences suggest a multi-step model for miRNA expression. miR-138 is spatially

restricted in the certain cell types but its precursor, pre-miR-138-2, is ubiquitously expressed

through the tissues examined. pre-miR-138-2 can be exported from nucleus to cytoplasm,

indicating the processing at Dicer step could be delayed or inhibited (Obernosterer et al.,

2006). The Hammond lab recently showed that many miRNA primary transcripts including

Pri-*let-7* are expressed at a high level but not processed by Drosha in mouse early

development, suggesting the suppression of miRNA production is achieved at or before the

Drosha processing step (Thomson et al., 2006).
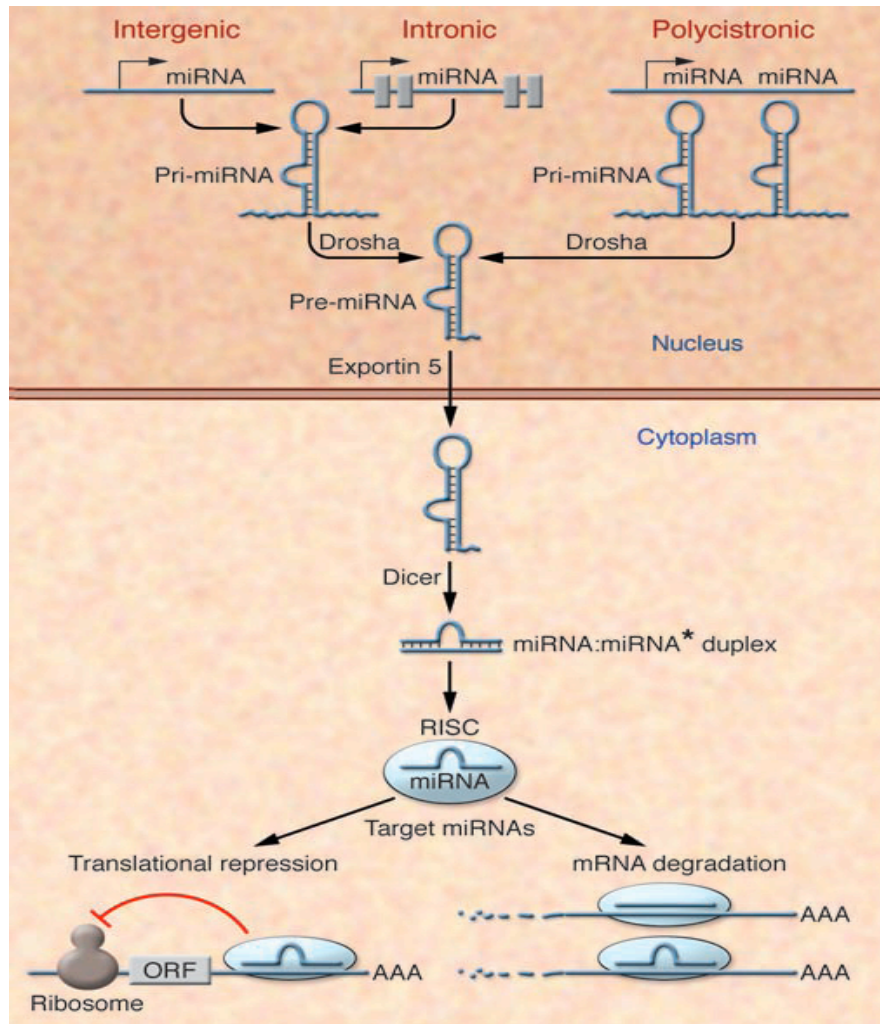
Figure 1 The animal microRNA biogenesis pathway (Van RooijE, Olson EN. JCI. 2007)

The current model of miRNA maturation is mainly based on two observations (Lee et

al., 2002). First, miRNA primary transcripts are converted into hairpin intermediates (pre-

miRNAs) and subsequently processed into mature miRNAs. Second, the catalytic activities

are compartmentalized into the nucleus and the cytoplasm, respectively. RNase III enzyme

can be divided into three classes based on their domain structure. Bacterial RNase III contains a catalytic domain and a dsRNA-binding domain. Drosha has two tandem catalytic domains and one dsRNA-binding domain. The third family, Dicer, also contains dual catalytic domains and additional helicase and PAZ motifs. RNase III Dicer is localized in the cytoplasm and has well characterized functions in the late stage of miRNA maturation (Billy et al., 2001; Provost et al., 2002). Drosha is located in the nucleus and was previously implicated in ribosomal RNA processing (Wu et al., 2000). pre-miR-30a forms a stem-loop like structure and contains a 2nt overhang at its 3' end, which is a characteristic of products from RNase III mediated reaction. Narry Kim's group demonstrated that the nuclear RNase III Drosha initiates miRNAs processing by cutting primary transcripts into precursors (Lee et al., 2003). Drosha forms a large complex of ~500 kDa in *D. melanogaster* or ~650 kDa in humans, known as the microprocessor complex. In this complex, Drosha interacts with its cofactor, DGCR8 (the DiGeorge syndrome critical region gene 8) in humans (also known as Pasha in *D. melanogaster*) (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004). DGCR8, but not Drosha, functions as the molecular anchor that measures the distance in between the dsRNA-ssRNA junction and cleavage site (Han et al., 2006).

Nuclear export of the pre-miRNA is mediated by exportin-5, a nuclear transport receptor (Lund et al., 2004; Yi et al., 2003). In general, members of the nuclear export receptors bind to cargo and the GTP-bound form of Ran in the nucleus. Once inside the cytoplasm following exportation, hydrolysis of GTP to GDP releases the cargo from the export complex. The pre-miRNAs and mature miRNAs in the cytoplasm are reduced when cells are depleted of exportin-5. Interestingly, pre-miRNA levels do not accumulate in the

nucleus upon exportin-5 depletion, which indicating that pre-miRNAs are stabilized by the interaction with exportin-5 (Yi et al., 2003).

miRNA precursors are further processed by endonuclease Dicer in the cytoplasm (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001). The processing of pre-miRNAs by Dicer yields about 21 nt-length-duplexes with 2 nt 3' overhang; each strand has a 5' phospho- and 3' hydroxyl. Several organisms have multiple Dicer genes and each Dicer processes dsRNAs from specific origin (Duchaine et al., 2006; Lee et al., 2004c). *D. melanogaster* has two Dicer isoforms: Dicer-1 (DCR-1) and Dicer-2 (DCR-2). DCR-1 preferentially converts pre-miRNAs into miRNA duplexes, whereas Dicer-2 (DCR-2) is responsible for siRNA maturation (Lee et al., 2004c; Pham et al., 2004). The processing of miRNA precursors by DCR-1 requires a RNA-binding protein Loquacious (Loqs), a homolog of human TRBP that was originally identified to bind to HIV trans-activator RNA (TAR) (Forstemann et al., 2005; Jiang et al., 2005). DCR-2 requires a dsRNA-binding protein R2D2 to mediate the integration of siRNAs into the RNA-induced gene silence complex (Liu et al., 2003). In *C. elegans*, only one Dicer has been found; recently, studies suggest that this Dicer interacts with different proteins and functions in distinct silencing pathway (Duchaine et al., 2006).

Following processing in the nuclus and nucleocytoplasmic export, miRNAs share the same biogenesis pathway with siRNAs. The siRNA pathway starts with long double-strand RNAs, which are either artificially introduced into cells or animals in knock down experiments (Fire et al., 1998), or are naturally produced from sense and antisense transcripts due to RNA dependent RNA polymerase activity (detected in plants, fungi, and nematodes, but not in flys, mice and humans) (Aravin et al., 2001; Cogoni and Macino, 1999; Dalmay et

al., 2000; Ketting et al., 1999; Mourrain et al., 2000; Smardon et al., 2000). In addition, long dsRNAs could derive from intermediates of viral replication (Li et al., 2002). Long dsRNAs are processed by Dicer into ~22nt RNA duplexs and one strand is selectively incorporated into the RISC complex to guide the complex to target mRNAs. RISC recognizes target mRNAs based on perfect (or nearly perfect) match between siRNA (or miRNA) and target mRNAs. RISC complex cleaves the site between the nucleotides pairing to the residue 10 and 11, measuring from siRNA 5' end (Elbashir et al., 2001a; Elbashir et al., 2001b).

The RISC complex has been purified from fly and human cells and it contains siRNAs/miRNAs and Argonaute proteins (Hammond et al., 2001; Hutvagner and Zamore, 2002; Martinez et al., 2002). This fits nicely with previous genetic data in which Argonaute proteins RDE-1, AGO-1 and QDE-1 are essential for RNAi and analogous activity in worms, plants and fungi, respectively (Fagard et al., 2000; Tabara et al., 1999). The single strand siRNA/miRNAs residing in the RISC complex is extremely tightly bound to the Ago proteins because salt concentration as high as 2.5 M KCL does not affect the binding of small RNAs with the Ago during RISC purification (Martinez and Tuschl, 2004). Ago proteins contain PAZ domains and PIWI domains (Carmell et al., 2002). Structure analyses revealed striking similarity between this PIWI domain and a member of RNase H family (Ma et al., 2005; Parker et al., 2005; Song et al., 2004). Further biochemical studies supported that Ago2 confers the RISC endonuclease activity, known as Slicer (Liu et al., 2004). PAZ is a RNA binding domain (RBD) that specifically binds to the terminus of a siRNA or miRNA helix (Lingel et al., 2004; Ma et al., 2004). Different organisms have different number of Ago proteins, ranging from one in *Schizosaccharomyces pombe*, four in mouse, five in *D. melanogaster*, eight in humans to more than 20 in *C.elegans* (Carmell et al., 2002). A recent

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study in *D. melanogaster* showed that AGO1 is required for miRNA accumulation whereas AGO2 is critical for siRNA-triggered mRNA cleavage (Okamura et al., 2004). AGO1-AGO4 all associate with siRNA and miRNA but only AGO2-containing RNPs have RISC activity in human cells (Meister et al., 2004b).

When miRNA dupexes (miRNA::miRNA*) are loaded into the RISC complex, only the guide strand is selectively incorporated into RISC while the other stand (miRNA*) appears to be degraded. The strand with the less stably paired 5' end enters into RISC complex and the thermodynamic properties of siRNAs and miRNAs determine their functional specificity (Khvorova et al., 2003; Schwarz et al., 2003).

In general, plant siRNAs or miRNAs guide the sequence-specific cleavage of target mRNAs based on perfect match between siRNAs and target mRNAs (Elbashir et al., 2001a), whereas most animal miRNAs repress gene expression through translational inhibition or slicer-independent mRNA degradation (Bartel, 2004). The endonuclease cleavage, referred to as "slicer" activity, is conferred by specific Argonaute proteins within the RISC complex. RISC cleaves the target mRNAs at a position corresponding to the tenth nt from the guide siRNA 5' end (Elbashir et al., 2001a). The cleavage products appear to be degraded by the same enzymes that process bulk cellular mRNA and the 3' fragment is degraded by the 5' to 3' exonucleases (Orban and Izaurralde, 2005). The mechanisms of miRNA-guided translational repression are not as well understood as that of siRNA-mediated cleavage. One possibility is that miRNA could increase target mRNA degradation in a slicer-independent mechanism. *let-7* and *lin-4* caused their target mRNAs to be degraded but none of the expected mRNA intermediates from slicer activity was found in *C. elegans*, indicating this degradation is not due to slicer activity (Bagga et al., 2005). Ectopic expression of miR-1 and

miR-124 caused a decrease of a population of transcripts that contain the binding sites for these two miRNAs (Lim et al., 2005).

The mechanisms underlying miRNA-mediated mRNA degradation are not well defined. Studies from P-bodies support the hypothesis that miRNAs might promote mRNA decapping and 5' to 3' degradation (Jakymiw et al., 2005; Liu et al., 2005a; Liu et al., 2005b; Sen and Blau, 2005). P-bodies are cytoplasmic foci in which untranslated mRNAs can be stored, decayed or translationally reactivated. P-bodies contain mRNAs not engaged in translation as P bodies and polysomes are mutually exclusive in their locations, indicating P-bodies do not contain the translational machinery (Brengues et al., 2005; Kedersha et al., 2005). In additon, P-bodies also contain the 5' to 3' mRNA degradation machinery (including the decapping enzyme DCP1 and DCP2) and proteins involved in translational repression (including Rck and Rap55). The major evidence implicating P-bodies in miRNA-mediated gene regulation is the co-localization of Argonaute proteins with P-bodies components. Although the majority of miRNAs and Argonautes are distributed diffusely in the cytoplasm, a fraction of them are localized to the P-bodies. All four mammalian Argonaute proteins can co-immunoprecipitate with the decapping enzymes in culture cells (Jakymiw et al., 2005; Liu et al., 2005a). Moreover, the target mRNAs are concentrated into P-bodies in a miRNA -dependent manner during miRNA triggered translational repression (Liu et al., 2005a). The silencing of GW182 protein, a major component of P-bodies, delocalizes those resident proteins of P-bodies and impairs the miRNA-guided repression (Liu et al., 2005b). These studies suggest that miRNAs direct target mRNAs into P-bodies and increase their association with the decapping machinery and eventually lead to mRNA degradation.

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In addition to slicer-dependent cleavage and slicer-independent mRNA degradation, miRNAs regulate gene expression through translational repression. Initial studies in worms showed that the polysomal distribution of lin-14 and lin-28, targets of *lin-4* miRNA, remain unchanged with or without the presence of miRN-mediated gene repression. These observations led to the suggestion that miRNAs repress target mRNA translational elongation but not initiation (Kim et al., 2004; Olsen and Ambros, 1999; Seggerson et al., 2002). However, miRNAs were recently implicated to inhibit target mRNA translation at early initiation step (Meister, 2007; Pillai et al., 2005; Wakiyama et al., 2007). Translation initiation was inhibited by miRNA *let-7* within the first 15 minutes of exposure to the cell extract, in which transcript destabilization was not detected (Mathonnet et al., 2007). Ago2 contains a highly conserved motif which bears significant similarity to the m(7) G cap-binding domain of eIF4E, an essential translation initiation factor, indicating that Ago2 represses target mRNAs translation initiation by binding to the m(7)G cap of target mRNAs and therefore prevent eIF4E recruitment (Kiriakidou et al., 2007). It has been reported that *Drosophila* miR-2 induces the formation of dense miRNPs (pseudo-polysomes) and inhibits translation initiation without affecting mRNA stability (Thermann and Hentze, 2007).

Methodologies for the studies of miRNA functions

The studies of miRNA funcion have been propelled by newly developed methods and conventional forward/reverse genetic approaches. The miRNA Northern blot has revealed interesting miRNA expression patterns. *lin-4* and *let-7* have stage-specific expression during development in *C. elegans* (Pasquinelli et al., 2000), whereas miR-206 is specifically expressed in skeletal muscle (Chen et al., 2006). The Sharp group showed by Northern blot

that ES cells express a specific miRNA gene cluster encoding miR-290 to miR-295 in mouse (Houbaviy et al., 2003). Microarray-based expression technology has been adapted to identify candidate miRNAs that correlate with biological pathways and to generate miRNA expression signatures of diseases (Thomson et al., 2004). To gain more spatial information of miRNA expression, in-situ hybridization is used to detect miRNA expression using locked nucleic acid-modified oligonucleotide probes (Obernosterer et al., 2007; Wienholds et al., 2005). In addition, sensitive RT-PCR method is used to detect and quantify miRNA expression (Varkonyi-Gasic et al., 2007). Very few miRNAs are detected during zebrafish and mouse early development, whereas large amount of miRNAs are induced during mid- to –late embryonic stage in a temporal and spatial fashion (Wienholds et al., 2005). Most miRNAs are down-regulated in cancer, indicating loss of miRNAs is critical for cancer formation (Lu et al., 2005). A subset of miRNAs are also dys-regulated during cardiac hypertrophy and heart failure (van Rooij et al., 2006).

The most effective way to study gene function is to knock down or knock out the gene of interest and examine the resulting consequences. However, RNAi-mediated knockdown is not possible to miRNA functional studies because miRNAs are also loaded into RISC. Based on the idea that oligonucleotides complementary with miRNAs can act as competitive inhibitors to block activity, two groups developed chemically modified 2'-O-methyl oligonucletides to inhibit miRNA functions (Hutvagner et al., 2004; Meister et al., 2004a). Utilizing the same principle of miRNA competitive inhibitor, a long-term miRNA "knockdown" approach was recently developed using an mRNA containing several miRNA target sites at its 3'-UTR to sequester miRNAs (Ebert et al., 2007).

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miRNA overexpression is another way to assess their unique functions. Transient

miRNA expression in a cell-based assay can be done by miRNA duplex transfection (Chen et

al., 2006). Long-term miRNA expression in cell culture can be achieved with RNA pol II-

based vectors (Chen et al., 2006), which allow overexpression of different miRNAs

simultaneously. miRNAs have been systemically introduced into hematopoetic and fetal liver

cells using *ex vivo* transfer and reconstituation (Chen et al., 2004a; He et al., 2005).

Transgenic approaches were used to express miRNAs in animal. miR-1 is specifically

expressed in the hearts using α-MHC promoter (Zhao et al., 2005). However, ectopic

miRNA expression could have "off-target" effects and miRNA overexpression results should

be ideally confirmed by loss-of-function analyses. The homologous recombination based

approaches were also used to inactivate miRNA activity such as miR-208 knockout study

((van Rooij et al., 2007). In additon, chemically modified oligonucletides, called antagomirs,

was used to effectively inactivate miRNA function through perfect base pairing with miRNA

sequence (Krutzfeldt et al., 2005).

Several independent computational approaches have been developed to identify

miNRA targets in hope of determining miRNA functions based on known roles of those

targets (John et al., 2004; Kiriakidou et al., 2004; Krek et al., 2005; Lewis et al., 2003). A

key criteria in those miRNA target prediction is that the residues 2-8 of miRNA at the 5' end

should match perfect complimentary sequences within target mRNA 3' UTR (Bartel, 2004;

Doench and Sharp, 2004). On average, 200 genes are predicted to be the targets for a

particular miRNA (Lewis et al., 2003). The most commonly used method to verify predicted

targets is the luciferase reporter assay coupled with Western blot analyses. A complementary

approach is the loss-of-function study using antisense 2'-O-methyl-modified

oligoribonucleotides (Chen et al., 2006). Proteomic approaches have been used to identify miRNA regulatory targets in Dicer deficient Drosophila oocytes (Nakahara et al., 2005). Since miRNA can lead to mRNA degradation in a slicer-independent manner, microarrays have been applied to identify miRNA targets (Lim et al., 2005). miRNP immunopurification coupled with microarray analysis has also been used for target identification (Beitzinger et al., 2007; Easow et al., 2007; Zhang et al., 2007). miRNP complexes associated with miRNAs and cognate mRNA targets were purified and microarray analysis was used to determine these target mRNA identity. Collectively, the studies of miRNAs functions require unique technologies and conventional approaches.

The functions of animal microRNAs

miRNAs regulate signaling pathways critical for early development. Zebrafish miR-430 dampens and balances the expression of squint and lefty, the TGF-beta Nodal agonist and the antagonist, respectively (Choi et al., 2007). Signaling by TGF-beta ligand Nodal is crucial for the dorsal induction of the Spemann's organizer in early *Xenopus* embryos. Ventral ly enriched miR-15 and miR-16 are negatively regulated by dorsal Wnt/beta-catenin pathways and restrict organizer size by targeting the Nodal type II receptor Acr2a (Martello et al., 2007).

miRNAs regulate cell fate determination through the interactions between miRNAs and signal transduction networks. Notch signaling in vulvase precursor cells (VPCs) induces miR-61 expression in response to neighboring signals. miR-61, in turn, represses Vav-1 that functions to repress Notch activity, therefore providing a reciprocal negative loop that perpetuates and amplifies cell fate decision (Yoo and Greenwald, 2005). Zebrafish miR-214

modulates Hedgehog signaling to specify muscle cell fates in early development (Flynt et al., 2007). In *Drosophila*, miR-9a inhibits neuronal cell fate in non-sensory organ precursor cells by down-regulating Sens, a component in Notch signaling (Li et al., 2006). miRNAs also affect cell fates through regulating transcriptional factor expression. In *C.elegan*, two asymmetric chemosensory neurons adopt either an ASE left (ASEL) or an ASE right (ASER) fate from a bi-potential ASE precursor. The fate choices are controlled by two miRNAs, lsy-1 and miR-273, which inhibit the expression of transcriptional factors involved in cell fate determination. (Chang et al., 2004; Johnston et al., 2005).

miRNAs play essential roles in cell proliferation and differentiation. Our studies showed that two muscle-specific miRNAs, miR-1 and miR-133, modulate skeletal muscle development in an antagonistic manner. miR-1 promotes skeletal muscle differentiation by represing HDAC4 and miR-133 enhaces cell proliferation by inhibiting SRF (Chen et al., 2006). In *Drosophila*, progenitor cells turn on miR-7 expression by inhibiting Yan, a repressor of miR-7, in response to EGF receptor signaling. miR-7, in turn, inhibits Yan expression when cells start to differentiate into photoreceptors. In contrast, the absence of EGF signaling leads to the stabilization of Yan and then the inbition of miR-7 transcription (Li and Carthew, 2005). Therefore, miR-7 participates in a negative feedback regulatory loop that generates a robust and stable gene expression change during cell differentiation.

miRNAs can switch off a small set of gene regulators, fine-tune the expression of a large number of genes and safeguard against promiscuous gene expression. Individual miRNAs can switch off a small number of key developmental regulators in lower organisms (Brennecke et al., 2003; Chang et al., 2004; Johnston et al., 2005; Lee et al., 1993; Wightman et al., 1993). For example, *lin-4* and *let-7* turned off lin-14 expression to control

developmental timing in *C. elegans* (Lee et al., 1993; Wightman et al., 1993). In *Drosophila*, the *bantam* locus encodes a miRNA, which regulates programmed cell death and cell proliferation by repressing pro-apoptotic gene *hid* (Brennecke et al., 2003). However, studies from vertebrates suggested that miRNAs more likely function to fine-tune gene expression to optimal levels (Chen et al., 2006; Karres et al., 2007; Sokol and Ambros, 2005; Thai et al., 2007; Xiao et al., 2007). miR-1 levels are increased during muscle differentiation, which occurs concurrently with lower HDAC4 protein levels but without a reduction of HDAC4 mRNA levels, indicating miR-1 regulates HDAC4 into an optimal level for muscle differentiation (Chen et al., 2006). In addition, some miRNAs also act as safeguards to inhibit inappropriate mRNA expression during gene program transitions (Farh et al., 2005; Hornstein et al., 2005; Stark et al., 2005). Those predicted targets of miRNAs are often highly expressed in the early developmental stages and their expression levels decrease as miRNAs begin to accumulate, indicating that stage-specific miRNAs tend to dampen the output of pre-existing messages to facilitate more rapid and robust transitions (Farh et al., 2005). miRNAs are generally excluded from tissue compartments where their target mRNAs are expressed, indicating that tissue or cell type-specific miRNAs repress unwanted messages to inconsequential levels and ensure tissue identity (Stark et al., 2005). In hindlimb development, miR-196 functions to safeguard against inappropriate Hox expression, which is controlled primarily at transcriptional levels (Hornstein et al., 2005). Therefore, some miRNAs prevent inappropriate gene expression in domains where target genes are already repressed at transcriptional levels. Together, recent studies have started to reveal roles for miRNAs in a wide range of biological processes, including cell proliferation, differentiation, apoptosis and others.

microRNAs and disease

miRNA expression analyses have revealed the connection between miRNAs and cancer (Iorio et al., 2005; Volinia et al., 2006). Most miRNAs are strongly decreased in tumors; this is interesting in light of the observation that most miRNAs are not expressed in early development and are remarkably induced after mid-gestation (Thomson et al., 2004). These findings indicate that lower miRNA expression might favor a less differentiated state, a hallmark of cancer. The miR-15-16 is frequently deleted in patients with chronic lymphocytic leukemia (Calin et al., 2002) and many miRNA genes are located at fragile sites and genomic regions involved in cancers (Calin et al., 2004). Some miRNAs are abundantly expressed in certain cancers and have oncogenic potentials (He et al., 2005; O'Donnell et al., 2005; Voorhoeve et al., 2006). miR-17-92 cluster has been implicated as proto-oncogene in B- cell lymphomas and miR-372-373 in testicular cancers. In addtion, emerging evidences suggests that miRNAs may act as tumor suppressors (Johnson et al., 2005; Mayr et al., 2007). let-7 was suggested to play tumor suppressive role in various cancers possibly through repressing key oncogenic components such as Ras and HMGA2. Studies from five independent groups connected miR-34 with p53 where miR-34 induces cell cycle arrest and promote apoptosis in response to p53 activation. (Bommer et al., 2007; Chang et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007). Although it remains unclear what the exact roles of miRNAs are in cancers, it is apparent that miRNAs are intimately integrated into the regulatory networks normally disrupted during oncogenic cellular transformation.

miRNAs have been implicated in cellular response to hypoxia (Kulshreshtha et al., 2007), DNA damage and oncogenic stress (Bommer et al., 2007; Chang et al., 2007; He et

al., 2007; Raver-Shapira et al., 2007), cardiac injury or cardiac pressure overload (van Rooij et al., 2006). As mentioned previously, miR-34 is up-regulated in response to DNA damage or oncogenic stress. miR-34, in turn, enhances cell cycle arrest and promotes apoptosis to prevent inappropriate cell proliferation (Bommer et al., 2007; Chang et al., 2007; He et al., 2007; Raver-Shapira et al., 2007). Cells adapt to stress conditions by changing gene expression program, including up- or down-regulation of miRNAs. In additon, not all miRNAs involved in stress change their expression pattern and the existing miRNAs, without any de novo synthesis, could selectively regulate certain mRNAs in response to stress. miR-208 expression remains unchanged upon pressure overload by thoracic aortic constriction or signaling by calcineurin (van Rooij et al., 2006). However, loss of miR-208 in mice results in pronounced expression of numerous fast skeletal muscle contractile protein genes, which are normally not expressed in the hearts (van Rooij et al., 2007). Thus, the cardiac cells reprogram mRNA metabolism such as the reactivation of fetal cardiac genes to adapt the changed conditions in stress. Existing miR-208 selectively inhibits inappropriate expression of fast skeletal muscle gene to control the quality of mRNA reprogramming.

miRNA-mediated gene regulation is suggested to be associated with "stress granules", the reversible cytoplamic foci induced under stress conditions (Leung et al., 2006). In response to stress, eukaryotic cells selectively redistribute mRNAs into transient non-membranous organelles, called stress granules (SGs), to repair stress-induced damages and adapt to changed conditions. The translation of mRNAs encoding "house-keeping" proteins is inhibited and the translation of mRNAs encoding proteins involved in damage repair is enhanced. SGs are considered to be the centers where mRNAs are sorted, remodeled and exported for further storage, decay or reinitiation (Anderson and Kedersha, 2006). Some SGs

components including FXR1, HuR, p54/rck and TTP are associated with Argonaute proteins (Bhattacharyya et al., 2006; Chu and Rana, 2006; Jing et al., 2005; Vasudevan and Steitz, 2007). miRNAs and Argonautes diffusely distribute in the cytoplasm and alos localized to P-bodies under normal conditons. However, miRNAs and all four Argonautes become localized to newly assembled SGs upon the exposure to stress such as oxidative stress in a miRNA-dependent manner (Leung et al., 2006). Therefore, the co-localiztion of Argonaute complex with other components recruited into SGs during stress provides a new opportunity of modulating miRNA-mediated regulation. Together, recent studies connect miRNAs with a spectrum of diseases, including cancers, cardiovascular diseases, muscle disorders and others, based on miRNAs expression profiles and functional studies. Interestingly, although miRNAs normally act as gene repressors, they can also activate gene expression in response to external stimuli (Vasudevan and Steitz, 2007; Vasudevan et al., 2007). Therefore, it will be a big challenge to determine the function of miRNAs in normal and pathological conditions.

CHAPTER 2

FUNCTIONAL STUDY OF MICRORNA-1 AND -133 IN SKELETAL MUSCLE DEVELOPMENT

INTRODUCTION

Skeletal muscle development begins with the multipotent progenitors located at the dermomyotome in the mouse embryo. The progenitors from the dorsal medial lip (DML) of the dermomyotome migrate, exit cell cycle and differentiate to give rise to the deep back muscle, whereas the progenitors from the ventral lateral lip (VLL) of the dermomyotome ultimately contribute to lateral trunk muscle. In additon, the progenitor cells from VLL of the dermomyotome also form the muscle in the limb, ventral body wall, diaphragm and tongue. The progenitors at the lip of the dermomyotome do not undergo extensive proliferation but instead withdrawal from cell cycle and quickly differentiate into multinucleated myoyubes in response to cues from adjacent tissues (Parker et al., 2003; Shi and Garry, 2006). The major muscle mass is derived from a population of progenitors, named Pax3$^+$/Pax7$^+$ cells, which migrate directly from the central dermomyotome to the myotome and undergo extensive proliferation before the induction of the differentiation gene program (Relaix et al., 2005).

The myogenic regulatory factors (MRFs) including MyoD, Myf5, Myogenin, and Mrf4 (Myf6) have well defined functions in muscle differentiation. MyoD and Myf5 are required for the muscle specification, whereas myogenin is essential for the progression of muscle differentiation. In addition, Mrf4 is critical for muscle terminal differentiation and

myofiber maintenance. Sonic hedgehog (Shh), which is derived from the notochord and the floor plate, plays a positive role for muscle specification by directly activating Myf5 expression. Wnt ligands, including Wnt-4, Wnt-6 and Wnt-7a, from the dorsal ectoderm function through Wnt signaling to activate MyoD expression (Parker et al., 2003). A PKA signaling effector, CREB, is identified as a novel nuclear target of the Wnt pathway and CREB-responsive elements are found on the regulatory sequence of Myf5, therefore providing the mechanism by which Wnt activates myogenesis (Chen et al., 2005). Mesoderm precursors are specified into myogenic lineage by the activation of Pax3 and Pax7 expression. Pax3 is necessary and sufficient for MyoD expression in the absence of Myf5 in the progenitor cells, whereas Pax7 ensures progenitor cells survival (Relaix et al., 2006). Collectively, the progenitors are specified into myogenic cell fate by the activation of transcriptional factors in response to the cues from surronding tissues.

After MyoD or Myf5 is turned on and myogenic cell fate is specified, how do myoblasts maintain the muscle identity without activating differentiation program? Multiple strategies are employed to maintain proliferating muscle precursors without the induction of the differentiation process. Homeoprotein Msx1 recruits the linker histone H1b to MyoD regulatory elements, thereby keeping MyoD expression at low levels (Lee et al., 2004a). Id expression is up-regulated in the presence of serum and then sequestrates more E proteins, the MyoD heterodimeric partners, therefore represses MyoD activity (Benezra et al., 1990). In proliferating myoblasts, the muscle regulatory regions of MRFs are occupied by repressor complexes, containing histone deacetylases (HDACs), histone lysine methyltransferase Ezh2 and others, therefore resulting in the repression of MRFs activity (Caretti et al., 2004). In order to initiate the differentiation program, negative repressors, such as HDACs and lysine

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Methyltransferases, need to be displaced and the negative modifications on histone residues should be removed. In addition, transcriptional co-activators need to be recruited to the regulatory regions of muscle genes.
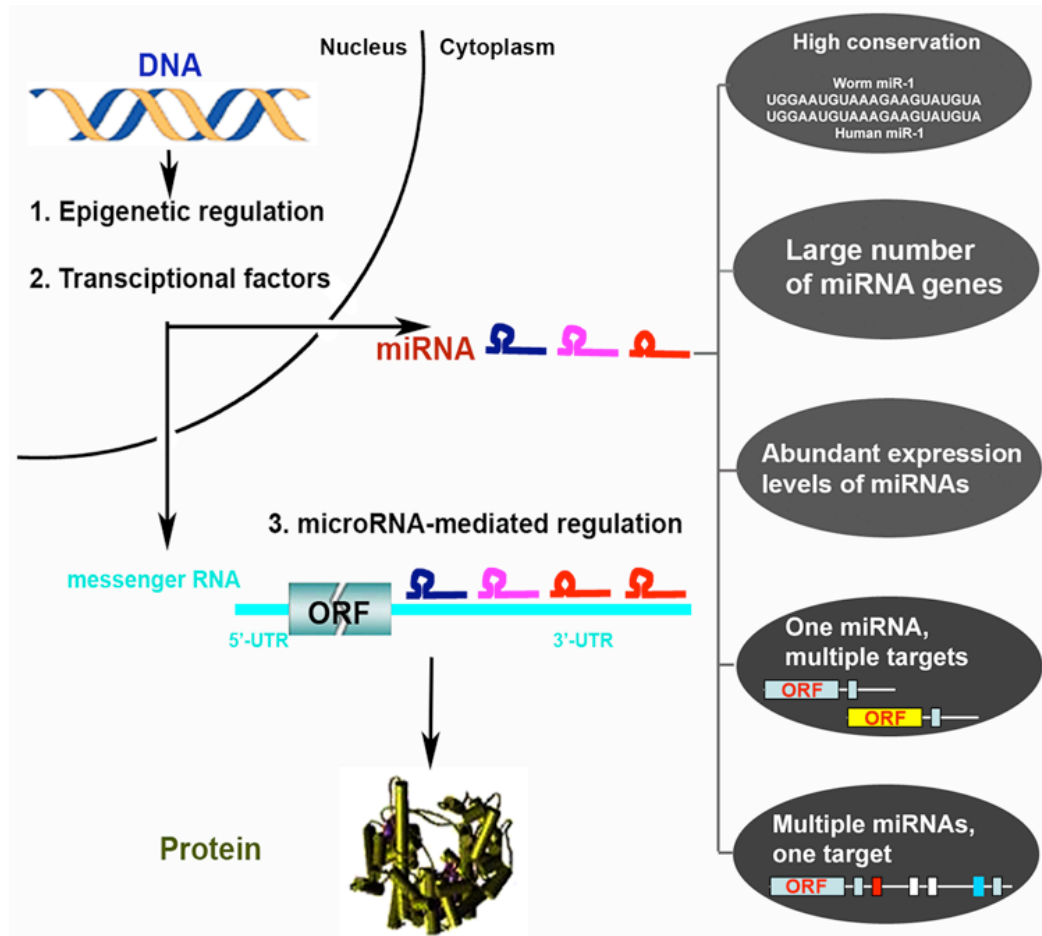
Figure 2 Gene regulatory networks involving microRNAs (miRNAs), and miRNAs' unique features. Gene regulatory networks involving microRNAs (miRNAs) and miRNAs' unique features. The selective expression of primary miRNAs and regular messenger RNA (mRNA) is controlled by epigenetic regulation (1) and transcriptional factors-mediated gene regulation (2). The mRNAs are regulated by miRNAs post-transcriptionally through base pairing to complementary sequences within the 3' untranslated regions (UTRs) of the mRNAs (3), resulting in mRNA degradation and/or translational repression. Large number of miRNAs, which are highly conserved across species, have been identified and many of them are abundantly expressed. A single miRNA is though to have dozes of targets and one particular mRNA can be regulated by multiple miRNAs simultaneously.

Myogenic cells actively expand under growth conditions, and proliferating myoblasts quickly exit cell cycle in response to growth factor depletion and form terminal differentiated

multinucleied myotubs. The down-regulation of cyclin/cdk activity in response to mitogen depletion results in the displacement of HDAC1 from MyoD regulatory elements, through the recruitment of HDAC1 by hypo-phosphorylated pRb, therefore promotes muscle differentiation (Puri et al., 2001). In addition, CaMK phosphorylates HDAC4 and stimulates its interaction with chaperon protein 14-3-3, therefore disrupting the association between HDAC4 and MEF2. HDAC4 represses the activity of MEF2, a potent activator for muscle differentiation. Therefore, the diruption of the interaction between HDAC4 and MEF2 results in the activation of muscle differentiation (McKinsey et al., 2000). It has been reported that p38 recruits the ATPase- dependent SWI/SNF chromatin-remodeling complex to muscle gene promoters through direct phosphorylation of the SWI/SNF subunit BAF60 (Simone et al., 2004). Therefore, BAF60 receives signals from cytoplasmic cascades such as p38 signaling and transmits them to other chromatin regulators, thereby allowing myogenic transcriptosome to adopt permissive conformation of transcription.

Gene regulations mediated by chromatin regulators and transcriptional factors are essential for skeletal muscle development. Emerging as a new layer of regulators of gene expression at the post-trancriptional level, microRNAs (miRNAs) are ~22 nucletides in length and generally inhibit translation or promote mRNA degradation by base pairing to complementary sequences within the 3' untranslated regions (UTRs) of regulatory target mRNAs (Figure 2). Hundreds of miRNAs have been identified and many of them are highly conserved across a number of species, including plants, worms and humans (Berezikov et al., 2006; Griffiths-Jones, 2004). Bioinformatic analysis indicates that miRNAs might target more than a third of human protein-coding genes (John et al., 2004; Lewis et al., 2003). Individual mRNAs can be targeted by several miRNAs, and a single miRNA can regulate

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multiple target mRNAs. More importantly, miRNAs can coordinately regulate a set of genes encoding proteins with related functions, providing enormous complexity and the potential of gene regulation (Esau et al., 2006; Leung and Sharp, 2007). Given the vast number of miRNAs, it's likely that miRNAs play essential roles for skeletal muscle differentiation. However, it remains unknown how miRNAs regulate muscle development. In this chapter, I will focus on two muscle- specific miRNAs, miR-1 and -133, and study their functions during skeletal muscle proliferation and differentiation.

METHODS

Analysis of microRNA expression by microarray

Total RNA was isolated from C2C12 cells cultured in growth medium comprising DMEM (Sigma) plus 10% fetal bovine serum (Sigma) and 1% penicillin or streptomycin (Invitrogen) or differentiation medium comprising DMEM (Sigma) plus 2% horse serum (Sigma) at different time points (days 0, 1, 3 and 5, where day 0 was the first day after transfer into differentiation medium). Microarray hybridization and data analysis were done as described9. In brief, 2.5 g of isolated RNA was labeled with 5'-phosphate-cytidyl-uridyl-Cy3-3' (Dharmacon) using RNA ligase and hybridized with a 0.5-mM mixture of oligoribonucleotide probes for 124 miRNAs labeled with Alexa 647 (Cy5) in SLF-0601 disposable chambers (MJ Research). Normalized log2 data were hierarchically clustered by gene and plotted as a heat map.

Northern blot analysis

Total RNA was extracted from C2C12 cells, mouse embryonic tissue or adult tissue with Trizol Reagent (Invitrogen). To aid analysis of miRNA, polyethylene glycol (PEG) was applied to remove large-sized RNAs. In brief, 30 g of each total RNA sample was mixed 1:1 with 5 x PEG solution and placed on ice for 10 min. After 10 min of centrifugation at maximum speed at 4 °C, the supernatant was transferred to a fresh tube. RNAs were then precipitated by adding 2.5 volumes of 100% ethanol, followed by centrifugation for 30 min at maximum speed. Genomic fragments of miR-1 and miR-133 were cloned by PCR and used as probes.

Cloning and expression of miR-1 and miR-133

Genomic fragments of miR-1 and miR-133 precursors from mouse chromosomes 2 and 18 were amplified by PCR using mouse genomic DNA as a template. The PCR products were cloned into the pDNA(+)3.1 vector (Invitrogen) and miRNA expression was detected by RNA blot analysis.

Cell culture, *in vitro* myogenesis differentiation and luciferase reporter assay

C2C12 myoblast cells were cultured and myogenesis was induced under differentiation medium. miRNA duplexes and 2'-O-methyl antisense oligoribonucleotides targeted towards miR-1, miR-133, miR-208 and miGFP were purchased from Dharmacon and introduced into mammalian cells by either Lipofectamine (Invitrogen) transfection (200 nM) or electroporation with the Nucleofector (Amaxa) system (5 g).

For construction of the 3'-UTR-luciferase reporter, the multiple cloning site of the pGL3-Control vector (Promega) was removed and placed downstream of the luciferase gene. We amplified the 3' UTRs of mouse HDAC4 and SRF by PCR and cloned them into the modified pGL3-Control vector, resulting in the constructs SRF-3'-UTR and HDAC4-3'-UTR.

Immunoblotting and immunostaining

Immunoblotting was done using antibodies to myogenin, SRF, MEF2, HDAC4 and -tubulin (Santa Cruz Biotechnology), and to phospho–histone H3 (Upstate Biotechnology). The MF20 antibody, which recognizes striated muscle-specific MHC, was obtained from the DSHB (University of Iowa). For immunostaining, C2C12 cells treated in 12-well plates were fixed with 4% formaldehyde for 5 min at 37 °C and washed in 0.1% Nonidet P40 (NP40) in PBS for 15 min at room temperature. Primary antibodies were incubated in 0.1% NP40 in PBS plus 3% bovine serum albumin (BSA) for 2 h in the following concentrations: anti-myogenin, 1:20 dilution; anti–phospho–histone H3, 1:100 dilution; MF20, 1:10 dilution. The secondary antibodies, fluorescein-conjugated anti-mouse and anti-rabbit (1:100 dilution, Vector Laboratories), were added in 0.1% NP40 plus 3% BSA in PBS for 1 h at 37 °C. 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) was then added for 5 min at room temperature. After several washes with PBS, cells were subjected to fluorescence microscopy.

RT-PCR analysis

Total RNA was extracted from C2C12 cells with Trizol Reagent (Invitrogen), and 2.0-g samples were reverse-transcribed to cDNA by using random hexamers and MMLV reverse transcriptase (Invitrogen). In each analysis, 2.5% of the cDNA pool was used for amplification and PCR was done for 24–28 cycles.

X. laevis **embryo injections and transgenesis**

Standard methods were used to obtain and culture X. laevis embryos. DNA constructs were linearized with KpnI and transgenic embryos were generated as described28. Expression of the transgene was analyzed under an MZFLIII microscope (Leica). Preparation and injection of X. laevis with miRNAs was done essentially as described29, except that RNA was not capped before injection.

RESULTS

1. The expression patterns of miR-1 and -133.

1.1 Genomic structures and primary transcripts of miR-1 and -133. miR-1 (miR-1-1 and miR-1-2) and miR-133 (miR-133a-1 and miR-133a-2) are clustered in the mouse chromosome 2 and 18, respectively. miR-206, which is specific to skeletal muscle not
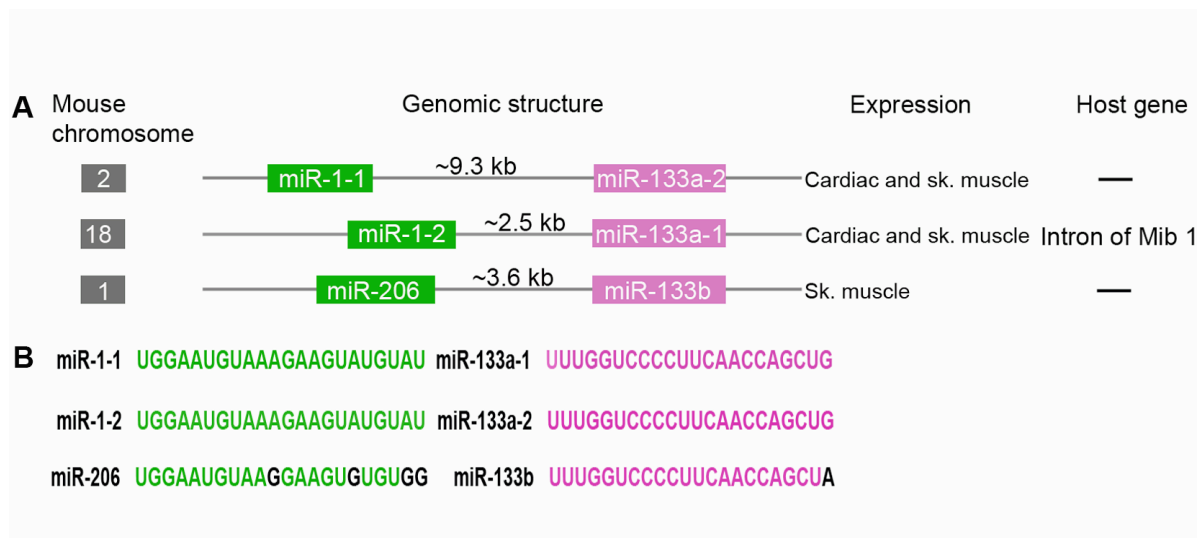

Figure 3 Genomic structures of muscle-specific miRNAs and their sequence homologies. (A) Schematic diagrams show the genomic locations of muscle-specific miRNA genes including, miR-1-

1/miR-133a-2, miR-1-2/miR-133a-1 and miR-206/miR-133b on mouse chromosomes. Their expression and host genes in which these miRNAs reside are also indicated. (B) The comparison of muscle-specific miRNA sequences. The same color indicates the homology of miRNA gene families whereas black color marks nucleotide differences.

cardiac muscle, is clustered with miR-133b in the mouse chromosome 1. In the chromosome 2, the cluster resides in a non-coding region with a 9.2 kb separation between miR-1-1 and miR-33a-2. The other cluster miR-1-2/miR-133a-1 is separated by 2.5 kb in the chromosome 18 and is located in the intron between exon 11 and 12 of the gene encoding a ubiqutin ligase Mib1 (Figure 3). miR-1-1 and miR-1-2 are identical in sequence, while miR-206 has a four nucletide difference with miR-1-1 and miR-1-2 at the 3' end. miR-133a-1, miR-133a-2 and miR-133b have same sequence except for one nucletide at the 3' end of miR-133b (Figure 3).
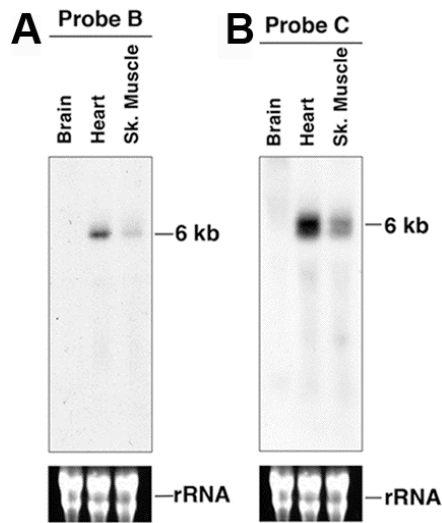
Figure 4 Northern-blot analyses of primary transcripts of miR-1-2 and miR-133a-1. Northern blot analyses of the expression of primary transcripts for miR-1-2 (A) and miR-133a-1 (B) from chromosome 18. 20 μg of total RNA from the indicated adult mouse tissues were used.

We performed a Northern blot analysis using ~300 bp genomic probe including the miR-1-2 or miR-133a-1 sequence (Figure 4). Both probes from chromosome 18 detected a ~ 6kb primary transcript from total RNAs isolated from heart and skeletal muscle, indicating that miR-1-2 and miR-133a-1 are indeed transcribed together. While both miR-1-1 and miR-133a-2 probes from chromosome 2 detected a transcript of ~10 kb from the heart and skeletal muscle, the miR-133a-2 probe also hybridized to two additional transcripts of ~ 4.5 kb and ~ 2.2 kb, while the miR-1-1 probe also detected a major transcript of ~ 6 kb (data not shown), suggesting the potential involvement of post-transcriptional processing. Together, our data indicates that

cardiac and skeletal muscle-specific expression of miR-1 and miR-133 is dictated at the primary transcription step.

1.2 Cardiac and skeletal muscle specific expression pattern of miR-1 and miR-133. It has been shown that miR-1 specifically expressed in heart in tested mouse tissue (Lagos-Quintana et al., 2002). To examine whether miR-1 also expresses in other tissues and miR-133, clustering with miR-1 at the same chromosome in mouse genome, has similar expression pattern with miR-1, we performed miRNA-Northern probing with miR-1 and/or miR-133 antisense sequence.
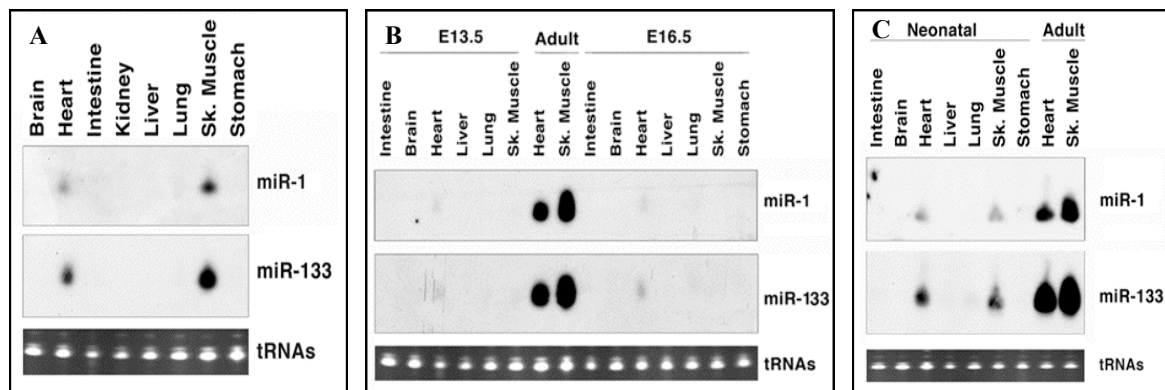
Figure 5 miR-1 and miR-133 expression pattern at mouse E13.5, E16.5, neonatal and adult stages. Different tissues as listed above from E13.5 (B), E16.5 (B), neonatal (C) and adult stage (A) mice were isolated and RNA was extracted for miRNAs-Northern analysis probing with miR-1 or miR-133 antisense sequence.

Experimental results show that miR-1 and miR-133 both specifically expressed in adult heart and skeletal muscle tissues. miR-1 and miR-133 spatial and temporal expression examination shows that miR-1 and miR-133 express very low early at E13.5 and E16.5 embryo and have relatively higher expression at neonatal stage, although it's significantly

lower than that of adults (Figure 5). This expression pattern suggests that miR-1 and miR-133 may play a role in development and/or maintenance of cardiac and skeletal muscle.

1.3 A miR-1 and -133 enhance directs reporter gene expression in the cardiac and skeletal muscle. Utilizing computational approaches, we identified an enhance element upstream of the primary transcripts of miR-1 and -133, and the sequence contains typical MEF2, SRF and MyoD binding sites, suggesting primary mir-1/miR-133 transcripts are controlled by those typical muscle transcriptional factors.
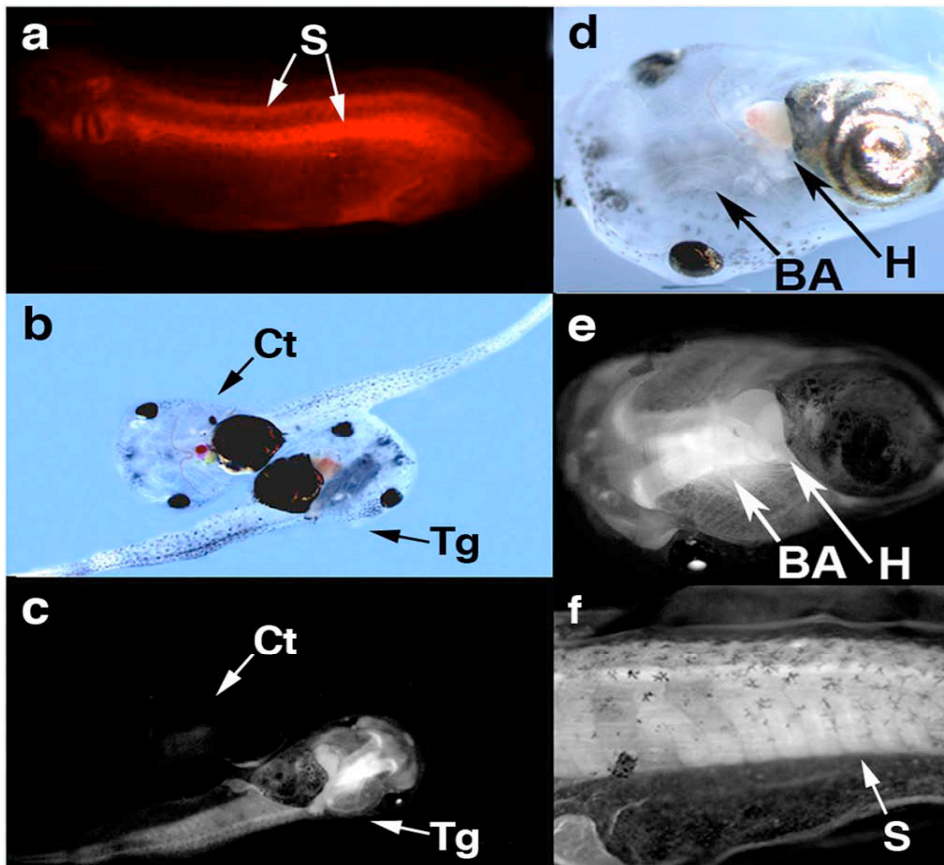
Figure 6 A miR-1 and miR-133 enhancer directs dsRed expression in the cardiac and skeletal muscle. (a) *Xenopus laevis* transgenic for mouse miR-1 and miR-133 genomic sequence linked to dsRed shows somite (S, arrows) expression at stage 28. (b) Transgenic (Tg) *Xenopus laevis* carrying a miR-1 and miR-133-containing transgene at stage 46 (lower embryo) and negative control (non-transgenic, Ct, upper embryo) under bright field. (c) Same embryos as b under fluorescence. (d) High power magnification of transgenic embryo in panel g under bright field and under fluorescence (e) showing expression of the trangene in the heart (H, arrows) and branchial arches (BA, arrows). (f) High magnification of stage 46 transgenic embryo showing expression of the transgene in the somites (S, arrows).

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The enhance sequence is cloned within the same frame with dsRed reporter gene and then this fusion gene is injected into single cell *X. laevis* embryos. The results showed that this regulatory sequence can direct dsRed expression specifically in the cardiac and skeletal muscle. Moreover, the mutations of MEF2, SRF, MyoD binding sites completely abolish dsRed expression in both caridaca and skeletal muscle (Figure 6).

2. The *in vitro* and *in vivo* function of miR-1 and -133 in the skeletal muscle development.

2.1 The C2C12 cell can faithfully mimic skeletal muscle differentiation *in vitro*. We used C2C12 cell to study miR-1 and -133 function in muscle development because C2C12 cell line can faithfully mimic skeletal muscle differentiation *in vitro*, as shown by the induction of differentiation markers when serum is withdraw from culture medium (Figure 7a, b). MicroRNA microarray results show that miR-1 and -133 are markedly up-regulated during C2C12 cell differentiation (data not shown) and that is confirmed by RNA blot analysis (Figure 7c).
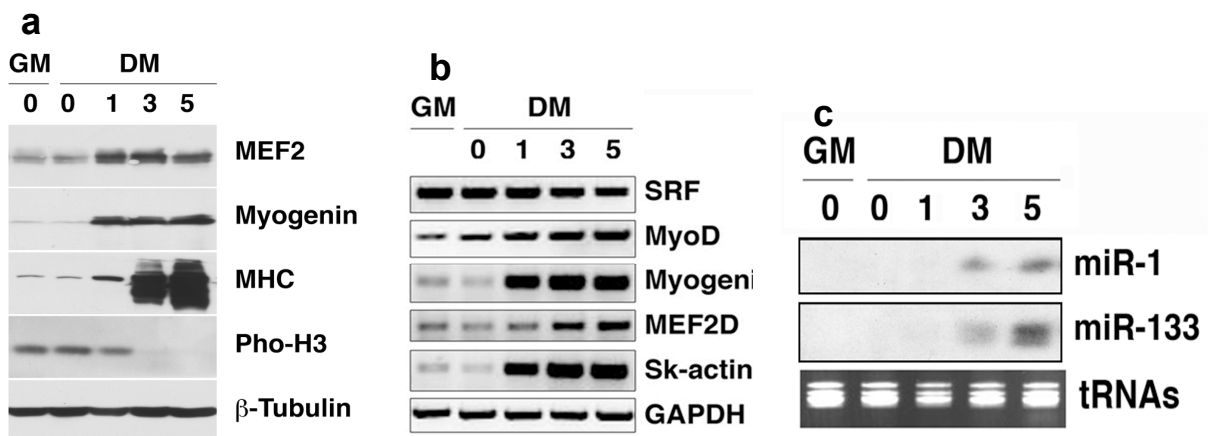
Figure 7 Expression of miR-1, miR-133 and skeletal muscle differentiation marker genes in C2C12 cells.

(**a**) Expression of skeletal muscle differentiation markers. C2C12 myoblasts were cultured in growth medium (GM) or in differentiation medium (DM) for 0, 1, 3 and 5 days, and western blots performed with cell extracts using the indicated antibodies. β-tubulin serves as a loading control. (**b**) Semi-quantitative RT-PCR analysis of skeletal muscle differentiation marker genes. GAPDH was used as a

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control for equal loading. (**c**) Northern blot analysis of the expression of miR-1 and miR-133 using total RNA isolated from C2C12 myoblasts cultured in GM or in differentiation medium (DM) for 0, 1, 3 and 5 days, respectively. tRNAs were used as a loading control.

2.2 Develop experimental tools to overexpress and/or knock down miR-1/miR-133

function. To understand miR-1 and miR-133 function in skeletal muscle, we overexpress them by cloning miRNAs precursor plus ~150 flanking sequence into pCDNA vector or by synthesizing miRNA duplexes directly. The primary transcripts will be made by RNA pol II and processed by miRNA biogenesis machine into mature miRNAs, which bind to their targets for translation repression. The overexpression of mature miRNAs and their activities were validated by Northern blot (Figure 8A) as well as "miRNA sensor" (Figure 8B). To generate the "miRNA sensor", we adapted a retroviral expression system in which the complementary sequence of miR-1 or miR-133 is cloned ownstream of a dsRed coding sequence. We transfected the construct into retrovirus packaging cell line to generate retrovirus for C2C12 cell infection. One miRNA dsRed sensor cell line can monitor the expression and function of this particular miRNA because overexpressed miRNA will bind to complementary sequence at 3'-UTR of dsRed mRNA and result in disruption of dsRed expression.

Figure 8 The overexpression and knock down of miR-1 and miR-133. (A) Northern blot analysis of miR-1 and miR-133 expression and maturation in cell culture. Expression vectors

designed to express miR-1 and miR-133 genomic fragments from two chromosome loci (mir-133a-1, mir-133a-2, mir-1-1 and mir-1-2) were transfected into COS-7 cells. Total RNA was isolated and 20 µg were hybridized 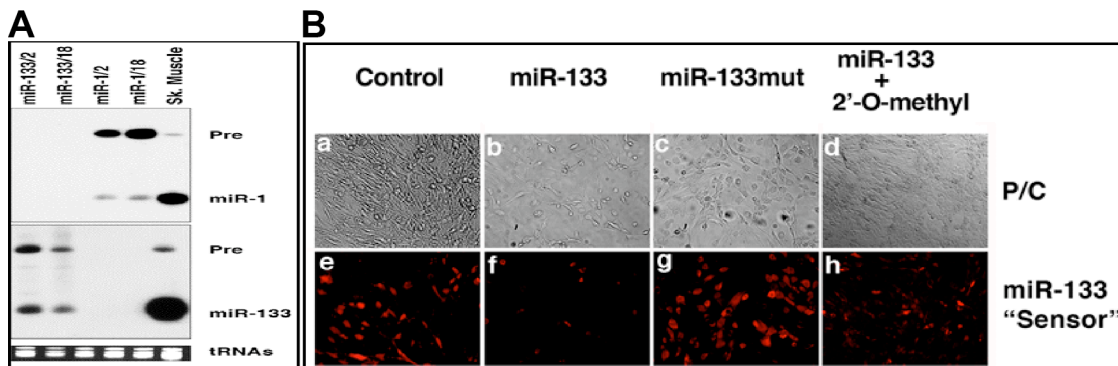to miR-1 or miR-133 probes. 20 µg total RNA isolated from adult mouse skeletal muscle was used as a control. Both mature miRNAs and their precursors (Pre) are indicated. tRNAs were used as a loading control. (B) C2C12 cells stably expressing the miR-133 sensor were transfected with expression vectors for GFP (control), wild –type miR-133 (miR-133), mutant miR-133 (miR-133mut) in which the "seed" sequence has been mutated, or a combination of miR-133 expression vector and 2'-O-methyl antisense oligos (miR-133 + 2'-O-methyl). Cells were transferred into differentiation medium for 36 hr and images were obtained using phase-contrast (P/C) (a, b, c, d) or fluorescence to show expression of the dsRed reporter gene (e, f, g, h).

To inhibit miRNA function, we synthesize 2'- O- methyl antisense oligos for miR-1 or miR-133 to specifically knock down their function. 2'-O-methyl antisense inhibitory oligos associate with endogenous mature miRNA with high affinity, therefore prevent mature miRNA to be incorporated into a functional RISC complex (Hutvagner et al., 2004; Meister et al., 2004a). We validated the function and specificity of miR-133 2'-O-methyl oligos using "miR-133 sensor" (Figure 8B). In miR-133 sensor stable cell line described above, dsRed was highly expressed when the cells were maintained on GM medium, in which miR133 expression is not induced yet. Once C2C12 cells were switched to differentiation medium, dsRed expression was completely abolished due to the induction of miR-133 expression. When 2'-O-methyl oligo antisense miR-133 was introduced into those cells, they can reverse dsRed gene expression by specifically knock down endogenous miR-133 function. These dsRed sensor allow us know whether or not miRNAs are overexpressed and function. Given knockdown of miRNAs can not be detected by miRNA northern-blot, dsRed sensor provides us information whether or not 2'-O-methyl oligos knock down corresponding miRNA activity.

2.3 miR-1 promotes cell differentiation and miR-133 enhances cell proliferation in skeltetal muscle development. In order to assess miR-1 and -133 functions in skeletal

muscle development, we transfected C2C12 myoblasts with miR-1 or miR-133 and then either maintained cells in growth medium (GM) or transferred them to differentiation medium (DM) after transfection. Experimental results shows that miR-1 strongly enhanced myogenesis, as indicated by an increase in expression of the respective early and late myogenic markers myogenin and myosin heavy chain (MHC), as well as other myogenic markers including MyoD, MEF2 and skeletal α-actin (Figure 9). miR-1 induced the expression of myogenic marker genes in cells maintained in the log-phase growth condition (Figure 9c) and in the differentiation condition (Figure 9d, e). Accelerated myogenic differentiation induced by miR-1 was accompanied by a decrease in cell proliferation, as marked by a significant decrease in expression of phosphorylated histone H3 (Figure 9c, e). We found that miR-1-induced myogenesis is specific, because overexpression of a green fluorescent protein control RNA duplex (miGFP) or miR-208, which is not endogenously expressed in skeletal muscle cells, showed no effect (Figure 9a-e). In addition, mutations introduced into miR-1 'seed' sequences abolished its ability to activate myogenic gene expression (Figure 9d, e). By contrast, overexpression of miR-133 repressed the expression of myogenin and MHC (Figure 9a-e) and promoted myoblast proliferation (Figure 9c, e). Again, we found that the effect of miR-133 on myoblasts proliferation is specific, because controls showed no effect and the mutation introduced abolished the function of miR-133

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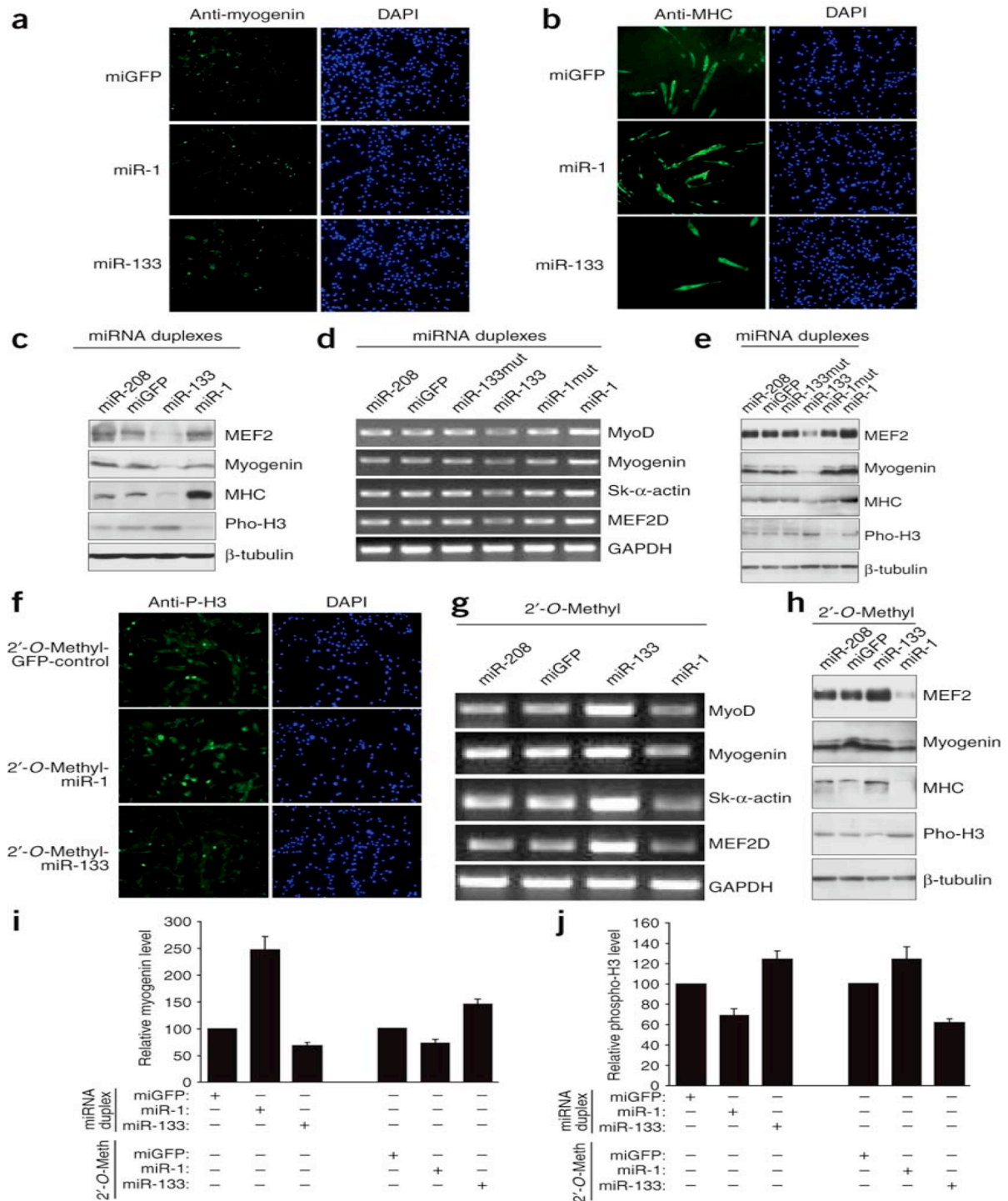(Figure 9a-e, j)

Figure 9 Regulation of myoblast proliferation and differentiation by miR-1 and miR-133.

C2C12 myoblasts cultured in growth medium were electroporated with double-stranded miR-1, miR-133 or control miGFP. (**a**,**b**) Cells were continuously cultured in growth medium for 24 h after transfection and then transferred to differentiation medium for either 12 h before immunostaining for

myogenin (**a**) or 36 h before immunostaining for MHC (**b**). (**c–e**) C2C12 myoblasts cultured in GM were electroporated with double-stranded miR-1, miR-133 (or their mutants as indicated) or control miR-208 or miGFP and cultured for 24 h before being either subjected to immunoblotting with the indicated antibodies (**c**), or transferred to differentiation medium for 24 h and subjected to RT-PCR for the indicated genes (**d**) or to immunoblotting with the indicated antibodies (**e**). (**f–h**) C2C12 myoblasts cultured in GM were electroporated with a 2'-*O*-Methyl antisense oligonucleotide inhibitors of miR-1, miR-133, miR-208 or miGFP as controls. Cells were cultured in growth medium for 24 h after transfection and then transferred into differentiation medium for 12 h before immunostaining for phospho–histone H3 (**f**), 24 h before RT-PCR for the indicated genes (**g**) or 24 h before immunoblotting with the indicated antibodies (**h**). (**i,j**) C2C12 myoblasts cultured in growth medium were electroporated with miRNA duplexes or with 2'-*O*-Methyl antisense oligonucleotide inhibitors as indicated. Cells were cultured in growth medium for 24 h after transfection and then transferred into DM for 12 h before immunostaining for myogenin (**i**) or phospho–histone H3 (**j**). Positive stained cells were counted and data are presented as the expression level relative to a miGFP control (100%).

We carried out a reciprocal experiment wherein we transfected C2C12 myoblasts with 2'-O-methyl antisense inhibitory oligoribonucleotides, which have been shown to inhibit the function of miRNAs targeted towards miR-1 or miR-133 (or control miGFP and miR-208). Cells transfected with the miR-1 inhibitor showed inhibition of myogenesis and promotion of myoblast proliferation, as indicated by a decrease in myogenic markers and an increase in phospho–histone H3 (Figure 9f-i). Consistent with the role of miR-133 in promoting myoblast proliferation and repressing differentiation, inhibition of miR-133 caused an opposing effect, whereby myogenesis was enhanced and cell proliferation was repressed (Figure 9f-j). By contrast, the control 2'-O-methyl inhibitors showed no effects (Figure 9f-j). We conclude that miR-1 and miR-133 have distinct roles in skeletal muscle proliferation and differentiation: miR-1 promotes myoblast differentiation, whereas miR-133 stimulates myoblast proliferation.

2.4 miR-1 and -133 regulate muscle development *in vivo*.

Both miR-1 and miR-133 have been found in most animal species, from *Drosophila* to human, suggesting that they are evolutionary conserved. Moreover, the high conservation of

miR-1 and miR-133 across differenent species allow us to assess their functions in different

systems. miR-1 and miR-133 have identical sequence on different mouse chromosome,

respectively, and mir-206 has a four nucletide sequence difference with miR-1. In addition,

miR-133b has same sequence with miR-133a except one nulcetide at the 3' end. These

sequence similarity provide substantial challenge to functionally study miR-1 and miR-133

using knockout approaches. To test the effects of miR-1 and miR-133 on skeletal muscle and

heart development *in vivo*, we identified copies of miR-1 and miR-133 in *X. laevis* and tested

their function through misexpression.
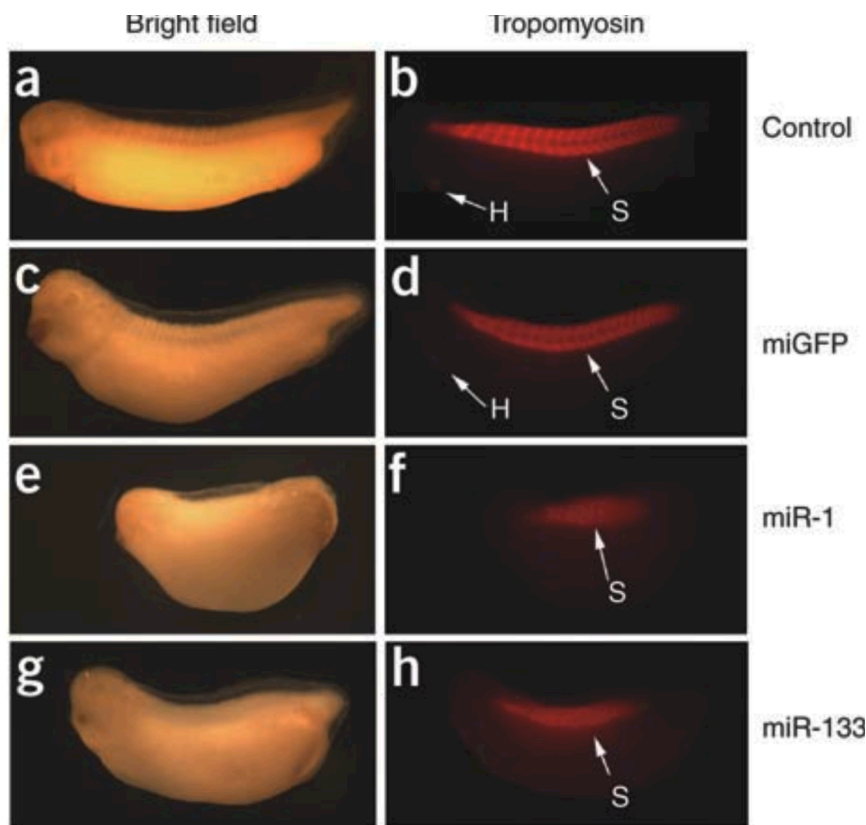
Figure 10 Wholemount staining analyses miR-1 and -133 overexpressing *X. laevis* embryos. (a–h) Images of uninjected (a,b), control miGFP-injected (c,d), miR-1-injected (e,f) and miR-133-injected (g,h) X. laevis embryos stained with anti-tropomyosin and shown at stage 32 under bright-field (a,c,e,g) or fluorescence (b,d,f,h) microscopy. Note the lack of staining for heart tissue (H, arrows) and the disruption of segmented somites (S, arrows) in f and h.

Introduction of miR-1 at the one-cell stage led to a markedly shortened axis with an accompanying reduction in

anterior structures and an increase in body size along the dorsal-ventral axis, as compared

with either uninjected or miGFP-injected controls (n > 50, two independent experiments)

Although somites formed in embryos injected with miR-1, whole-mount antibody staining

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and serial sectioning showed that the tissue was highly disorganized and did not develop into segmented structures (Figure 10e, f). Cardiac tissue was completely absent, as judged by histology, staining for tropomyosin (Figure 10f) and staining for cardiac actin (data not shown). In addition to these defects, there was a marked decrease in phospho–histone H3 staining (Figure 11a-c), consistent with the notion that miR-1 is essential in regulating muscle cell proliferation and differentiation. Misexpression of miR-133 also led to a reduction in anterior structures and defects in somite development but, in contrast to misexpression of miR-1, there was only a modest reduction in anterior-posterior length and somitic defects were most severe in the more anterior or posterior aspects of the embryo where somites failed to form (Figure 10g, h). In addition, cardiac tissue frequently formed in miR-133-injected embryos, although it was highly disorganized and did not undergo cardiac looping or chamber formation (Figure 10h and data not shown). Collectively, these data suggest that correct temporal expression and amounts of both miR-1 and miR-133 are required for proper skeletal muscle and heart development.
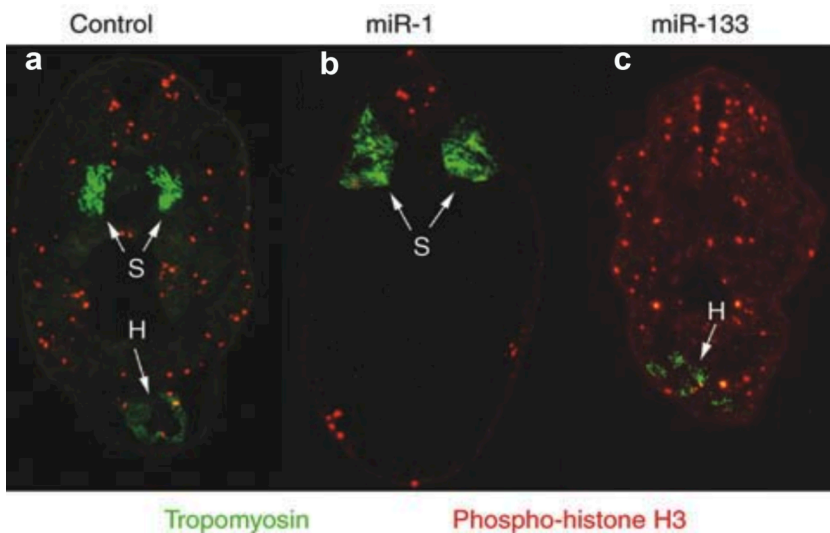
Figure 11 Section immnostaining miR-1 and 133 overexpressing *X. laevis* embryos. (a-c) Transverse sections corresponding to the position of the heart at stage 32 in uninjected (a), miR-1-injected (b) and miR-133-injected (c) X. laevis embryos stained with anti-tropomyosin to visualize somites (S, arrows) and cardiac tissue (H, arrows), and antibody to phospho–histone H3

(red) to visualize cells in S phase. Each set of injections was conducted at least twice independently, and the phenotype was observed in at least 90% of a minimum of 50 embryos scored by whole-mount immunostaining.

3. miR-1 and miR-133 regulate HDAC4 and SRF, respectively, to control muscle development.

3.1 Reporter assay to validate miR-1 and -133 targets. To identify target genes that might mediate the observed effects of miR-1 and miR-133 on skeletal muscle proliferation and differentiation, we next examined potential targets of these two miRNAs. Many computational and/or bioinformatics-based approaches have been used to predict numerous potential targets of miRNAs. Strikingly, many transcription factors have been suggested to be targets of miRNAs, raising the possibility that miRNAs might be involved in transcriptional regulation of gene expression (John et al., 2004; Kiriakidou et al., 2004; Krek et al., 2005; Lewis et al., 2003). Among the predicted targets of miR-1, HDAC4 has been shown to inhibit muscle differentiation and skeletal muscle gene expression, mainly by repressing MEF2C, an essential muscle-related transcription factor (Lu et al., 2000; McKinsey et al., 2000). HDAC4 contains two naturally occurring putative miR-1-binding sites at its 3' UTR, which are evolutionarily conserved among vertebrate species. Similarly, two conserved miR-133-binding sites are found in the 3' UTR of the mammalian gene encoding SRF, which has been shown to be important in muscle proliferation and differentiation *in vitro* and *in vivo* (Wang et al., 2002).

We fused the 3' UTRs of mouse SRF and HDAC4 to a luciferase reporter gene and transfected these constructs, along with transfection controls, into mammalian cells. Ectopic overexpression of miR-1 strongly repressed the HDAC4 3' UTR luciferase reporter gene, whereas overexpression of miR-133 inhibited the SRF 3' UTR luciferase reporter gene. By

contrast, mutations introduced into miR-1 or miR-133 seed sequences abolished this repression, indicating the specificity of the action (Figure 12a). When the above reporters were transfected into C2C12 myoblasts and luciferase activity was measured before and after the induction of cell differentiation, reporter gene activity was markedly repressed in differentiated cells (Figure 12b), indicating that the increase in endogenous miR-1 and miR-133 inhibited the reporter gene. By contrast, the activity of a luciferase reporter gene for MCK, an indicator of muscle differentiation, was increased in differentiated muscle cells.

3.2 Validate the targets of miR-1 and -133 at protein and mRNA level. In addition, overexpression of miR-1 led to the downregulation of endogenous HDAC4 protein in C2C12 cells in both the growth condition (Figure 12c) and the differentiation condition (Figure 12e), whereas overexpression of miR-133 repressed the expression of endogenous SRF proteins (Figure 12c, e). By contrast, the mRNA levels of SRF and HDAC4 were not altered by these miRNAs (Figure 12d), supporting the notion that miRNAs repress the function of their target genes mainly by inhibiting translation. The application of 2'-*O*-methyl-antisense oligoribonucleotides targeted towards miR-1 or miR-133 relieved the repression of HDAC4 or SRF protein, respectively (Figure 12g), but had no effect on their mRNA levels (Figure 12f).
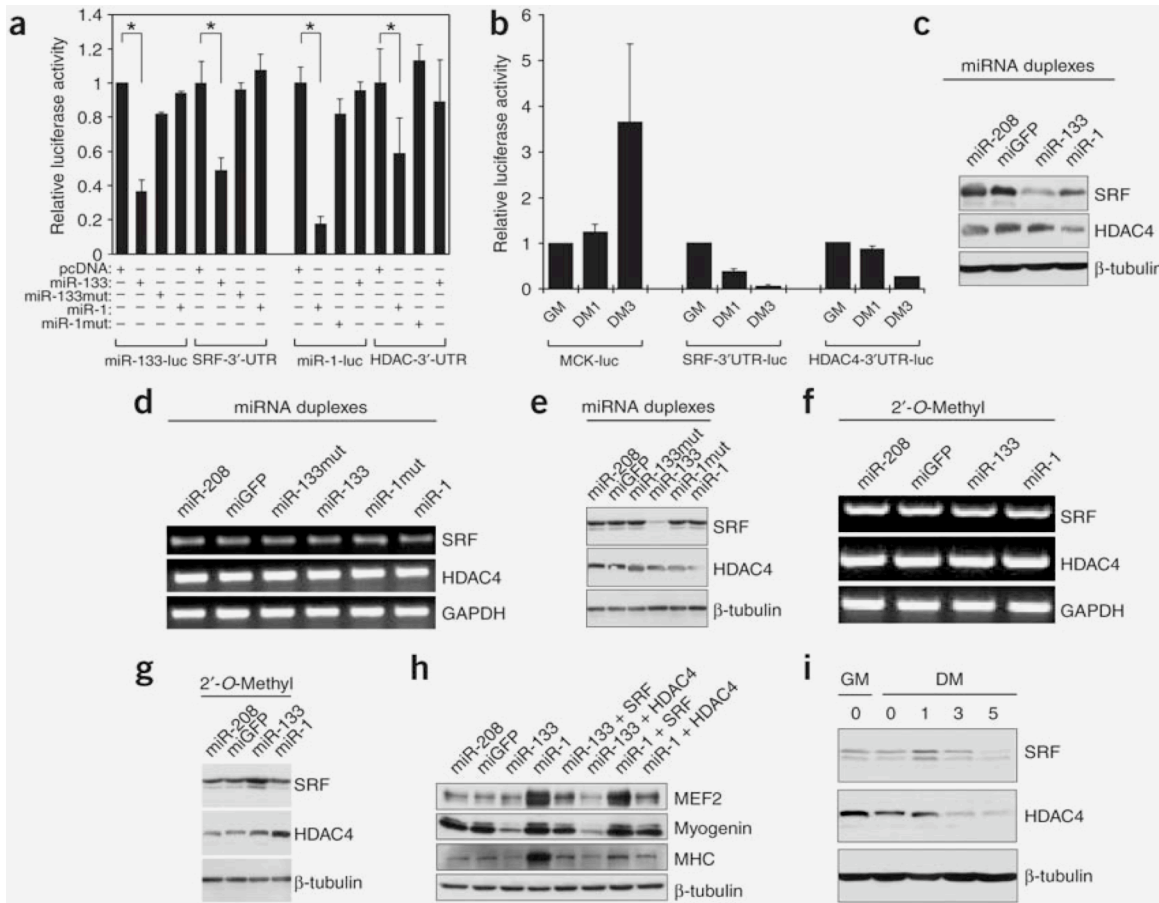
Figure 12 Identification of miR-1 and miR-133 target genes in skeletal muscle.

(**a**) Repression of SRF and HDAC4 3' UTRs by miR-133 and miR-1. (**b**) SRF-3'-UTR, HDAC4-3'-UTR and MCK-luc luciferase reporters were transfected into C2C12 myoblasts. Cells were maintained in growth medium for 24 h (GM) or transferred into differentiation medium for 1 d (DM1) or 3 d (DM3) before luciferase activity was determined. (**c–e**) C2C12 myoblasts cultured in growth medium were electroporated with the indicated miRNA duplexes (or their mutants), or miR-208 and miGFP as controls. Cells were cultured in growth medium for 24 h after transfection before being either subjected to immunoblotting with anti-SRF and anti-HDAC4 antibodies (**c**), or transferred into differentiation medium for 24 h and subjected to RT-PCR for the indicated genes (**d**) or to immunoblotting with the indicated antibodies (**e**). (**f,g**) C2C12 myoblasts cultured in growth medium were electroporated with the indicated 2'-*O*-Methyl antisense oligonucleotide inhibitors. Cells were cultured in growth medium for 24 h after transfection and transferred into differentiation medium for 24 h before being subjected to RT-PCR for the indicated genes (**f**) or to immunoblotting with indicated antibodies (**g**). (**h**) C2C12 myoblasts cultured in growth medium were electroporated with the indicated miRNA duplexes and/or expression plasmids for SRF or HDAC4, as indicated. Cells were cultured in growth medium for 24 h after transfection. Immunoblotting with the indicated antibodies was done 24 h after transfer into differentiation medium. (**i**) C2C12 myoblasts were cultured in growth medium or differentiation medium for 0, 1, 3 or 5 d and subjected to immunoblotting with the indicated antibodies.

To verify that HDAC4 and SRF are cognate targets of miR-1 and miR-133 in

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regulating skeletal muscle gene expression, we tested whether cotransfecting expression plasmids encoding SRF and HDAC4 could 'suppress' miRNA-mediated myogenesis. Indeed, overexpression of SRF partially reversed the myogenic gene repression induced by miR-133 (Figure 12h), whereas overexpression of HDAC4 counteracted the effects of miR-1 on skeletal muscle gene expression (Figure 12h). Consistent with the potential involvement of HDAC4 and SRF in miR-1- and miR-133-dependent skeletal muscle proliferation and differentiation, endogenous HDAC4 and SRF protein was downregulated in differentiated C2C12 cells, coupled with a concomitant increase in expression of myogenic differentiation markers and a decrease in expression of the mitotic index marker phospho–histone H3 (Figure 12i). The decrease in expression of SRF and HDAC4 protein was accompanied by an increase in expression of miR-1 and miR-133 (compare Figure 12i with Figure 12b). Together, these data show that miR-1 and miR-133 specifically repress HDAC4 and SRF protein, respectively, which in turn contributes to (at least in part) the regulatory effects of these miRNAs on myoblast proliferation and differentiation.

Summary

Taken together, we have characterized cardiac-specific and skeletal muscle–specific miR-1 and miR-133 and have shown their essential functions in controlling skeletal muscle proliferation and differentiation. Notably, we have shown that miR-1 and miR-133, which are clustered on the same chromosomal loci and transcribed together as a single transcript, become two independent, mature miRNAs with distinct biological functions achieved by inhibiting different target genes. This finding implicates miRNAs in complex molecular mechanisms.
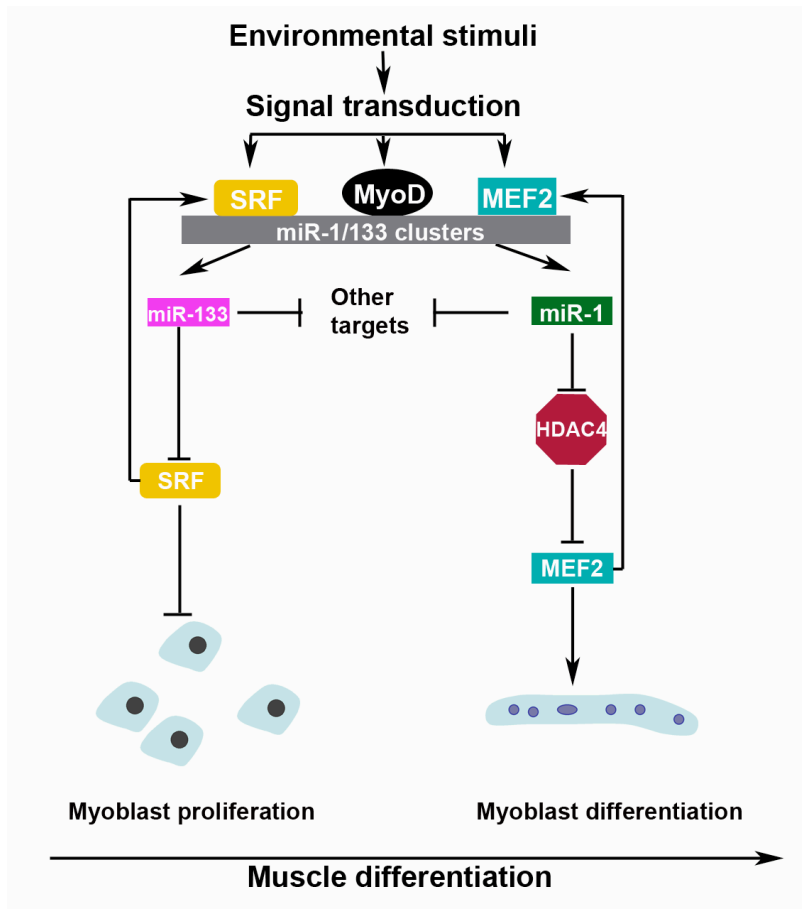
Figure 13 Model of miR-1 and miR-133-mediated gene regulation during muscle proliferation and differentiation. Tissue-specific expression of miR-1 and miR-133 cluster is controlled by transcriptional factors SRF, MEF2 and MyoD. miR-1 promotes muscle differentiation by repressing the expression of HDAC4 (histone deacetylase 4), a signal-dependent inhibitor of muscle differentiation that repressed MEF2 activity. MEF2, in turn, potently activates miR-1 expression in muscle. In contrast, miR-133 enhances myoblast proliferation and inhibits their differentiation, at least in part, by reducing SRF protein levels, a critical regulator of muscle differentiation

Although the tissue-specific expression of miR-1 and miR-133 is controlled by MyoD and SRF, expression of SRF is repressed by miR-133. miR-1 promotes muscle differentiation by repressing HDAC4, a signal dependent chromotin regulator that repress MEF2 activity. MEF2, in turn, potently activates miR-1 and miR-133 expression. Thus, these findings identify a negative regulatory loop in which miRNAs participate to control cellular proliferation and differentiation (Figure 13). In the future, it will be interesting to determine whether miR-1 and miR-133 are involved in cardiac-related and skeletal muscle–related human diseases.

CHAPTER 3

THE GLOBAL ROLE OF MICRORNAS IN HEART DEVELOPMENT

INTRODUCTION

Heart development and morphogenesis

Classically, the myocardial cells within the primary heart field are thought to originate from the anterior mesoderm, which emerges from the primitive streak during gastrulation. The precardiac mesoderm cells migrate and form a crescent-shaped epithelium, called the cardiac crescent, where the precardiac cells adopt cardiac fate in response to cues from adjacent endoderm around mouse E 7.5. As development proceeds, the cardiac crescent forms the cardiac tube, which subsequently undergoes looping around E 8.5 (Buckingham et al., 2005). The establishment of the heart position and cell fates is mainly based on lineage mapping experiments from chicken embryos. The cells in the anterior regions of the streak are pre-patterned along the arterial-venous axis in terms of their contributions to the heart tube. The cardiac progenitor cells fuse anteriomedially in the ventral midline to form the first segment of the heart tube, and additional segments are added to the end of the first in a sequential fashion from the posteriolateral heart forming field mesoderm (Buckingham et al., 2005). These observations suggest that all the myocardium originates from a single heart field and supports the segmental model of cardiogenesis.

A second heart field from pharyngeal mesoderm was recently found in chicken and mouse (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). The secondary heat field marked by the Fgf10-LacZ transgene is located anteriorly and dorsally to heart tube. As heart morphogenesis proceeds, these secondary heart-field cells ultimately contribute to the right ventricle, whereas those more anterior cells assume, slightly later, the outflow-tract myocardium identity (Waldo et al., 2001). The contributions of second heart progenitors to heart formation was further confirmed by the studies of Isl1 (a LIM homeodomain transcriptional factor) (Cai et al., 2003). The lineage tracing analyses showed that the secondary heart field cells also contribute to the venous and arterial pole. Two waves of cell migrations are important to bring heart progenitors to the ventral midline where the heart tube is formed. During gastrulation, precardiac mesoderm cells are mobilized from the node and primitive streak to embryo cranial regions and form the cardiac crescent, in which FGF signaling pathway is crucial. The mutation of earliest cardiac marker MESP1/MESP2 (mesoderm posterior 1/2) led to Fgf4 down-regulation (Kitajima et al., 2000). In response to FGF signaling, transcriptional factor snail induces the epithelial-mesenchymal transition in the epiblast cells by repressing the expression of E-cadherin, a calcium-dependent cell-adhesion molecule (Ciruna and Rossant, 2001). Subsequently, heart progenitor cells form the heart tube along ventral midline in the second wave of migration, in which the graded distribution of fibronectin along the cranio-caudal axis is essential. Fibronectin (Fn1) deletion led to the failure of cardiac cell fusion, a condition termed cardia bifida (George et al., 1997). Gata4 in the endoderm is also required for the migration of heart progenitor cells in mouse and zebrafish, indicating that correct differentiation is essential for the second movement (Narita et al., 1997).

The mammalian heart contains three major cell lineages, including myocardial, endothelial, and smooth muscle cells. Lineage tracing studies from chicken embryos support an early separation of endocardial lineage and myocardial lineage, most likely prior to or during gastrulation (Coffin and Poole, 1988). Lineage tracing studies in mouse embryos using Cre/Lox system revealed that all three cell types activate Nkx2.5 expression, indicating their common origins (Lints et al., 1993). Multipotential progenitor cells have been identified by using the isl1 marker in hearts and these progenitor cells can differentiate into cardiomyocytes, smooth muscle cells and endothelial cells *in vitro* (Moretti et al., 2006). The development of vascular smooth muscle cells takes place much later and remains much more diverse in terms of their origins than that of myocardial and endothelial cells (Majesky, 2007). The smooth muscle cells at outflow tract and aorta have dual origins in the neural crest and the lateral plate mesoderm (Waldo and Kirby, 1993). The lineage tracing studies of smooth muscle cells at the base of aorta and pulmonary artery have shown an unexpected origin of the secondary heart field, which is thought to give rise to only the cardiac cells of the outflow tract, right ventricle and ventricular septum (Waldo et al., 2005). Cardiac SMCs arise from an extracardiac source, proepicardium, which attaches to looping hearts and migrates as a sheet to cover the entire surface of the heart. Once the heart is completely coverd, the epicardium undergoes epithelial-to-mesenchymal transformation (EMT) and generate the endothelial and smooth muscle cells of the coronary arteries (Mikawa and Gourdie, 1996). The retrospective clonal analysis revealed, through genetically tracing single cell-derived clonies during mouse development, a common origin of aortic smooth muscle and skeletal muscle cells (Esner et al., 2006). Those studies suggest that smooth muscle cells have less distinct origins in general, and smooth muscle progenitors are recruited locally

from those mesenchymal precursors and differentiate into smooth muscle cells in response to local cues. The inhibition of Notch signaling in the neural crest cells led to the defects of smooth muscle cell differentiation in the cardiac outflow tract (High et al., 2007). It remians a big challenge to define the molecular nature of the interactions between the secondary cardiac cells, neural crest cells and endothelial cells during early heart formation.

Our fundamental understanding into cardiac cell specification is attributed to those critical advancements in the field. We identified those molecular makers for cardiac fate specification, including Nkx2.5, Gata4, and their enhancer elements. Using those enhancer elements, we were able to define those signaling effectors and transcriptional factors that bind to the enhancer elements and turn on the expression of these cardiac marker genes. In additon, explant culture, including posterior lateral plate mesoderm in chicken and ventral marginal zone mesoderm in *Xenopous laevis,* were established to test their responses to the inducing signals of cardiac specification. Lastly, the genetically accessible model organisms were available to functionally study specific genes *in vivo*. Genetic and biochemical studies from different organisms demonstrated that BMPs, FGF8 and Wnt antagonists have positive influence on cardiac induction, whereas Wnt signaling has negative effect on early cardiac specification (Marvin et al., 2001; Schneider and Mercola, 2001; Schultheiss et al., 1997). BMPs signaling effectors, the Smad family, directly activate genes encoding cardiac transcriptional factors (Liberatore et al., 2002; Lien et al., 2002). The formation of the second heart field, defined by the Isl1 expression, is under positive regulation by canonical Wnt pathway (Ai et al., 2007; Cohen et al., 2007; Lin et al., 2007). In response to those inductive signals, the cardiac crescent activates a set of cardiac-specific transcriptional factors, including Nkx2.5, Gata4, Tbx5 and SRF, which work cooperatively to activate cardiac

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structural genes (Olson and Schneider, 2003). Nkx2.5 and Gata4 are also auto-regulated by themselve and form a positive feedback loop that activates and maintains their expression and function after cardiac specification (Molkentin et al., 2000). Recent studies indicted that an early function of Nkx2.5 in cardiac development is to dampen the expression of Bmp2, the immediate upstream inducer of Nkx2.5 expression. This negative feedback loop between Bmp2 and Nkx2.5 provides fine-control the number of cardiac progenitors through opposing effects on cardiac induction and proliferation (Prall et al., 2007).

Transcriptional factors are essential for cardiac muscle proliferation and differentiation. Genetic studies demonstrated that Nkx2.5, Hand1 and Tbx5 mainly affect the first lineage (Bruneau et al., 2001; Lyons et al., 1995; Riley et al., 1998). The Nkx2.5 mutant mice didn't have ventricles and lost Hand1 expression at the heart tubes (Lyons et al., 1995). Hand1 null mice die due to extra-embryonic defects, and cardiac progenitor cells lacking Hand1 failed to contribute to the left ventricle in Hand1 chimaeras (Firulli et al., 1998; Riley et al., 1998). The use of Cre-recombinase under the control of the Nkx2.5 promoter to delete Hand1 in the early embryonic hearts resulted in left-ventricle hypoplasia (McFadden et al., 2005). Tbx5 null mice exhibited severe defects in the atria-inflow region as well as left ventricle hypoplasia, whereas TBX5 haploinsufficiency caused cardiac and forelimb defects seen in the Holt-Oram syndrome (Bruneau et al., 2001). Genetic evidences also support the view that Tbx1, Isl1 and Mef2c mainly affect the second heart field. Tbx1 null mice have a similar cardiac phenotype with Fgf8 hypomorph, and Tbx1 haploinsufficiency is sufficient to partially phenotype the DiGeorge syndrome (Lindsay et al., 2001). Loss- and gain-of-function studies suggest that Fgf8 and Fgf10 could be the targets of Tbx1 (Hu et al., 2004; Kume et al., 2001), which is potentially activated by the transcriptional factor FOXC1 and

FOXC2 (Kume et al., 2001). FOXH1 positively regulates Fgf8 expression and Foxh1 null

mice displayed no right ventricular and outflow-tract, although a truncated outflow region is

present (von Both et al., 2004). Consistent with the fact that Isl1 is a molecular marker of the

secondary heart field, Isl1 null mice do not have the outflow tract, right ventricle and much

of the atria. In those isl1 mutant hearts, isl1- expressing progenitors are progressively lost

possibly due to the down-regulation of FGF and BMP growth factors (Cai et al., 2003). Isl1

directly regulates mef2c through the Isl1 binding enhancer element in mef2c promoter region

(Dodou et al., 2004). Mef2c null mice reveal secondary heart field defects, including a

reduced outflow region and a loss of the right ventricle (Lin et al., 1997).

After the three lineages of heart are specified, they undergo cell proliferation,

differentiation and extensive morphogenesis during further development. Around E9.5 in

mouse, a subset endothelial cells overlying the future valve regions are stimulated by signals

from underlying myocardium to undergo EMT and form endocardial cushions. Those

endocardial cushions within the AV canal and the outflow tract are the precursors of the

tricuspid/mitral valves and the aortic/pulmonic valves, respectively. Epithelial cells in EMT

acquire mesenchymal characteristics, including loss of cell-cell contact, cytoskeleton

rearrangement, ECM degradation and remodeling, and cell migration within developing

hearts. Those studies from expression pattern analyses, *ex vivo* cushion explants and mouse

genetic manipulation provide a molecular and cellular understanding of EMT (Bernanke and

Markwald, 1982). A unique subset of endocardial cells could be patterned to undergo EMT

before heart tube formation. Altanatively, the Notch signaling could generate a small

population of cells competent for EMT via lateral induction of specific cell fates during early

development (Timmerman et al., 2004). Consistent with the role of Notch signaling in EMT

initiation, the disruption of Notch signaling results in hypoplastic cardiac cushions and the reduced expressions of TGF-β and snail/slug. In response to myocardium-derived BMP/TGF-β signals, a sub-population of endocardial cells initiates EMT through the up-regulation of snail/slug, which inhibits the expression of cell-cell adhesion molecules (Sugi et al., 2004). After the initiation of EMT, endothelial cells trans-differentiate into mechenchyme cells that migrate away from the endocardium. VEGF positively regulates endothelial cell proliferation via increasing NFATc1 nuclear localization during valve development, therefore maintaining endocardium integrity. (de la Pompa et al., 1998; Johnson et al., 2003). Three-fold increase of VEGF levels results in the embryonic lethality due to delayed outflow tract septation, excessive endothelial cell proliferation and cardiac enlargement (Miquerol et al., 2000). Constitutively active Wnt signaling leads to excessive endocardial cushion, whereas elevated expression of Dkk1, a secreted Wnt inhibitor, blocks cushion formation in Zebrafish (Hurlstone et al., 2003). Therefore, Wnt/β-catenin signaling plays a positive role for EMT during cardiac valve formation. β-catenin is phosphorylayed in response toVEGF signaling, and phosphorylated β-catenin co-localizes with VEGF receptor, PECAM-1 and Cadherin to epithelial cell-cell junctions, therefore providing a cellular "sink" to regulate cytosolic β-catenin levels (Ilan et al., 1999). These studies suggest that classic signaling pathways including VEGF and Wnt signaling regulate valve development.

The epicardial cells originating from the proepicardium (PE) migrate as a continuous sheet of mesothelial cells to cover the AV canal first and then the inflow and outflow tract (Manner et al., 2001). The epicardial cells maintain cell-cell contact and do not loose the epithelial organization of their cytoskeleton during migration. Therefore, epicaridial cells do not convert into other potential cell types. By E11.5~12.5, myocardium-derived signals

stimulate the epicardial cells to execute EMT to form the epicardial-derived mesenchymal cells (EPDCs), the precursors of coronary vessels. EPDCs not only contribute to the coronary vessel but also give rise to heart ECM cells and participate in valve and septal development (Dettman et al., 1998; Poelmann et al., 2002). The essential myocardium-derived signals that stimulate epicardium EMT haven't been fully defined. FGF-1, -2 and -7 stimulate epicardial EMT whereas TGFβ-1, -2, -3 inhibit this process in organ cultures (Morabito et al., 2001). Slug, Wilms's tumor-1 (WT1) and c-ets (ets1) are found to be critical for epicaridal cell EMT (Carmona et al., 2001; Macias et al., 1998). WT-1 is required for retinoic acid synthesis in the EPDCs and retinoic acid could provide growth-promoting signals for developing myocardium (Xavier-Neto et al., 2000). The Ets1 can activate a number of genes critical for extracellular matrix degradation and cell migration. It remains a challenge to identify those critical genes and pathways that control the movement and fates of EPDCs during heart development.

Fly and mouse genetic studies suggest an intimate relationship between the cellular architecture and cell proliferation during EMT process (Bilder, 2004; Perez-Moreno and Fuchs, 2006). EMT leads to the translocation of those components involved epithelial cell-cell contact from the cell surface to the nucleus, where they can regulate gene expression. Loss of cell-cell adhesion results in the translocation of β-catenin, a building component of cell-cell junction, from epithelial cell surface to the nucleus and therefore regulate gene expression (Gottardi and Gumbiner, 2001). Loss of cell-cell contact can trigger signaling transduction pathways to regulate gene expression. Specific deletion of α-catenin in the epidermal cells results in massive hyperproliferation due, at least partially, to elevated Ras and MAPK activity (Vasioukhin et al., 2001). Loss of cell adhesion affects NF-κB

transcription through a mechanism not fully understood but RhoGTPase, α-catenin and

p120-catenin are thought to be involved (Kobielak and Fuchs, 2006; Perez-Moreno et al.,

2006). The subcortical actins are predisposed to be assembled into stress fibers during the

loss of cell adhesion in EMT, therefore releasing those actin-binding proteins from actins for

their redistributions in the cells. Cadherin engagement inhibits RhoA activity via the

activation of p190rhoGAP, and reduced RhoA activity prevents stress fiber formation (Noren

et al., 2003). RhoA-actin signaling activation in response to serum stimulation leads to an

accumulation of stress fibers and a decrease of monomeric actin in the cytoplasm of cells.

Subsequently, SRF coactivator MAL, which normally is sequestered by monomeric actin in the

cytoplasm, will accumulate in the nucleus to regulate SRF activity (Miralles et al., 2003;

Vartiainen et al., 2007).

In addition to physically contributing cells to the developing heart, the epicardium

provides signalings for myocardium growth. Thin ventricular myocardium is frequently

observed from the disruption of those genes essential for epicardium formation, including

VCAM-1 (Kwee et al., 1995), GATA-4 (Watt et al., 2004), α4- integrin (Yang et al., 1995),

and RXRα (Stuckmann et al., 2003). RXRα exerts its effects on the compact zone of

myocardium in a non-cell autonomous manner (Chen et al., 1998). Recent studies have

shown that RA induces the expression of Fgf9 and regulates myocardial proliferation (Lavine

et al., 2005). Myocardial FGF signaling regulates coronary vascular development by

triggering a wave of Hedgehog (HH) signaling (Lavine et al., 2006). Mutations in Fog2

(Friend of GATA-2) produce mice with a thin myocardium, yet an intact epicardium is

present in those mice. However, endothelial markers, PECAM-1, ICAM-2, and flk-1, which

are normally expressed in the subepicardial space, cannot be detected in Fog2 null mice.

Chimeric and rescuing studies suggested that Fog2 in the myocardium has non-automatic role for coronary vessel development (Tevosian et al., 2000).

Trabecular myocytes are intimately associated with the endocardium and cross-talk between myocardium and endocardium is essential for heart development. Trabecular formation is dependent on ephrin B2 (EPHB2) and its receptor EPHB4, neuregulin-1 and its receptor ERBB2/4 (Gassmann et al., 1995; Lee et al., 1995), and BMP10 (Chen et al., 2004b). Activated Notch signaling in endocardial cells regulates BMP expression and the proliferation of those adjacent trabecular myocytes, whereas Notch activity in the endocardium controls myocyte differentiation by regulating EPHB2-NRG1 (Grego-Bessa et al., 2007).

Hypertrophic and dilated cardiomyopathy

Heart failure is induced by a number of common disease stimuli including cardiomyopathy (genetic or acquired disease from cardiac infarction or ischaemia, hypertension, or diabetes), valve disease, congenital malformation, or arrhythmias (Ahmad et al., 2005). The two major and distinct remodeling patterns of cardiomyopathy are hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM).

Hypertrophy is anatomically defined as an increased cardiac ventricular wall thickness due to enlargement of myocyte size and/or increased fibrosis (Ahmad et al., 2005). Hypertrophy is a compensatory response to increase cardiac output. Prolonged cardiac hypertrophy is associated with numerous adverse effects with distinct molecular and pathological characteristics causing hearts decompensate, through myocyte degeneration and death, culminating sudden death or heart failure (Marx, 2003). Mouse models for genetic

HCM have been recapitulated by mutations in genes that encode sarcomere proteins (Ahmad et al., 2005). Myosin binding protein C (MYBPC3), β-myosin heavy chain (MYH7), cardiac troponin I (TNNI3) and cardiac troponin T (TNNT2) are the most prevalent genes mutated in HCM, while the mutations were also found in α-tropomyosin (TPM1), cardiac actin (ACTC), cardiac troponin C (TNNC1), essential myosin light chain (MYL3), regulatory myosin light chain (MYL2), α-cardiac myosin heavy chain (MYH6), and titin (TNN). The cellular and molecular events triggered by sarcomere protein gene mutation remain undefined. The heterogeneity of mutant and wild-type proteins within the same sarcomere may compromise overall sarcomere function. In addition, unmet metabolic demand caused by increased sarcomere contents can result in premature myocyte death and fibrosis within hearts. Calcium cycling between sarcomere and sarcoplasmic reticulum is frequently altered in the HCM (Fatkin et al., 2000; Semsarian et al., 2002).

The initial stimuli for acquired HCM can be segregated into biomechanical stimuli and neurohumoral one associated with a release of hormones, cytokines, chemokines and peptide growth factors (Heineke and Molkentin, 2006). Myocytes can directly detect mechanical stretch through an internal sensory apparatus involved in Integrin, which can activate downstream signaling effectors such as FAK, Ras and Rho. The integrin-interacting protein melusin has been implicated as a stretch sensor and is essential for the phosphorylation of glycogen synthase kinase-3β (GSK3β) in the myocyte (Brancaccio et al., 2003). A second stretch sensor is suggested to be the small LIM-domain protein MLP (muscle LIM protein) localized in the Z-disc and MLP mediates signaling through the calcineurin-NFAT signaling pathway (Knoll et al., 2002). The neurohumoral and endocrine hormones, including angiotension II, endothelin-1 and α-adrenergic, can bind to specific

membrane receptors coupled to heterotrimeric G proteins of the Gq/11 subclass (D'Angelo et al., 1997; Rockman et al., 2002) and therefore lead to the mobilization of internal Ca2+ through PLCβ-PKC pathway (Wu et al., 2006).

Multiple interconnected signaling modules culminate in the nucleus in response to hypertrophy stimuli and control gene expression at multiple levels, including transcriptional initiation, transcription elongation, and protein translation. The MAPK signaling is activated by G -protein coupled receptor (GPCR), receptor tyrosine kinase (IGF-I, FGF receptor), receptor serine/threonine kinase (TGFβ), gp130 receptor, and by stress stimuli such as stretch. MAPK signaling phosphorylates MEF2 factors, GATA factors and ATF transcriptional factors through three terminal MAPK families (ERK, JNK and p38) (Heineke and Molkentin, 2006). Calcineurin dramatically activates cardiac growth in response to a specific wave of Ca^{2+}, in which NFATs are among those major substrates of calcineurin. NFAT dephosphorylation by calcineurin results in its translocation into nucleus where dephosphorylated NFAT interacts with MEF2 and GATA transcriptional factors, providing a link between calcium signaling and fetal cardiac gene expression. NFATs are rephosphorylated by GSK-3 and translocate back to the cytoplasm. GSK-3 suppression by Akt/protein kinase B (PKB) provides a link between the calcineurin and IGF-PI3K pathway (Crabtree and Olson, 2002). The MEF2-HDAC module is a critical mediator for cardiac hypertrophy. Nuclear exportation of ClassII HDACs is mediated by a stress response protein kinase that leads to the derepression of MEF2 activity. MEF2, in turn, potently activates muscle differentiation. In the absence of signals, classII HDACs accumulate in the nucleus and compete with HATs, such as p300, for MEF2 binding sites and thereby repress gene expression for hypertrophic growth (Backs and Olson, 2006; Zhang et al., 2002).

Transcriptional elongation is also essential for cardiac hypertrophy. Numerous hypertrophic signals stimulate the activity of cyclin-dependent protein kinase 9 (cdk9), which phosphorylates pol II C-terminal domain (CTD) to induce transcription elongation. In addition, Cdk9 induces the dissociation of pol II CTD with its endogenous inhibitor, a small nuclear RNA inhibitor 7SK (Sano et al., 2002). The IGF-PI3K pathway has critical role in hypertrophy through regulating protein translation. AKT activation, mediated by IGF-PI3K signaling, results in GSK3 inactivation and protein synthesis stimulation. In addition, AKT has been shown to activate mammalian targets of rapamycin (mTOR) (Schmelzle and Hall, 2000), which stimulate protein synthesis by phosphorylating p70 S6 kinase and the elongation factor 4E (eIF4E). Hypertrophy can be attenuated by rapamycin, an inhibitor of mTOR activity (Shioi et al., 2003). The cardiac-specific deletion of PTEN in mice leads to cardiac hypertrophy (Crackower et al., 2002).

Dilated cardiomyopathy (DCM) is defined by left/bi-ventricle dilation in association with the diminished myocardial contractility. The histopathology of DCM usually comes with a minor increase of ventricular wall thickness and modest fibrosis (Ahmad et al., 2005). However, pathological hypertrophy frequently progresses to DCM due to myocyte apoptosis. In certain circumstances, DCM can happen without intermediate hypertrophy stage (Olson and Schneider, 2003). Genetic DCM caused mainly by the disruption of myocyte sarcomere architecture or the cytoskeletal scaffold proteins, which connect the nucleus with the extracellular matrix (ECM) through sarcolemma. Human DCM mutations have been identified in multiple sacomere components, including actin, cardiac troponin T, β-myosin heavy chain, and α-tropomyosin. Mutations in many cytoskeletal protein genes including desmin, δ-sarcoglycan, β-sarcoglycan, dystrophin, also cause human DCM (Ahmad et al.,

2005). One of the major functional consequences from these mutations is the defection of force generation and/or force transmission from sacomere to the extracellular matrix (ECM). Mutations in the phospholamban (PLN) gene, which encodes the reversible inhibitor of the sarcoplasmic reticular Ca2+-ATPase (SERCA2), were found to cause DCM. One mutation of PLN prevented its phosphorylation by PKA and resulted in calcium uptake delay from the cytosol to sarcoplasmic reticulum (SR), implying that precise regulation of calcium cycling is required for normal heart function (Minamisawa et al., 1999; Schmitt et al., 2003). Altered metabolic process can also cause DCM. Mutations in the regulatory SUR2A subunit of KATP channel, which participates in decoding metabolic signaling during stress adaptation, were identified in human DCM (Bienengraeber et al., 2004). Mutations in nuclear envelope protein lamin A/C resulted in human HCM while the underlying pathophysiological mechanisms are unclear (Nikolova et al., 2004).

Vast number of miRNAs and the diverse functions in different biological processes observed from a relative small number of miRNAs studied thus far suggest that miRNAs could play essential roles during heart development and function. However, genetic evidence for miRNAs function in the heart is slowly coming. In this chapter, we will study the global roles of miRNAs in heart development using mouse genetic approaches. Cardiac-specific deletion of Dicer, a ribonuclease III enzyme responsible for miRNAs maturation, was performed using Cre-recombinase under the control of the promoter of α-myosin heavy chain (αMHC), which direct Cre recombinase expression specifically in cardiac muscle.

MATERIALS AND METHODS

Generation of Dicer conditional null mice

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Dicer floxed and Cre double heterozygous mice (MHC$^{Cre/+}$; Dicer$^{flox/+}$) were generated by crossing Dicer$^{flox/flox}$ mice with MHC$^{Cre/+}$ mice. The genotypes of offspring obtained from crossing MHC$^{Cre/+}$; Dicer$^{flox/+}$ mice with Dicer$^{flox/flox}$ mice were determined by PCR using these primers: Cre1: 5'GTT-CGC-AAG-AAC-CTG-ATG-GAC-A 3' Cre2: 5'CTA-GAG-CCT-GTTTTG-CAC-GTT-C 3'. Dicer forward: 5' ATTGTTACCAGCGCTTAGAATTCC, Dicer reverse: 5' GTACGTCTACAATTGTCTATG.

Histological analysis, cell number and size measurements, and TUNEL staining

After euthanasia, animal hearts were dissected and fixed in 4% paraformaldehyde (pH 8.0) overnight. After dehydration through a series of ethanol baths, samples were embedded in paraffin wax according standard laboratory procedures. Sections of 5 mm were stained with haematoxylin and eosin (H&E) for routine histopathological examination with light microscopy. For cell size measurement, cross-section areas (CSA) of cardiomyocytes, which have been stained for laminin to mark the cell surface, were measured. Two independent methods were applied for the cell number determination. First, the cardiomyocyte number was determined by quantifying the number nuclei in the heart, using 5-mm heart sections stained with H & E, and assuming one nucleus per cardiomyocyte. Pairs of comparable fields of left ventricle, right ventricle, intraventricular-septum and ventricular apex of wild type and Dicer knockout hearts were photographed at a 200x magnification. The total number of cardiomyocyte nuclei was obtained by combining nuclear density from at least six different section areas of three independent hearts each from wild type and Dicer knockout hearts. Second, to ensure that the results of above cell counting indeed reflect that of cardiomyocytes, we perform a similar nuclei counting using DAPI staining from ventricle sections which are costained with muscle markers. No significant difference was detected in cardiomyocyte numbers from Dicer knockout heart and controls. Fibrosis was analyzed with Masson Trichrome staining.

Immunohistochemistry and Western blot analysis

For Western blotting analyses were performed using protein extracts from frozen mouse hearts. Primary antibodies include Dicer (Abcam); a-tubulin, Connexin 43, vinculin (Sigma); phospho-histone H3 (Upstate Biotechnology); SRF, HDAC4 (Santa Cruz Biotechnology), Vinculin (gift from Jim Bear lab, University of North Carolina); Desmin (DakoCytomation); Connexin 45, Connexin 40 (Zymed Labratories); antibodies

against cardiac Troponin T and cardiac a-tropomyosin (a-TM) are generous gifts from Dr. Jim Lin (University of Iowa); the MF20 antibody, which recognizes striated muscle-specific MHC, was obtained from the DSHB (University of Iowa, Iowa City, IA). Anti-MHC antibody, which recognizes both a-and b-MHC isoforms, was kindly provided by Dr. J.-P. Jin (Northwestern University). Anti-b-MHC antibody was purchased from Sigma. For immunohistochemistry analysis, hearts were dissected, fixed in the 4% paraformaldehyde (pH8.0) for overnight and then embedded in paraffin before 5 mm sectioning or in Optimal Cutting Temperature compound (OCT) before 10 mm cryosectioning. After rehydration for paraffin sections, slides were heated in the 89°C in retrievagen A solution (BD PharMingen) for 10 min and washed in 0.1% Nonidet P-40 in PBS for 5 min at room temperature. Sections were examined and photographed using regular fluorescent or laser scanning confocal microscopy.

Determination of cardiomyocyte proliferation

Antibodies against phospho-histone H3 (Upstate Biotechnology) and Ki-67 (BD Biosciences) were used to determine the proliferation index at P2 and E18.5 wild-type and Dicer mutant hearts. Cardiac cell proliferation at E13.5 was determined by bromodexyuridine (BrdU) labeling, in which pregnant mice were administrated 100 mg of BrdU (Sigma)/kg of body weight i.p. One hour later, embryos were harvested and hearts were dissected, fixed and embedded in paraffin. Genotypes of E13.5 wild-type and Dicer mutant embryos were determined using genomic DNA isolated from yolk sacs. BrdU incorporation was detected by immunohistochemistry using BrdU antibody (Invitrogen). Cell proliferation were quantified by scoring the number of BrdU-positive nuclei in the muscular apex, IVS and LV region by use of NIH Image J software.

Transthoracic echocardiography

Echocardiography was performed on control and Dicer knockout neonatal mice. The mice were gently taped to a temperature-controlled mouse board (Indus Instruments), and a layer of ultrasonic gel was applied over the mouse body. Using a Vevo 660 ultrasound machine (VisualSonics) equipped with 40MHz probe, measurements were obtained from the M-mode display by an echocardiographer blind to the animal genotypes. The following measurements were obtained in both systole and diastole: interventricular septal thickness (IVS), left ventricular posterior wall thickness (LVPW), left ventricular internal diameter (LVID), heart rate and fractional shortening.

Measurements were done from the long-axis imaging plane using 4 cardiac cycles and averaged. Mean, standard error of the mean and standard deviation were calculated for each genotype, and pairwise statistical comparisons were made using Student's t tests.

Transmission electron microscopy (TEM)

Hearts were immersed after dissection in a fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.15M sodium phosphate buffer (pH 7.4). After fixation, the hearts were cut in half and processed for electron microscopy. Following three rinses with phosphate buffer, the samples were postfixed for 1 h in 1% osmium tetroxide/1.25% potassium ferrocyanide in sodium phosphate buffer. The tissues were rinsed in deionized water and dehydrated through increasing concentrations of ethanol (30%, 50%, 75%, 100%, and 100%, 10 min each) and put through two changes of propylene oxide (15 min each). Tissue samples were infiltrated with a 1:1 mixture of propylene oxide: Polybed 812 epoxy resin (1A:2B formulation, Polysciences) for 3 h followed by an overnight infiltration in 100% resin. The tissue pieces were embedded in fresh epoxy resin and polymerized for 24 h at 60°C. The blocks were trimmed, and 1-micrometer sections of the tissue were mounted on glass slides and stained with 1% toluidine blue O in 1% sodium borate. Representative areas were selected by light microscopy and 70 nm ultrathin sections were cut using a diamond knife. Ultrathin sections were collected on 200 mesh copper grids and stained with 4% aqueous uranyl acetate for 15 min, followed by Reynolds' lead citrate for 7 min. Sections were observed using a LEO EM910 transmission electron microscope at 80kV (LEO Electron Microscopy) and photographed using a Gatan Orius SC 1000 CCD Digital Camera (Gatan).

MicroRNA microarray, Northern blot analyses and RT-PCR

MicroRNA microarray and Northern blot analyses are essentially as described. Total RNAs isolated using Trizol Reagent (Invitrogen) from control or Dicer mutant hearts were used. Total RNAs isolated from liver was also included in the miRNA microarray experiments to serve as a control. RT-PCR was performed as described.

Human patient's samples

Under a UNC-CH IRB-approved protocol, left ventricular tissue was taken from patients with end-stage heart failure (failure hearts) requiring mechanical circulatory support as a bridge to transplantation. In brief, left ventricular assist devices were inserted after excising a circular core of LV apical tissue that was subsequently snap-frozen in liquid nitrogen. After a period of clinical recovery (range 2-10 months) and when a suitable donor heart was available, the patient's heart was removed at the time of transplantation. LV tissue was subsequently dissected and snap-frozen. We used hearts samples from patients with aortic stenosis who have not developed heart failure (non-failure) to serve as controls.

RESULTS

1.Cardiac-specific deletion of Dicer.

To understand the global involvement of miRNAs in heart development and function, we applied the Cre-loxP system to disrupt the Dicer tissue-specifically in the heart. Mice homozygous for the floxed Dicer alleles (Dicerflox/flox), which are viable and fertile without
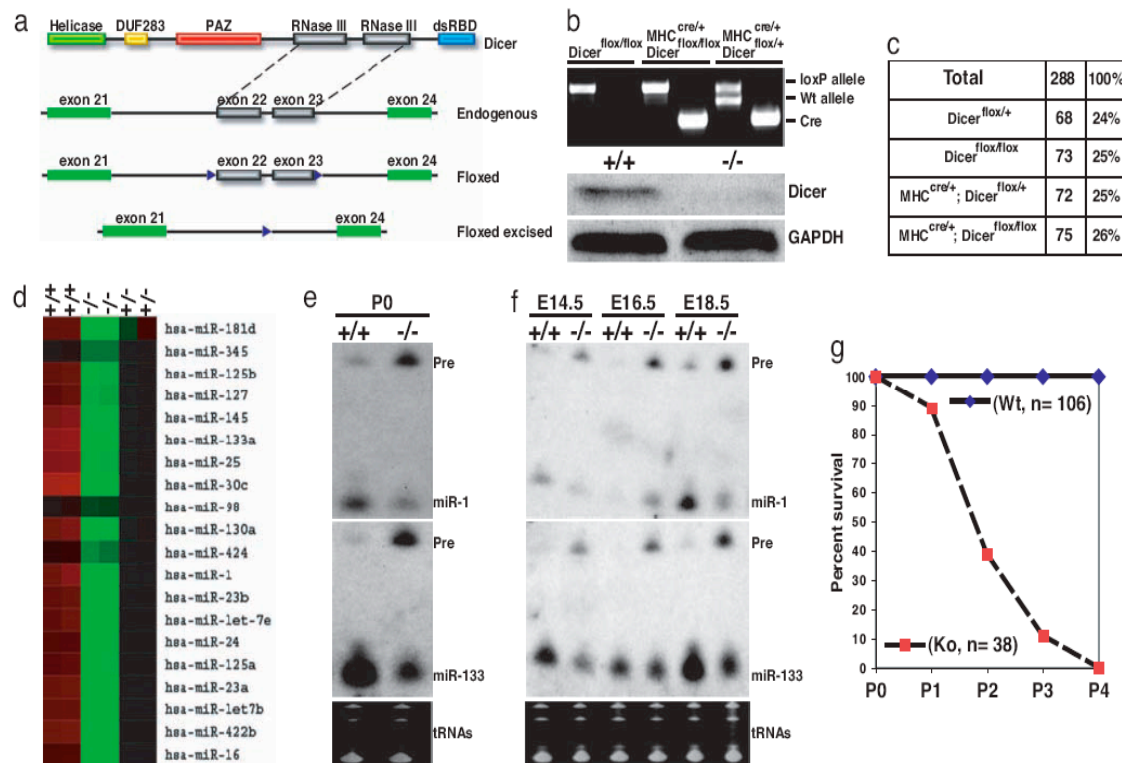

Figure 14 Generation of cardiac-specific Dicer knockout mice.

(*a*) The gene targeting strategy for cardiac-specific deletion of Dicer. (*b*) PCR genotyping analysis of P0 mice. Wild-type loxP alleles and Cre products are indicated (*Upper*). Western blot analysis of Dicer protein from wild-type (+/+) and Dicer mutant (–/–) hearts. GAPDH serves as a loading control (*Lower*). (c) Genotyping results of P0 offspring from MHCCre/+; Dicerflox/+, mice and Dicerflox/flox mice intercrossing. (d) miRNA microarray analysis of miRNA expression in P0 wild-type (+/+), Dicer heterozygous (+/–), and homozygous (–/–) mutant hearts. Red denotes high expression and green denotes low expression. (e and f) miRNA Northern blot analysis to detect the expression of precursor (Pre-) and mature miR-1 and -133 in P0 or embryonic wild-type (+/+) and Dicer mutant (–/–) hearts. tRNAs were used as a loading control. (g) Kaplan–Meier survival curves for wild-type (Wt) and Dicer knockout (KO) mice. All Dicer mutant mice die before P4.

any apparent abnormalities, were crossed to a transgenic mouse line in which the Cre

recombinase is expressed under the control of alpha myosin heavy chain (-MHC) promoter

(MHC$^{Cre/+}$), which directs cardiac-specific expression (Figure 14a). Double-heterozygous

progenies (MHC$^{Cre/+}$; Dicer$^{flox/+}$) were identified and mated with Dicer$^{flox/flox}$ to obtain

cardiac-specific Dicer mutant animals (Figure 14b). Genotyping of offspring from the latter

mating did not identify any Dicer mutant mice (MHC$^{Cre/+}$; Dicer$^{flox/flox}$) at weaning age (data

not shown), indicating that cardiac-specific deletion of Dicer caused premature lethality. We

then genotyped postnatal day 0 (P0) mice from the above intercrossing and found all possible

genotypes in expected Mendelian frequency (Figure 14c), suggesting that cardiac-specific

Dicer deletion did not cause embryonic lethality. We confirmed, using Western blot analysis,

that Dicer protein was indeed removed from the hearts of knockout mice (Figure 14b). To

confirm that cardiac-specific disruption of Dicer indeed results in the loss of mature miRNAs

in the hearts, we performed miRNA microarray analysis using total RNA samples isolated

from P0 wild-type Dicer heterozygous and homozygous hearts. There is a dramatic reduction

in mature miRNA expression in Dicer mutant hearts compared with wild-type control hearts.

The very low level of mature miRNA expression detected in the Dicer knockout hearts is

likely due to the accumulation of miRNAs processed before the functional Dicer was

effectively deleted. A substantial reduction of mature miRNA expression was also observed

in heterozygous hearts (Figure 14d). In contrast, no difference in mature miRNA expression was observed in the livers of wild-type and Dicer mutant mice (data not shown), confirming cardiac-specific depletion of mature miRNAs. Northern blot analysis of Dicer mutant hearts further demonstrated a marked decrease in the expression of mature forms of miR-1 and -133, two of the most abundantly expressed muscle miRNAs (Figure 14e, f). Notably, miRNA precursors accumulated in the Dicer mutant hearts, consistent with the view that Dicer is required for processing of mature miRNAs from their precursors. We have examined miRNA expression in the embryonic hearts and found decreased expression of mature miRNAs in Dicer mutant hearts as early as embryonic day 14.5 (E14.5) (Figure 14f).

2. Phenotypic characterization of cardiac-specific Dicer knockout mice.

Newborn Dicer mutant mice are externally indistinguishable from their control littermates. However, all mutant mice die within 4 days after birth (Figure 14g). Preceding death, mutant animals become very fragile and exhibit decreased spontaneous activity. The hearts of mutant mice are substantially larger than that of their littermates (Figure 15a), even though there is no difference in body size. Histological analyses of mutant hearts indicate dramatic left ventricle and left atria dilation (Figure 15b). We always observe accumulation of blood clots and/or thrombin in the left ventricle and left atrium of mutant hearts (Figure 15b) indicating that Dicer mutant hearts do not have sufficient force to pump blood out to the left ventricle and suffer from impaired cardiac function. The myocardium is less organized, and the integrity of cardiomyocytes is impaired in Dicer mutant hearts (Figure 15c). However, no substantial increase in fibrosis was observed in the mutant heart (data not shown). Consistent with the observed cardiomyocyte cyto-architectural defect, a profound decrease in the

expression of contractile proteins was observed in Dicer mutant ventricles (Figure 15d and

e). Isolated neonatal cardiomyocytes from Dicer mutant hearts also show disarrayed
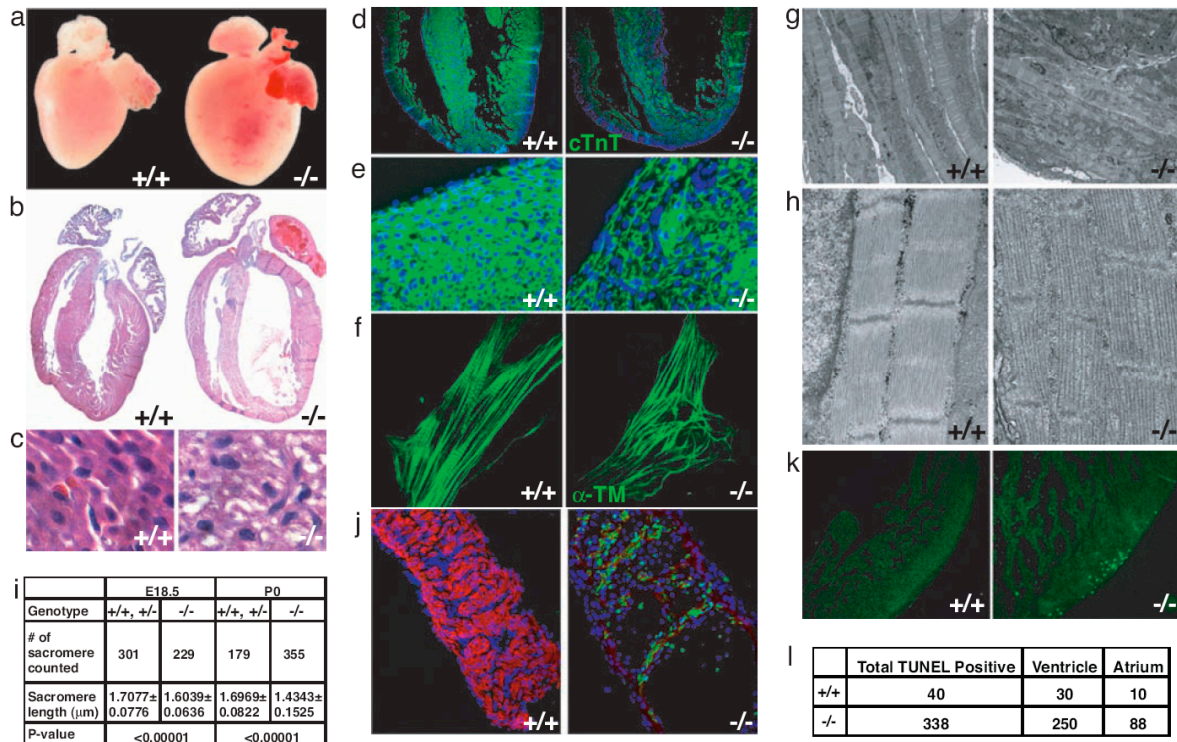
myofibrils (Figure 15f)

	E18.5		P0	
Genotype	+/+, +/-	-/-	+/+, +/-	-/-
# of sacromere counted	301	229	179	355
Sacromere length (µm)	1.7077± 0.0776	1.6039± 0.0636	1.6969± 0.0822	1.4343± 0.1525
P-value	<0.00001		<0.00001	

	Total TUNEL Positive	Ventricle	Atrium
+/+	40	30	10
-/-	338	250	88

Figure 15 Cardiac-specific Dicer deletion leads to DCM.
(*a*) Gross morphology of P0 wild-type (+/+) and Dicer mutant (–/–) hearts. (*b*) H&E staining of sagittal sections of P0 wild-type (+/+) and Dicer mutant (–/–) hearts. (*c*) Higher-magnification images from H&E stained left ventricular myocardium of P0 wild-type (+/+) and Dicer mutant (–/–) hearts. (*d*) Immunohistology of P0 wild-type (+/+) and Dicer mutant (–/–) hearts for cTnT (green). DAPI stains nuclei. (*e*) Higher-magnification images from cTnT immunohistology-stained left ventricular myocardium of P0 wild-type (+/+) and Dicer mutant (–/–) hearts. (*f*) Confocal microscopic images of cultured neonatal cardiomyocytes from wild-type (+/+) and Dicer mutant (–/–) hearts stained for cardiac -TM. (*g*) Electron microscopic analysis of ventricular myocardium of wild-type (+/+) and Dicer mutant (–/–) hearts. There is substantially less organized myofibril in Dicer mutant hearts. (*h*) Higher magnification of electron micrographies of wild-type (+/+) and Dicer mutant (–/–) myocardium to show disarrayed sarcomere in mutant hearts. (*i*) Quantitative measurement of sarcomere length in wild-type (+/+) and Dicer mutant (–/–) hearts. (*j*) TUNEL staining (green) detected increased apoptosis in the left atrium and ventricle of P2 Dicer mutant (–/–) heart. Antibody against cTnT was used to label cardiomyocytes (red). DAPI stains nuclei (blue). (*k*) Increased apoptosis in the left ventricular wall of P2 Dicer mutant (–/–) hearts as revealed by TUNEL staining. (*l*) Quantitative measurement of TUNEL positive cells in P2 wild-type (+/+) and Dicer mutant (–/–) hearts.

Ultrastructural analyses using transmission electronic microscopy show striking alterations in the myocardial structure of Dicer mutant hearts. Foremost, there are substantially less sarcomeres in the mutant ventricles (Figure 15g). Second, the sarcomeres that are present are dramatically disarrayed and substantially shorter in the mutant hearts (Figure 15h). These observations were confirmed by quantitative measurement of sarcomere length of Dicer mutant cardiomyocytes from E18.5 and P0 Dicer mutant hearts versus the controls (Figure 15i). However, we did not detect a significant difference in the structure of intercalated discs between Dicer mutant and control hearts (data not shown). These defects in the cardiac muscle contractile apparatus likely account for the abnormalities in heart morphogenesis and the dysfunction in cardiac contraction found in the Dicer mutant hearts (see below). There is no significant difference in the proliferation of cardiomyocytes of control compared with Dicer mutant hearts, as determined by phosphorylated histone H3 and Ki-67 staining, and bromodeoxyuridine (BrdU) labeling. A substantial increase in apoptosis, detected by TUNEL staining, was found in the left atrium of P2 mutant hearts (Figure 15j and l). In addition, restricted areas of the ventricular apex and left ventricle wall of P2 Dicer mutant hearts also show an increase in apoptosis (Figure 15k and l). However, we did not observe increased apoptosis in other regions of the Dicer mutant hearts examined. Neither did we detect any significant increase in apoptosis in the Dicer mutant hearts before P0, before obvious signs of cardiac dysfunction (data not shown). We measured the cell number and size of cardiomyocytes of Dicer mutant heart. Overall, no substantial change in the cell size or cell number in P0 Dicer knockout hearts was observed.

3. Dicer knockout mice exhibit features of DCM and lead to heart failure.

Cardiac function of Dicer mutant mice was analyzed by noninvasive transthoracic echocardiography and compared with littermate controls (Figure 16a and Table 1). Dicer mutant mice exhibit severe left ventricle dilation coupled with a dramatic decrease in fractional shortening. Notably, the left ventricle posterior wall thickness at end diastole was significantly increased, without significant change at end systole, indicating a significant loss of ventricle force generation and cardiac malfunction in Dicer mutant hearts. Interestingly, Dicer mutant mice display remarkable decrease in heart rate (Table 1).

Table 1. Dicer mutant mice are defective in cardiac function

Genotypes	Heart rate, bpm	IVSd, mm	IVSs, mm	LVPWd, mm	LVPWs, mm	LVIDd, mm	LVIDs, mm	FS, %
+/+ (n = 10)	555.9 ± 42.33	0.348 ± 0.06	0.604 ± 0.12	0.355 ± 0.07	0.624 ± 0.13	1.248 ± 0.13	0.696 ± 0.15	44.63 ± 8.86
−/− (n = 7)	278.6 ± 64.38	0.40 ± 0.12	0.57 ± 0.15	0.571 ± 0.10	0.713 ± 0.10	1.517 ± 0.25	1.287 ± 0.16	19.77 ± 5.31
+/− (n = 8)	537.4 ± 48.28	0.335 ± 0.05	0.573 ± 0.09	0.295 ± 0.04	0.567 ± 0.17	1.306 ± 0.16	0.756 ± 0.16	42.34 ± 8.52
P value	<0.0001	0.296	0.629	0.0001	0.170	0.01	<0.0001	<0.0001

Echocardiographic analyses of P0 wild-type (+/+), Dicer heterozygous (+/-), and homozygous (-/-) mutant mice. Values were quantified from five independent M-mode measurements. IVSd, interventricular septal thickness at end diastole; IVSs, interventricular septal thickness at end systole; LVPWd, left ventricular posterior wall thickness at end diastole; LVPWs, left ventricular posterior wall thickness at end systole; LVIDd, left ventricular internal diameter at end diastole; LVIDs, left ventricular internal diameter at end systole; and FS, fractional shortening.

Table 1 Echocardiographic analyses of P0 wild-type, Dicer heterozygous, and homozygous mutant mice.
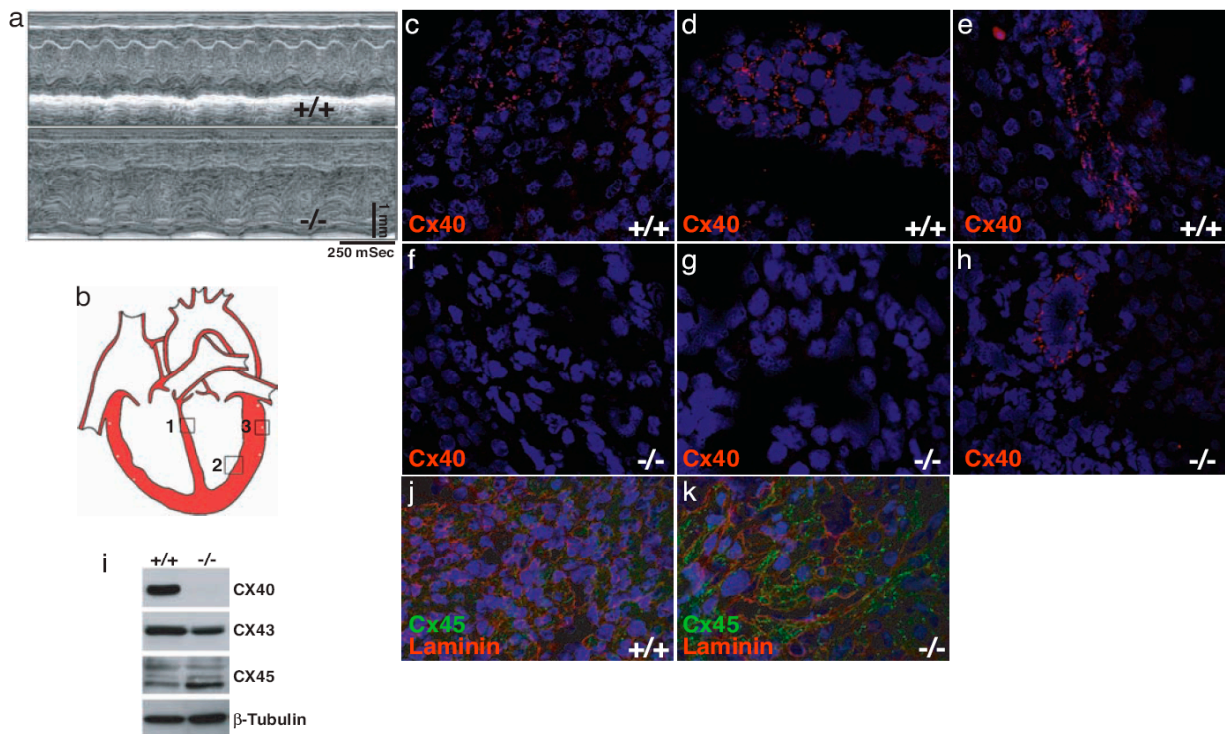
Figure 16 Impaired cardiac function in Dicer mutant hearts.
(*a*) M-mode echocardiograms of P0 wild-type (+/+) and Dicer mutant (–/–) mice. (*b*) Diagram to show the areas of ventricles examined in confocal immunohistochemistry images: area 1 for *c* and *f*; area 2 for *d* and *g*; area 3 for *e* and *h*. (*c–h*) Confocal microscopic images for Cx40 protein expression (red) in the ventricles of P0 wild-type (+/+) and Dicer mutant (–/–) mice. DAPI stains nuclei. (*i*) Western blot analyses of connexin protein expression in P0 wild-type (+/+) and Dicer mutant (–/–) mice. β-Tubulin serves as a loading control. (*j* and *k*) Confocal microscopic images of Cx45 in P0 wild-type (+/+) and Dicer mutant (–/–) hearts. Laminin marks the cell surface and DAPI stains nuclei.

Although wild-type and heterozygous hearts contract at 550 beats per minute (bpm), the

mutant hearts only beat about half of the rate (278 ± 64 bpm), suggesting that Dicer mutant

mice may also suffer from conduction defects (Table 1). Together, these data demonstrate

that cardiac-restricted Dicer knockout mice exhibit several features of DCM and heart

failure. Many DCMs are also associated with defects in cardiac conduction. We examined

the expression of connexin (Cx) 40 (Cx40), Cx43, and Cx45, three major gap junction

proteins expressed in the heart and are responsible for the proper function of the cardiac

conduction system. The expression of Cx40 was dramatically decreased in the cardiac

conduction system of the Dicer mutant hearts (Figure 16b-d, f and g). However, the

expression of Cx40 in the coronary vessels was not disturbed in Dicer knockout hearts

(Figure 16b, e, and h), which is not surprising because Dicer was specifically removed in

cardiomyocytes, not coronary vessels. Western blot analyses quantitatively demonstrated a

significant decrease of Cx40 protein in Dicer mutant hearts (Figure 16i). Remarkably, the

expression of Cx45 was significantly increased in Dicer mutant hearts (Figure 16i-k),

whereas there was no significant change in the expression of Cx43 (Figure 16i and data not

shown). The change of connexin protein levels in Dicer mutant hearts could be detected as

early as E16.5 and is evident at E18.5.

4. Dys-regulation of cardiac contractile protein expression in Dicer mutant hearts.

Our observations suggest that Dicer mutant hearts suffer from a deficiency in contractile force generation and/or transmission, as well as a likely defect in conduction. Failure to provide sufficient contraction is the hallmark of human heart failure and is often associated with defects in the assembly of the contractile apparatus, the sarcomere, and/or the expression of contractile proteins. Therefore, we examined the expression of cardiac contractile proteins in control and Dicer mutant hearts. Indeed, decreased expression of cardiac troponin T (cTnT), an actin-binding protein linked to DCM in human patients, was evident in the Dicer mutant hearts (Figure 15d and e), which was further confirmed by Western blot analyses (Figure 17g).
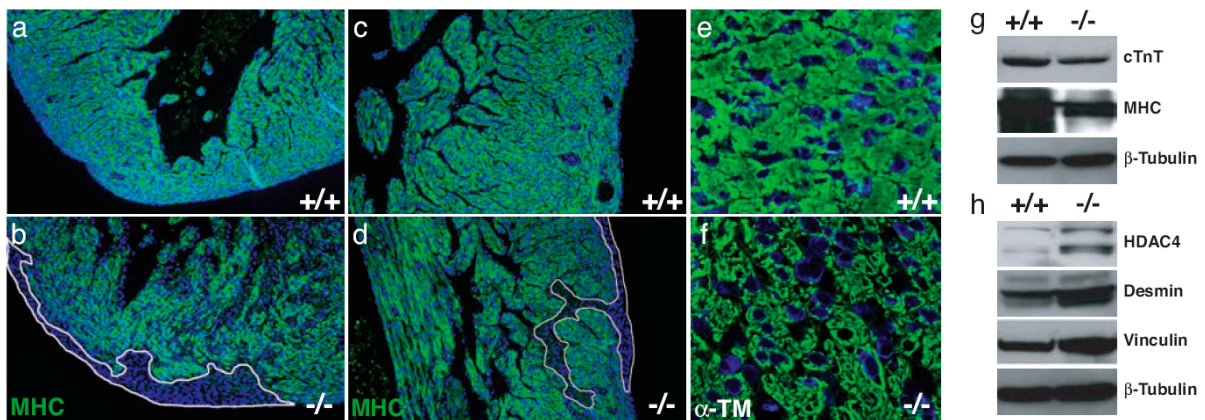
Figure 17 Expression of cardiac contractile proteins in Dicer mutant hearts.
(*a–d*) Confocal microscopic images of P0 wild-type (+/+) and Dicer mutant (–/–) hearts stained with MHC in apexes (*a* and *b*) or left ventricle (*c* and *d*). Note patches of myocardium that lack expression of MHC in Dicer mutant (–/–) hearts. DAPI stains nuclei. (*e* and *f*) Confocal microscopic images of P0 wild-type (+/+) and Dicer mutant (–/–) hearts stained with antibody against α-TM. Note dramatic decrease in the expression of -TM in Dicer mutant (–/–) hearts. DAPI stains nuclei. (*g* and *h*) Western blot analyses of indicated proteins using protein extracts from P0 wild-type (+/+) and Dicer mutant (–/–) hearts. β-tubulin serves as a loading control.

Human DCM is associated with mutations in genes important for myocyte contraction and cell integrity, including MHC and -tropomyosin (-TM). We examine the expression of those contractile proteins and desmin, an intermediate filament found near the

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sarcomeric Z line, and vinculin, a cytoskeletal protein involved in cell adhesion, in Dicer

mutant hearts (Figure 17). Mutations in both desmin and vinculin proteins have also been

linked to DCM. Strikingly, the expression of MHC proteins was altered in Dicer mutant

hearts in a pattern where patches of ventricular myocytes lost the expression of those proteins

(Figure 17a–d). Decreased MHC protein level in Dicer mutant hearts was further confirmed

by Western blot analysis (Figure 17g). Interestingly, whereas the expression of total MHC

proteins decreased in Dicer mutant hearts, we noticed that the expression of β-MHC protein

appears unchanged (data not shown). We also examined the expression of MHC in

embryonic Dicer mutant hearts and found that its protein level decreased as early as E18.5.

Confocal microscopy images of ventricular myocytes demonstrate a decrease in α-TM

protein level in Dicer mutant hearts (Figure 17e and f). However, we did not detect any

decrease in the protein level from E16.5 or E18.5 Dicer mutant hearts. Interestingly, the

expression of vinculin and desmin was increased in Dicer mutant hearts (Figure 17h). In

addition, we examined protein levels of HDAC4 and SRF, two proteins previously identified

as regulatory targets for miR-1 and -133, respectively, in Dicer mutant hearts. Although the

expression of HDAC4 was increased in the Dicer mutant hearts, the protein expression level

of SRF was unchanged (Figure 17h and data not shown). Notably, the expression of those

proteins was unaltered in E16.5 or E18.5 Dicer mutant hearts.

5. Decreased Dicer expression in human patients with end-stage DCM and heart failure.
Having demonstrated that Dicer is essential for normal heart function in animals, by which

loss of Dicer led to acute DCM and heart failure, we sought to examine the expression of

Dicer protein in human patients with DCM and/or heart failure. Indeed, Dicer protein level is

very low in human failing hearts (Figure 18, lanes 1–4). Remarkably, the expression of Dicer

protein is significantly elevated in recovering hearts after installation of a left ventricular

assist device (LVAD) in those patients (Figure 18, lanes 5–8). Clinically, LVADs are used in

patients with end-stage failing hearts to mechanically support medically refractory hearts.

Recent evidence indicates that reverse remodeling can occur even in the most advanced

DCM after the application of LVAD (Dipla et al., 1998). In contrast, we found that Dicer was

expressed at a modest level in nonfailure patient hearts (Figure 18, lanes 9–11). These data

suggest that Dicer and therefore miRNAs are likely involved the progression and/or

regression of DCM and heart failure in human patients.
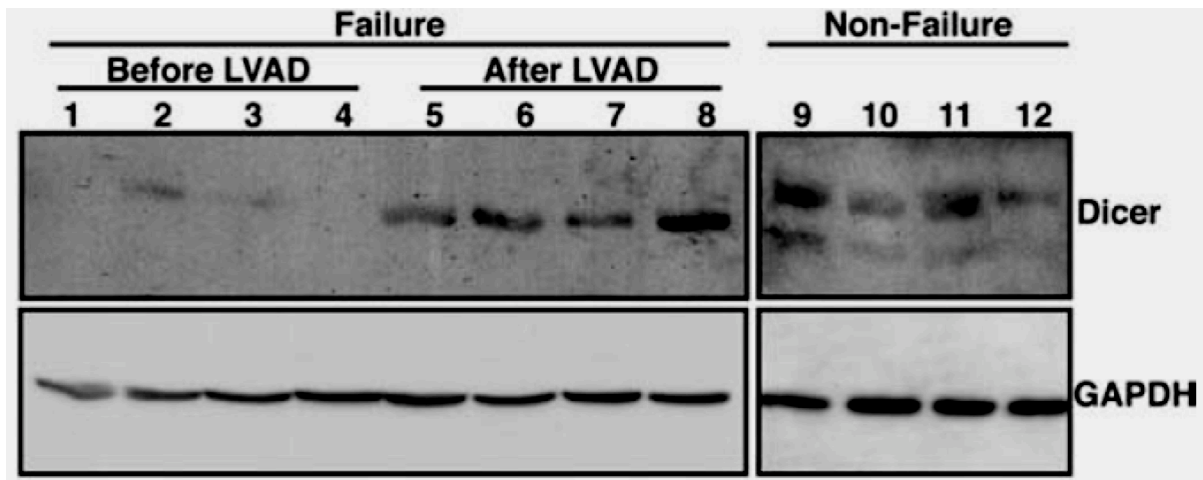
Figure 18 Dicer protein expression in patients with failure or nonfailure hearts.
Western blot analyses for Dicer from total protein extracts of ventricles of human subjects with end-stage heart failure before (lanes 1–4) or after (lanes 5–8) the application of LVAD, or nonfailure heart samples (lanes 9–11). GAPDH serves as a loading control.

Together, we assessed the global role of miRNAs in the heart, using a cardiac-

specific conditional Dicer knockout mouse model. We found that Dicer and miRNAs play a

critical role in normal cardiac function and animal survival. Dicer knockout leads to a severe

decrease in cardiac contractility and loss of contractile proteins in the hearts. Most

importantly, we report that heart failure patients lose normal Dicer protein expression.

CHAPTER 4

MICRORNA-1 AND -206 FUNCTION IN SATELLITE CELLS

INTRODUCTION

Muscle adult stem cells, named satellite cells, are responsible for muscle postnatal growth and muscle regeneration after injury. Satellite cells are localized along the surface of muscle fibers under the basal lamina(Schultz et al., 1978). Satellite cells are mitotically quiescent under normal conditions but actively proliferate and quickly differentiate into terminally differentiated multinuclei myotubes in response to stimuli or injury (Kuang et al., 2008; Morgan and Partridge, 2003; Sloper and Partridge, 1980). Satellite cell self-renewal, proliferation and differentiation are precisely regulated, and diminished satellite cell self-renewal or proliferation will adversely affect skeletal muscle postnatal growth and muscle regeneration after injury. Premature differentiation of satellite cells results in a reduced stem cell source and impairs skeletal muscle development and function. Therefore, perturbation of satellite cell proliferation and differentiation often results in a variety of muscle diseases including synovial sarcomas and muscular dystrophy (Haldar et al., 2007; Jejurikar and Kuzon, 2003; Luz et al., 2002). Although numerous efforts have been taken to study satellite cells (Collins et al., 2005; Montarras et al., 2005), it remains largely unknown how the balance between satellite cell proliferation and differentiation is controlled.

Satellite cells are characterized by the expression of paired/ homeodomain transcriptional factor Pax7 (Buckingham, 2007; Kuang et al., 2008). Pax7 ensures satellite cell survival, promotes satellite cell proliferation and prevents precocious differentiation of satellite cells (Kuang et al., 2006; Oustanina et al., 2004; Relaix et al., 2006; Zammit et al., 2006). Genetic studies in mouse have shown that the satellite cells lacking Pax7 and its close homolog, Pax3, die or switch to the other cell fates, indicating Pax3 and Pax7 are required for satellite cell fate specification (Relaix et al., 2005). The satellite cells from Myf5[GFP/GFP] mutant mice fail to differentiate into myofibers after infection with viruses expressing dominant-negative forms of Pax3 and Pax7, suggesting Pax3 and Pax7 activate myogenic program (Relaix et al., 2006). Pax3 and Pax7 have been shown to function upstream of MyoD to activate skeletal muscle gene expression and initiate myogenesis program (Relaix et al., 2006). However, sustained expression of Pax7 in satellite cells delayed the onset of myogenesis. Elevated Pax7 expression in primary myoblasts inhibits MyoD expression, and prevents myogenin induction and muscle terminal differentiation (McFarlane et al., 2007; Olguin and Olwin, 2004). Those studies suggest that Pax7 plays dual roles in satellite cell fate specification and myogenic progenitor cell differentiation. Therefore, the clearance of Pax7 is crucial for satellite cell transition from proliferation/self-renewal to differentiation.

MicroRNAs (miRNAs) are a class of ~ 22 nt non-coding RNAs that regulate gene expression at post-transcriptional level. Over 600 human miRNAs have been identified and most (if not all) protein-encoding genes are predicted potential regulatory targets of miRNAs (Ambros, 2004; Bartel, 2004; Farh et al., 2005; John et al., 2004; Lewis et al., 2003). The discovery and subsequent analysis of miRNAs has revealed another layer of complex mechanisms appearing to 'fine-tune' protein dosages of many key regulators. Recent studies

indicate that miRNAs participate many essential biological processes including cell proliferation, differentiation, apoptosis as well as important disease such as cancer and cardiovascular disease (Brennecke et al., 2003; Chen et al., 2006; He et al., 2005; van Rooij et al., 2006). The involvement of miRNAs in muscle biology has recently been reported. It has been shown that miRNAs regulate the expression of transcription factors and signaling mediators important for cardiac and skeletal muscle development and function (Callis and Wang, 2008; van Rooij et al., 2008). Aberrant miRNA expression has been observed during muscle diseases, including cardiac and skeletal muscle hypertrophy, heart failure as well as in muscular dystrophy (Eisenberg et al., 2007; Tatsuguchi et al., 2007; Thum et al., 2007). We have previously shown that the expression of muscle-specific miR-1 and miR-133 is induced during skeletal muscle differentiation. We further demonstrated that miR-1 and miR-133 play central regulatory roles in myoblast proliferation and differentiation (Chen et al., 2006). Interestingly, miR-1 and miR-133 are also indicated as important regulators of cardiomyocyte differentiation and heart development (Ivey et al., 2008; Zhao et al., 2007).

 As stem cells differentiate, they turn on lineage-specific genes while down-regulate stem cell maintenance genes in response to appropriate cues (Jaenisch and Young, 2008; Rossant, 2008). Although these maintenance genes are regulated mainly at transcriptional level, remaining transcripts that were highly expressed in the previous stage need to be silenced effectively for gene program transition. In that sense, miRNAs are well suitable for clearing these residual messages through simultaneously repression of many target genes. Genetic studies on the components in miRNA biogenesis pathway indicate that Dicer and miRNAs are essential for embryonic stem (ES) cell survival and differentiation (Murchison et al., 2005; Wang et al., 2007). Interestingly, it was recently reported that a skin microRNA (miR-

203) promotes the differentiation of skin progenitor cells by restricting cell proliferation and inducing cell-cycle exit. miR-203 achieved this function by repressing p63, a key factor for skin stem cell fate maintenance (Senoo et al., 2007; Yang et al., 1999; Yi et al., 2008). Interestingly, muscle-specific miR-1 and miR-133 appear to play a role in cardiac differentiation from embryonic stem cells (Ivey et al., 2008). Together, those studies indicate that miRNAs could be previously unrecognized regulators for stem cell fate maintenance and/or muscle progenitor cell proliferation and differentiation processes.

In this sectionn, we study microRNAs (miRNAs) function in skeletal muscle satellite cells and we find that miR-1 and miR-206 are highly induced during satellite cell differentiation. We show that miR-1 and miR-206 promote satellite cell differentiation and restrict their proliferative potential. We further identify Pax7 as one of the direct regulatory targets of miR-1 and miR-206. *In vivo* knockdown of endogenous miR-1 and miR-206 in neonatal mouse skeletal muscle substantially enhances satellite cell proliferation and increases Pax7 protein level. In contrast, loss of miR-1 and miR-206 repression leads to sustained Pax7 expression which results inhibition of myogenic precursor cell differentiation. Together, our studies suggest that miR-1 and miR-206 in satellite cells participate in a regulatory circuit that allows rapid gene program transitions from proliferation to differentiation by repressing the expression of critical stem cell maintenance genes.

MATERIAL AND METHODS

Antagomir synthesis and injection

Antogomir-1 and mm-antogomir-1, antogomir-206 and mm-antogomir-206 were designed and synthesized (Dharmacon) as described (Krutzfeldt et al., 2005). The antagomir-1 sequence was 5'-

*mA*mAmUmAmCmAmUmAmCmUmUmCmUmUmUmAmCmAmU*mU*mC*mC*mAChl-3' and mm-antogomir-1 sequence was

*mA*mAmUmcmCmAmUmgmCmUmcmCmUmUmUmAmCmAmg*mU*mC*mC*mAChl-3'. The antogomir-206 sequence was

*mA*mCmCmAmCmAmCmAmCmUmUmCmCmUmUmAmCmAmU*mU*mC*mC*mAChl-3' and mm-antogomir-206 sequence was

*mA*mCmCmCmcmCmAmCmgmCmUmcmCmCmUmUmAmCmAmg*mU*mC*mC*mAChl-3'. Low-case letters represent the mismatched sequence, "m" represents 2'-O-methyl modified nucleotide and "*" indicates a phosphorothioate linkage and "Chl" indicates cholesterol linked through a hydroxyprolinol linkage. Introperitoneal injection (IP) was performed on postanatal day 3 (P3) C57BL/6J mice. RNAs were administrated twice at dose of 80 mg per kg body weight in 0.01 ml per injection for every other day. Measurements of miRNA levels in tissue were performed 24h after the last injection.

Mice, Myoblast Cultures, Satellite Cell and Single-Fiber Isolations

Mice carrying the Dicer floxed allele were described previously (Murchison et al., 2005). Satellite cell-derived myoblasts were isolated from the skeletal muscle of hindlimb of adult mice as described previously (Rando and Blau, 1994). Single fibers and associated satellite cells were prepared from extensor digitorum longus muscles as described previously (Rosenblatt et al., 1995; Shinin et al., 2006). The identity of satellite cells was assessed using Pax7 antibody. Primary myogenic cells were plated on collagen coated dish in Ham's F-10 medium (Invitrogen) containing 20% FBS (Sigma), 1% (Pen/Strep) and fed with 5.0 ng/mL basic fibroblast growth factor (Gibco #13256-029). Satellite cell differentiation was induced by the depletion of bFGF as described previously (Frock et al., 2006). The C2C12 cell culture and differentiation follow previous described procedure (Chen et al., 2006).

Generation of Adenoviral Vectors, Retroviral Vectors and Cell Infection

Ad-Cre, Ad-LacZ and Ad-GFP adenoviruses were purchased from UNC vector core. The Ad-miR-1, Ad-miR-206 or Ad-miR-1+206 vectors were generated using standard molecular biology techniques (supplementary table 2). High-titer viruses were prepared as described previously (He et al., 1998) and purified following

ViraBind^{TM} Adenovirus Purification Kit (Cell Biolabs, INC.). Satellite cell-derived myoblasts were cultured for 2 days and then infected with adenoviruses at 37^0C at a multiplicity of infection of 20-80 for 12h before the replacement with new medium. The cells were cultured for 2 -3 days before immunofluorescence and flow cytometry, or were induced for differentiation in DMEM medium with 2% horse serum for the duration of the time indicated in the figure legends before immunofluorescence.

To generate retroviral miR-1, -206 and miR-1+206, the Murine Stem Cell Virus vector (MaRX^{TM}IVfPuro) was modified to carry splicing donor and splicing acceptor sequence where miRNA precursors were inserted. The retroviral vectors were generated using standard molecular biology techniques. The 293 LE cells were transfected with 6ug retroviral vector , 3ug env. and 3ug Gag-pol. plasmids using Fugene. Viral supernatants from 293T cells were filtered through 0.2 um syringe, added to cultured muscle cells for infection as described previously (Springer and Blau, 1997). Briefly, the exponentially growing muscle cells were immersed with viral supernatant to a final concentration of 20% FBS, 8ug/ml polybrene, and 5.0 ng/ml bFGF, returned to CO2 incubator for 15 minutes, and then centrifuged the dish at 32^0C for 30 minutes at 1,100 x g. After the centrifuge, the cells were replaced with fresh growth medium and cultured for overnight before selection using puromycin at 10ug/ml concentration.

MicroRNA Microarray, Northern blot and RT-PCR

MicroRNA microarray, Northern blot and RT-PCR were performed as described previously.

Proliferation and Differentiation Assay, Immunohistochemistry and Western blot Analysis

To measure cell proliferation, satellite cell -derived myoblasts infected with adenoviruses were maintained on GM for 48h and then pulsed with 10 uM BrdU (Sigma) for 1.5 h, fixed for immunostaining using anti-BrdU diluted at 1:1000 (A21300, Invitrogen). To examine the expression level of endogenous Pax7 in satellite cells after overexpression of miR-1 and -206, retroviral miR-1 and -206 infected satellite cells were cultured under puromycin (10ug/ml) selection for 4 days before immunostaining or western blot using Pax7 antibody diluted at 1:100 dilution (DSHB, University of Iowa). To examine myogenic differentiation of satellite cells after removal of bFGF, retroviral miR-1 and -206 infected muscle cells at different time points as indicated by figure legend were fixed for immunostaining using MF-20 antibody diluted at 1:20 (DSHB, University of Iowa). To

examine the effect of Pax7 expression on C2C12 cell differentiation, C2C12 myoblasts transfected with control, Pax7, Pax7-UTR or Pax7-UTR-M constructs were cultured for 12h under growth condition, and then switched to differentiation condition for 36h before immunostaining or western blot analysis. Antibodies used were as follows: MF20 (DSHB, University of Iowa), 1:20 dilution in immunostaining and 1:100 dilution in western blot; Myogenin (Santa Cruz Biotechnology), 1:200 dilution; Flag (F7425, Sigma), rabbit polyclonal at 1:400 dilution; PCNA (610664, BD Biosciences), 1:200 dilution.

TUNEL Assay and Flow Cytometry

Ad-LacZ or Ad-Cre infected cells were cultured under growth condition for 72h and then switched to differentiation condition for 5h before TUNEL assay. TUNEL assays were performed using ApopTaq$^{®}$ Fluorescein in situ Apoptosis Detection Kit (S7110, Chemicon) according to the manufacturer's instructions. For flow cytometry analysis, Ad-LacZ or Ad-Cre infected Dicer$^{flox/flox}$ myoblasts were cultured under growth condition for 48h, and then switch to differentiation medium for 24h before flow cytometry analysis. Cell death was quantified using FITC-Annexin V and PI staining of adenovirus-infected cells (Molecular probe) according the manufacture's instructions. Each measurement was from triplicate plates.

RESULTS

miRNAs are Required for Proper Differentiation of Satellite Cell

To study the potential involvement of miRNAs in muscle stem cell differentiation, we first established satellite cell *in vitro* differentiation system. Mouse skeletal muscle satellite cells were isolated from single myofibers of hind leg muscle of young adult mice (See material and methods for details).
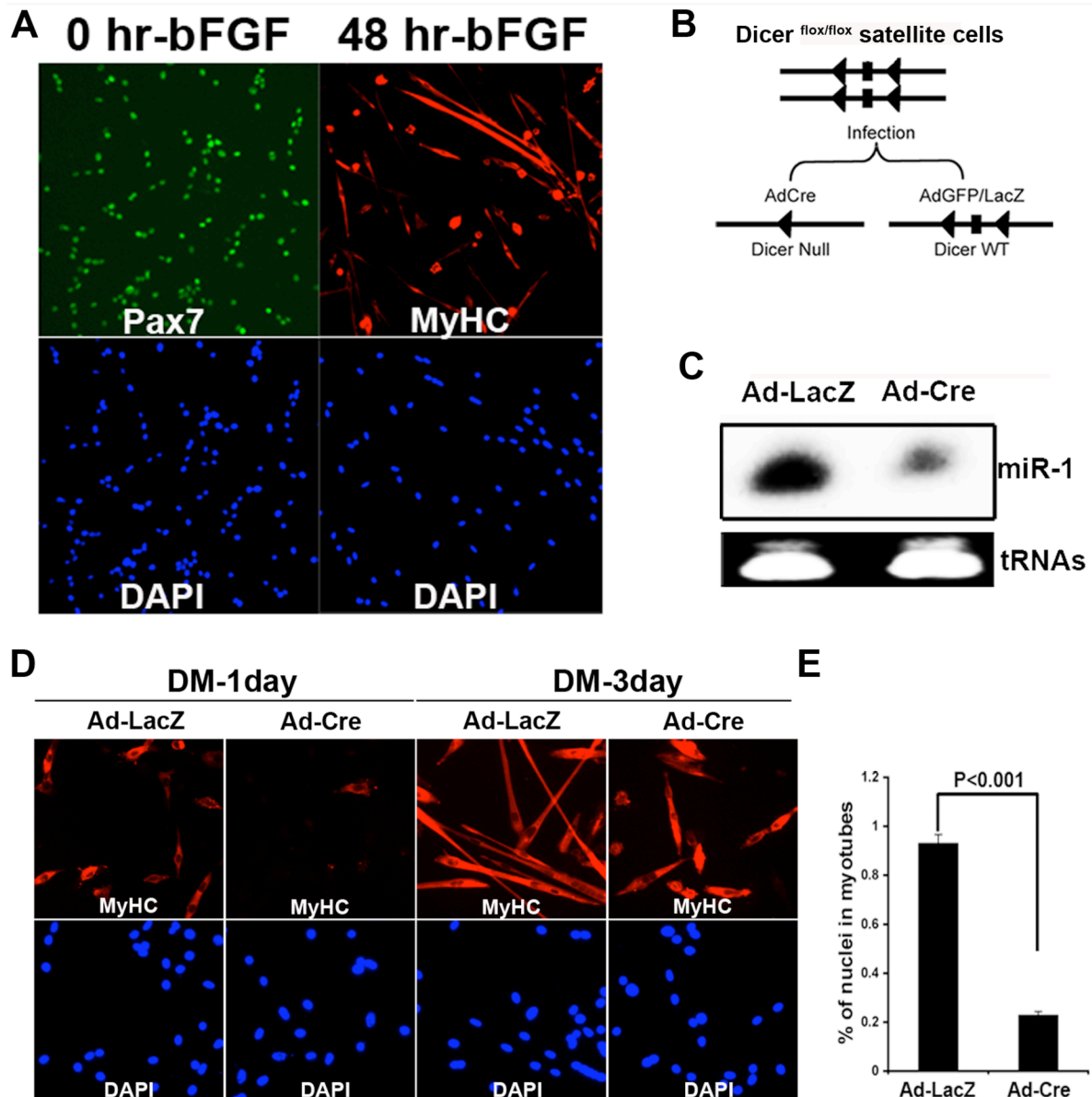

Figure 19 miRNAs are required for satellite cell differentiation.

(A) Isolation and differentiation induction of satellite cells. Satellite cells under growth (0h-bFGF) or differentiation condition (48h-bFGF) were fixed and stained with antibodies against Pax7 or MyHC. DAPI stained nuclei. (B) Scheme for the generation of Dicer nul satellite cells. LoxP sites (triangles) allow the deletion of Dicer following the infection of an adenoviral vector expressing Cre recombinase (Ad-Cre). Adenovirus expressing GFP (Ad-GFP) or LacZ (Ad-LacZ) serves as the control. (C) Northern blot analysis of miR-1 expression using RNAs isolated from Ad-Cre or Ad-LacZ infected Dicer$^{flox/flox}$ satellite cells at 48 h post-infection and differentiation induction (DM). tRNAs were used as a loading control. (D) Satellite cells infected with Ad-LacZ or Ad-Cre were switched into differentiation medium for 1 day (DM-1d) or 3 days (DM-3d) and myogenic differentiation was detected by immunostaining for MyHC. DAPI stained nuclei. (E) Quantification of fusion event of myoblasts infected with Ad-LacZ or Ad-Cre at 3 days in differentiation medium (DM). The fusion index is calculated as the percentage of nuclei in fused myotubes out of the total nuclei for each 20 x microscopic field. Myotubes with ≥ 2 nuclei were defined as fused myotubes. Each bar represents the mean ± standard error of ten 20 x microscopic fields from three independent experiments.

We constantly achieved greater than 95% purity of isolated satellite cells, as evidenced by the expression of Pax7 (Figure 19A) and the absence of MyoD expression (data not shown). Isolated satellite cells were maintained on collagen-coated plates. When cultured at the presence of bFGF, these satellite cells will continue to proliferate without differentiation. However, several hours after the removal of bFGF and exposed to low serum medium, satellite cells will quickly exit the cell cycle and initiate differentiation program, a process that faithfully mimics *in vivo* myogenesis process (Clegg et al., 1987; Sabourin et al., 1999; Templeton and Hauschka, 1992). The differentiation capacity of isolated satellite cells was confirmed by the expression of MyHC, a skeletal muscle terminal differentiation marker, upon differentiation induction (Figure 19A).
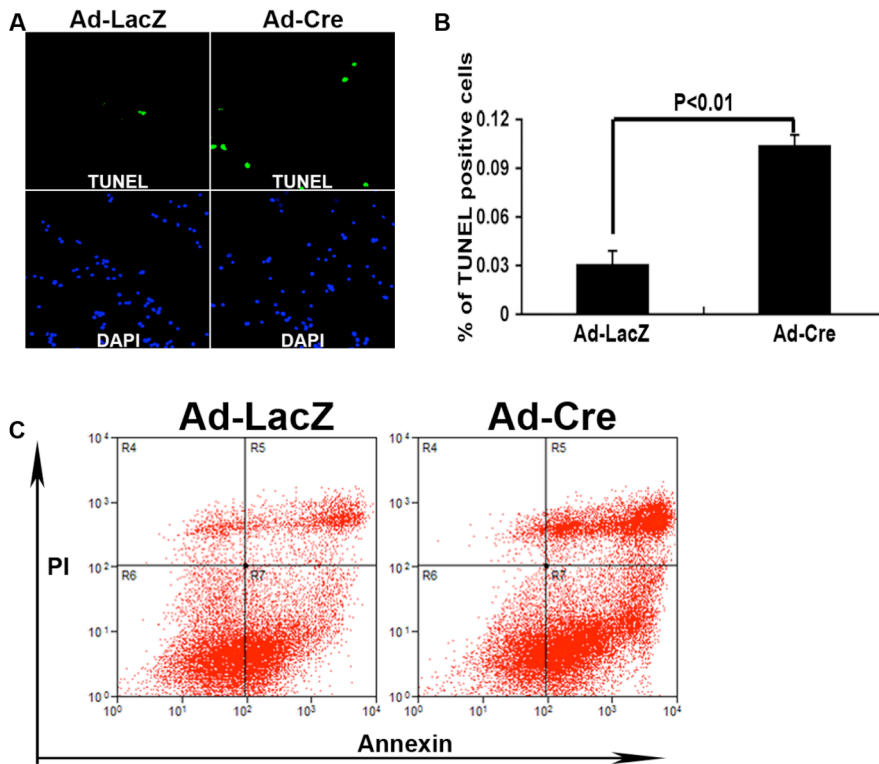
Figure 20 Dicer depleted myoblasts exhibit high rate of apoptosis in response to serum withdrawal. (A) Fluorescence-conjugated TUNEL analysis of Dicer$^{flox/flox}$ myoblasts infected with Ad-LacZ or Ad-Cre. (B) The quantification of TUNEL assay results in (A). The "% of TUNEL positive cells" is calculated as the percentage of TUNEL positive cells out of total number of nuclei indicated by DAPI staining for each 20 x field. Each bar represents the mean ± standard error of ten 20 x field from three independent experiments. (C) Flow cytometry analysis of Dicer$^{flox/flox}$ myoblasts infected with Ad-LacZ or Ad-Cre. The Dicer$^{flox/flox}$ myoblasts were cultured in growth condition 48 hr after infection and switched to DM for 24 hr before experiments. The experiment shown is one of three similar results.

To assess the requirement of miRNAs in satellite cell survival, proliferation and differentiation, we isolated and cultured satellite cells from Dicer$^{flox/flox}$ mice in which exon 22 and 23 of the Dicer gene has been flanked by flox sequences (Chen et al., 2008; Murchison et al., 2005). Dicer encodes a RNase III endonuclease responsible for miRNA maturation, therefore the deletion of Dicer will lead to a depletion of all mature and functional miRNAs (Bernstein et al., 2001; Grishok et al., 2001). We infected the Dicer$^{flox/flox}$ satellite cells with adenoviruses that express either the Cre recombinase (Ad-Cre), or the control LacZ- (Ad-LacZ) or GFP- (Ad-GFP) (Figure 19B). 48 hr after Ad-Cre infection, Dicer was deleted and the expression of miRNAs is significant decreased, as demonstrated by the decreased expression of miR-1, one of the most abundant miRNA in skeletal muscle

(Figure 19C). To assess the differentiation potential of Dicer null satellite cells, we switched

the Ad-Cre infected Dicer[flox/flox] satellite cells to differentiation medium in which bFGF was

removed and 2% of horse serum was added (Sabourin et al., 1999). Interestingly, we

observed a significant loss of cell viability in Dicer null satellite cells upon differentiation

induction, evidenced by the increase of TUNEL positive cells (Figure 20A and B).

Additionally, flow cytometry analyses also confirmed increased apoptotic satellite cells when

Dicer is deleted (Figure 20C). These observations are consistent with previous reports

demonstrating that Dicer is required for stem cell survival (Bernstein et al., 2003).

We next investigate the effects of Dicer mutation to satellite cell differentiation potential. We

examined the expression of Pax7, Myogenin and MyHC in Ad-Cre or Ad-LacZ transduced

satellite cells under growth or differentiation conditions. Under growth condition, in which

both the Dicer null and wild-type satellite cells were maintained at an undifferentiated

condition, we observed no difference in the expression of Pax7 level (data not shown). 12 hr

after switched to differentiation medium, satellite cells start to differentiate, we detected

similar Myogenin expression in both Dicer null and wild-type satellite cells (data not shown).

These observations indicate that miRNAs are unlikely required for the initiation of satellite

cell differentiation program. 24 hr after switched to differentiation medium, satellite cells

further differentiate into myoblasts in control wild-type satellite cells, as evidenced by the

expression MyHC proteins. However, very little MyHC expression was detected in Dicer

null cells (Figure 19D). 3 days after differentiation induction, myogenic differentiation is

profound in wild-type satellite cells and robust MyHC expression was detected in

multinucleated myotubes. However, Dicer null satellite cells were unable to complete the

terminal differentiation, evidenced by the much lower expression level of MyHC (Figure

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19D) and the absence of the multinucleated myotube formation (Figure 19D and 1E). These data suggest that Dicer, as a consequence of miRNAs, is essential for terminal differentiation of skeletal muscle satellite cells.

miRNA Expression During Satellite Cell Differentiation and Skeletal Muscle Regeneration

To define the signature of miRNA expression in satellite cells and identify miRNAs that are responsive to satellite cell differentiation, we performed miRNA microarray analyses using RNAs from satellite cells which are grown in growth medium and satellite cells which have been switched to differentiation medium for one day to induce differentiation. We also use RNAs from normal adult mouse skeletal muscle as well as injured muscle conferred by cardiotoxin injection, in order to identify miRNA expression signature during muscle regeneration *in vivo*. It has been well established that quiescent satellite cells will be activated to reenter cell cycle and undergo extensive proliferation and differentiation after injury (Morgan and Partridge, 2003; Yan et al., 2003). Molecular marker analyses confirmed that injured muscle was at the stage when satellite cells are actively proliferating (Figure 21B). miRNA microarray data revealed that a subset of miRNAs, including miR-1, miR-206, miR-133a and miR-133b, are highly induced during satellite cell differentiation. Intriguingly, the expression of these miRNAs is markedly down-regulated in injured skeletal muscle where an active muscle regeneration is occurring (Figure 21B). Conversely, another set of miRNAs are highly induced in regenerating skeletal muscle and their expression decreased in differentiating satellite cells (Figure 21B). In addition, we found that the expression of some miRNAs was moderately increased in both differentiating satellite cells and regenerating

skeletal muscle (Figure 21B). These observations suggest that miRNAs are likely involved in satellite cell proliferation, differentiation and skeletal muscle regeneration.
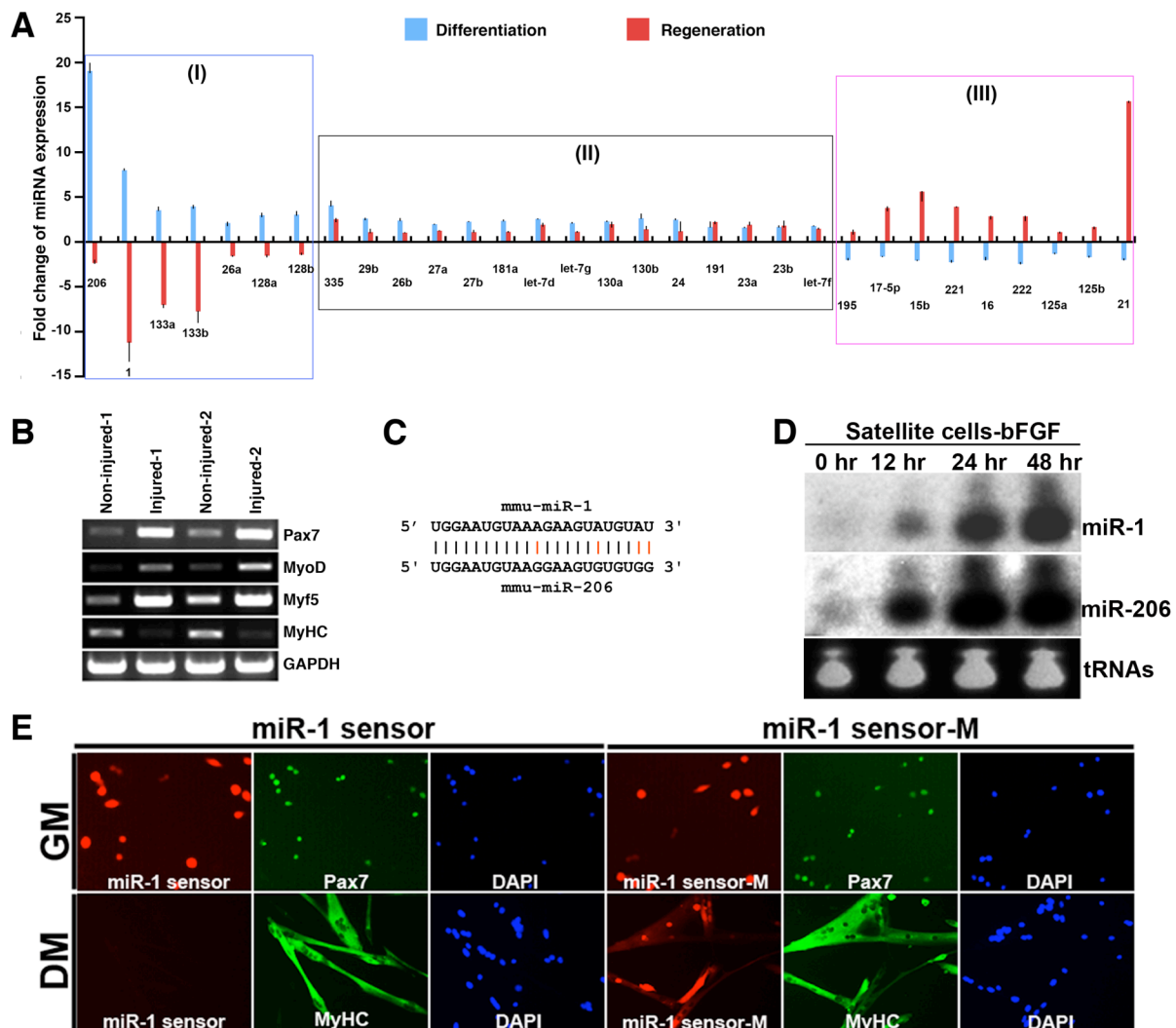

Figure 21 miRNA expression pattern during satellite cell differentiation and adult skeletal muscle regeneration.
(A) Microarray analysis of miRNA expression in differentiating satellite cells or regenerating skeletal muscle. Bar graph indicates the fold-change in miRNA expression during satellite cell differentiation and muscle regeneration compared to their respective controls. Group I represents miRNAs induced in differentiated satellite cells and repressed in regenerating muscle. Group II miRNAs are moderately induced in both differentiated satellite cells and regenerating muscle. miRNAs in group III are repressed in differentiated satellite cells and induced in regenerating muscle. Data represent two independent experiments in triplicates. $P<0.05$. (B) RT-PCR analysis of RNAs isolated from non-injured or injured skeletal muscle for the indicated genes. GAPDH serves as a loading control. (C) Alignment of mouse miR-1 and miR-206 sequences.
(D) Northern blot analysis of miR-1 and miR-206 expression using RNAs isolated from satellite cells at different time points of differentiation induction. tRNAs were used as a loading control.

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(E) Immunofluorescence of satellite cells expressing a sensor construct containing miR-1 complementary site (miR-1 sensor) or mutated miR-1 complementary site (miR-1 sensor-M) under growth condition (GM) or differentiation condition (DM). Note the expression of miR-1 is inversely correlated with dsRed. miR-1 sensor, but not the mutant sensor, was completely silenced in differentiation condition, in which miR-1 is highly expressed. Satellite cell identity and their differentiation status were confirmed by the expression of Pax7 and MyHC, respectively. DAPI counterstained nuclei.

Based on the level of miRNA expression and their dynamic change during satellite cell differentiation and muscle regeneration, we decided to focus on miR-1 and miR-206. Both miR-1 and miR-206 belong to the miRNA family of miR-1 and they share high homologue in their sequences (Griffiths-Jones, 2004) (Figure 21C). The fact that both miR-1 and miR-206 contain exact same seed sequences further suggests that they likely regulate same or very similar target genes. The induction of miR-1 and miR-206 expression during satellite cell differentiation is further confirmed by miRNA Northern blot analysis (Figure 21D). In order to precisely monitor the expression and action of these miRNAs at single cell level in satellite cells, we used a "miR-1 sensor", in which the complementary sequence for miR-1 was cloned downstream of a dsRed coding sequence (Chen et al., 2006). As a control sensor, the miR-1 binding sequence was mutated (miR-1 sensor-M). Under growth condition, similar dsRed expression level was detected in both miR-1 sensor expressed cells (miR-1 sensor) and control cells (miR-1 sensor-M), indicating there is not or only very weak miR-1 expression in undifferentiated satellite cells. However, when satellite cells were induced to differentiate, the expression of dsRed was undetectable in miR-1 sensor cells, while control cells exhibited strong dsRed (Figure 21E). These data further confirm that the expression of miR-1 and miR-206 is highly induced during satellite cell differentiation.
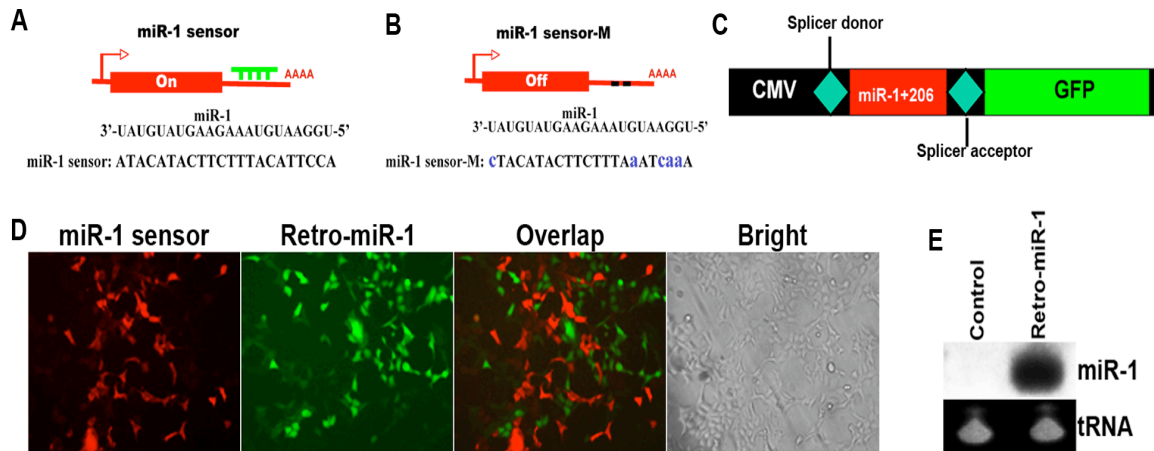
Figure 22 miR-1 sensor and retroviral vector expressing miR-1 and miR-206 expression. (A-B) Cartoons depicting the miR-1 sensor construct (miR-1 sensor) and mutated miR-1 sensor construct (miR-1 sensor-M). (C) Diagram miR-1 and miR-206 expression strategy in which the precursor of miRNAs flanking with splicing donor and receptor is upstream of GFP gene. Both miRNAs and GFP genes are under control of CMV promoter. (D) Immunofluorescence of 293 cells infected with both adenoviral miR-1 sensor (red) and retroviral vector expressing miR-1 and miR-206. (E) Northern-blot analysis of miR-1 expression using RNA isolated from 293 cells infected with retroviral miR-1+206.

miR-1 and miR-206 Restrict Satellite Cell Proliferative Potential and Promote Their Differentiation

The sharp induction of miR-1 and miR-206 expression during the transition of satellite cells from cell proliferation to differentiation prompted us to investigate the role of these miRNAs in satellite cells. We predict that miR-1 and miR-206 could repress satellite cell proliferation program, thus, to facilitate the progression of differentiation. To test this hypothesis we prematurely over-express miR-1, miR-206 or both in satellite cells using retroviral vectors. In order to overexpress and monitor the expression of miR-1 and miR-206 in satellite cells, we adapted a retroviral expression system (Grez et al., 1990; Miller and Rosman, 1989) in which the genomic sequences encoding miR-1 and miR-206 genes are cloned within the same frame of a GFP cassette and are under the control of the CMV promoter, which directs the expression of miR-1, miR-206 and GFP simultaneously (Figure 22C). We also validated the

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expression and activity of these miRNAs using both RNA Northern blot analysis, as well as the "miR-1 sensor" (Figure 22 D and E and data not shown). 5'-bromodeoxyuridine (BrdU) incorporation experiments were performed to assess the proliferation of satellite cells. miR-1 and miR-206 strongly inhibits satellite cell proliferation, as evidenced by significant less BrdU incorporation in miRNA overexpressed satellite cells comparison with control cells (A and B) (n=3 independent isolation and infection).
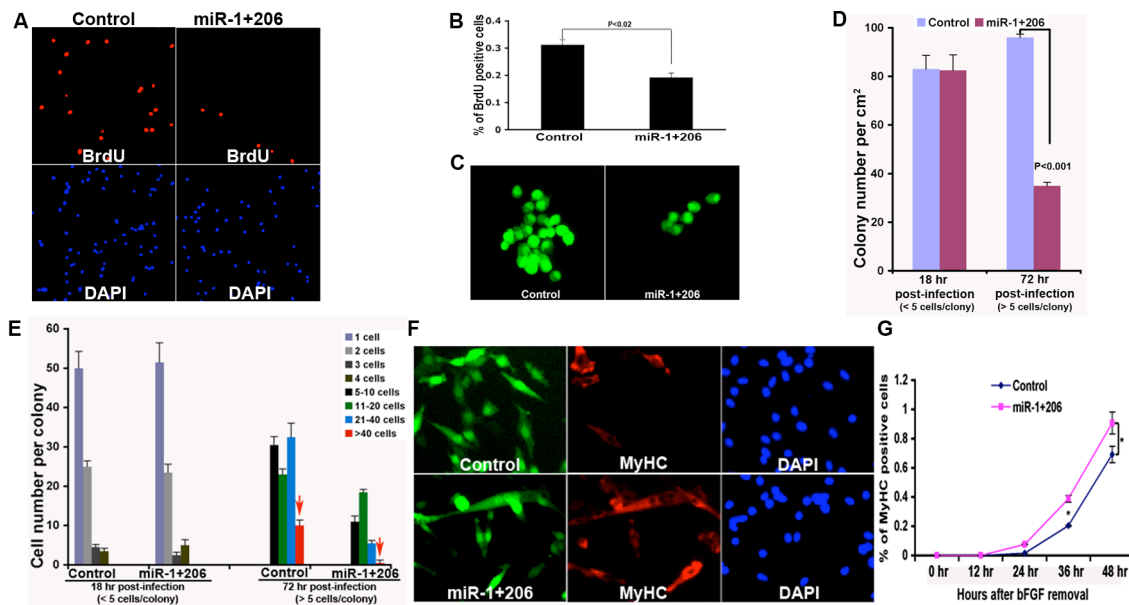

Figure 23 miR-1 and miR-206 restrict the proliferative potential of satellite cells and enhance their differentiation.
(A) Satellite cells were infected with either Ad-miR-1+206 or Ad-GFP (control) and pulsed with BrdU. Cell proliferation was determined using antibody against BrdU (red). DAPI counterstained nuclei. (B) Quantification of BrdU labeling experiments. The "% of BrdU positive cells" is calculated as the percentage of BrdU positive cells out of total number of nuclei indicated by DAPI staining for each 20 x microscopic field. Each bar represents the mean ± standard error of ten 20 x microscopic fields from three independent experiments. (C-D) Satellite cell colony formation assays. (C) Satellite cells infected with retroviral vectors expressing miR-1 and miR-206 (miR-1+206) or mutated miR-1 and miR-206 (control) and colony size determined. Note the small size of colony expressing miR-1 and miR-206 (miR-1+206) compared to controls, 72 h after infection. (D) Quantification of colony formation results. Data represent the mean ± s.d. from two independent experiments. (E-F) Satellite cells infected with retroviral vector expressing miR-1+206 or GFP (control were switched into differentiation medium for 48 h and myogenic differentiation was determined by immunostaining for MyHC. DAPI counterstained nuclei. The results were quantified in (F). Green marks infected cells, MyHC is red and DAPI is blue. Data represent the mean ± s.d. from three independent experiments (*P < 0.05).

To further characterize the inhibition of satellite cell proliferative potential by miR-1 and miR-206, we performed satellite cell colony formation assays. Indeed, overexpression of miR-1 and miR-206 significantly inhibits the colony formation of satellite cells; whereas control satellite cells form typical colonies that contain many cells, miR-1 and miR-206 overexpressed satellite cells form less colonies and the colonies are normally much smaller and contain much less cells (Figure 23C, D and E). ~12 hr after retroviruses infection, similar number of colonies and similar number of cells within individual colony were observed in the control and miR-1 and mir-206 infected satellite cells. At this stage, majority of GFP positive colonies exhibit single or two cell pattern when they are plated under low density. However, 72 hr after retroviral infection, significantly less number of colonies were formed in miR-1 and miR-206 infected satellite cells compared to controls. In addition, we found that ~50% colonies contain 20~40 cells and > 40 cells in the control satellite cells. In contrast, no single colony contains > 40 cells and the number of colonies that contain 20~40 cells was much less in miR-1 and miR-206 infected satellite cells (Figure 23E). Together, these data indicate that miR-1 and miR-206 restrict satellite cell proliferation and colony formation.

To study the effect of miR-1 and miR-206 on satellite cell differentiation, we performed immunoflurescent staining using antibody against MyHC at different time points after the initiation of satellite cell differentiation induction. miR-1 and miR-206 accelerate satellite cell differentiation process (Figure 23F). Quantitative analyses demonstrated that these changes are statistically significant (Figure 23G). Together, these data suggest that the primary function of miR-1 and miR-206 is to restrict satellite cell proliferative potential. miR-1 and miR-206 also positively regulate satellite cell differentiation.

Inhibition of miR-1 and miR-206 Results in Increased Satellite Cell Proliferation *in vivo*

To investigate whether miR-1 and miR-206 also restrict the proliferative potential of satellite cells *in vivo*, we knocked down both miR-1 and miR-206 in skeletal muscle of neonatal mice using miRNA antagomirs (Krutzfeldt et al., 2005; Yi et al., 2008). Administration of antagomir-1 and -206, but not PBS or mut-antagomir-1 and -206 (in which four mismatch mutations were introduced into each miRNA sequences), resulted in profound decrease of endogenous expression level of miR-1 and miR-206 in skeletal muscle (Figure 24A). Both intraperitoneal (IP) injection and intramuscular injection of antagomir-1 and -206 markedly abolished endogenous miR-1 and miR-206 in skeletal muscle (data not shown). The expression of miR-1 is also dramatically abolished in the heart after IP injection (but not intramuscular injection) of antagomir-1 (data not shown). Most importantly, the expression level of miR-133, which is clustered together with miR-1in mouse genome and is normally co-transcribed together with miR-1, was unaffected by antagomir-1 and -206 under the same conditions, indicating that the action of antagomirs is highly specific (Figure 24A).

We examined the effects of miR-1 and miR-206 knockdown on mouse skeletal muscle development and satellite cell proliferation. We first performed BrdU incorporation experiment to label proliferating cells. Quantification analysis reveals a significant increase in BrdU positive cells in antagomir-1 and -206 treated skeletal muscle compared to control mut-antagomir-1and -206 treated samples (Figure 24B and C), indicating that knockdown of endogenous miR-1 and miR-206 indeed enhanced cell proliferation. Consistent with the above observation, we found substantially more phospho-histone H3 (p-H3) positive cells in antagomir-1 and -206 treated muscle cells than in control cells (Figure 24D and E). In order

to confirm the satellite cell identity of these proliferating cells in skeletal muscle, we examined the expression of Pax7, a well-defined marker for satellite cells, and found a significant increase in Pax7 positive cells in antagomir-1 and -206 treated skeletal muscle (Figure 24F and G). These *in vivo* results, together with our previous findings in which miR-1 and miR-206 inhibit satellite cell colony formation and proliferation *in vitro*, further support the view that miR-1 and miR-206 are key regulators for satellite cell proliferation.
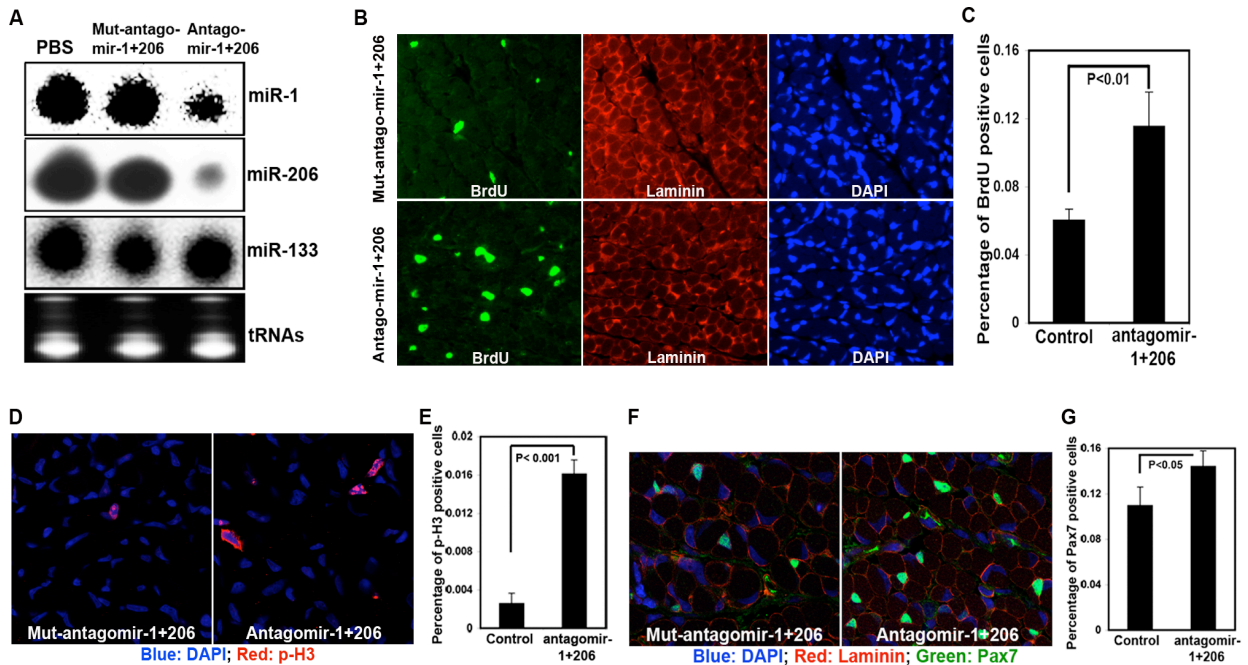

Figure 24 Knockdown of miR-1 and miR-206 increases the proliferation of satellite cells in vivo

(A) Northern blots of total RNAs (10 ug) isolated from skeletal muscle after injection of RNA antagomirs against miR-1 and miR-206 (Antagomir-1+206). Muscle injected with PBS or mutated miR-1 and miR-206 antagomirs (mm-antagomir-1+206) were used as controls. tRNAs were used as a loading control. (B) Confocal microscopic images of skeletal muscle 4 h after BrdU labeling from postnatal mice treated with antagomir-1+206 or mm-antagomir-1+206 (serves as a control). Cell proliferation was determined by anti-BrdU antibody (green), Laminin (red) marks cell surface and DAPI (blue) counterstains nuclei. (C) Quantitative measurement of BrdU positive cells from experiments in Figure 4B. (D) Confocal microscopic images of skeletal muscle from mice treated with antagomir-1+206 or mm-antagomir-1+206 (controls). Anti phospho histon H3 antibody (red) visualizes mitotic cells. DAPI (blue) counterstains nuclei. (E) Quantitative measurement of phospho histon H3 (p-H3) positive cells from experiments in Figure 4D. (F) Confocal microscopic images of skeletal muscle from antagomir-1+206 or mm-antagomir-1+206 treated mice. Anti-Pax7 antibody labels satellite cells (green). Laminin (red) outlines cell surface and DAPI (blu) marks nuclei. (G) Quantitative measurement of Pax7 positive cells from experiments in Figure 4F. Each set of

experiments was conducted at least twice independently, and at least ten 20 x microscopic fields were counted for each experiment. Data represent the mean ± s.d. and P values indicated.

Pax7 is a Direct Regulatory Target of miR-1 and miR-206 During Satellite Cell Differentiation

Pax7, a paired homeodomain transcriptional factor, has been well-established as a key regulator of satellite cell survival, self-renewal and proliferation (McFarlane et al., 2007; Oustanina et al., 2004; Relaix et al., 2006; Zammit et al., 2004). The observation that overexpression of miR-1 and miR-206 in satellite cells dramatically inhibited colony formation and decreased the size of individual colonies prompted us to closely examine the expression of Pax7 in miR-1 and miR-206 overexpressed satellite cells. Indeed, we observed an inverse correlation between the expression level of miR-1/miR-206 and Pax7 protein in satellite cell colonies (Figure 26A), suggesting that miR-1 and miR-206 may have participated the repression of Pax7 protein expression. Interestingly, whereas we observed a rapid down-regulation of Pax7 protein during satellite cell differentiation, the expression level of Pax7 mRNA appears not affected (Figure 25A and B), indicating a post-transcriptional regulation of Pax7 expression is likely involved.
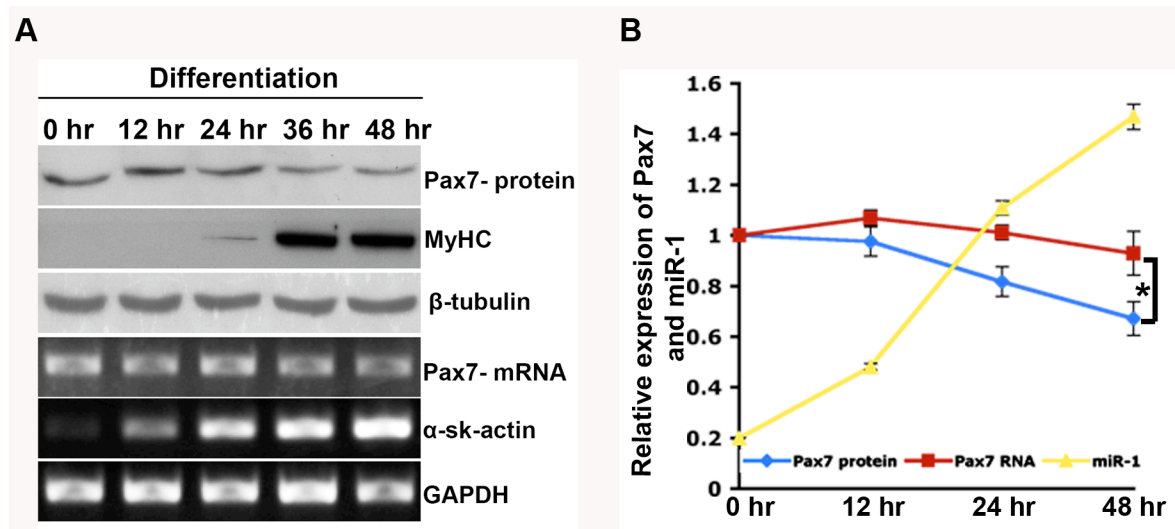
Figure 25 Pax7 mRNA and protein expression during satellite cell differentiation. (A) Western blot and RT-PCR analysis of Pax7 expression using protein or RNA isolated from satellite cells at different time points during their differentiation. (B) The quantification of Pax7 mRNA, protein and miR-1 expression (see Figure 2D) during satellite cell differentiation. Data represent the mean ± s.d. from three independent experiments (*P < 0.05).

The fact that the expression of miR-1 and miR-206 is sharply increased during satellite cell differentiation raises the possibility that miR-1 and miR-206 may directly inhibit the translation of Pax7 protein. To test whether miR-1 and miR-206 directly repress Pax7 expression, we performed bioinformatics analyses for potential miRNAs regulatory sites at the 3'-UTR of Pax7 transcripts. Indeed, two putative miR-1 and miR-206 binding sites at the 3'-UTR of Pax7 mRNA, were identified (data not shown). Next, we performed luciferase reporter assay using a partial sequence of the Pax7 3'-UTR that contains both miR-1 and miR-206 binding sites (data not shown). Not surprisingly, miR-1 or miR-206 or both miRNAs strongly represses the luciferase activity (Figure 26B). Introduction of mutations into these two miRNA binding sites completely abolished miR-1 and miR-206-mediated repression, demonstrating the specificity of repression (Figure 26B). Finally, we test whether miR-1 and miR-206 could repress the expression of endogenous Pax7 protein level in satellite cells. Western blot analyses clearly demonstrate that miR-1 and miR-206 repress Pax7 protein expression (Figure 26C). Together, these results demonstrated that miR-1 and miR-206 directly regulate Pax7 protein expression post-transcriptionally.
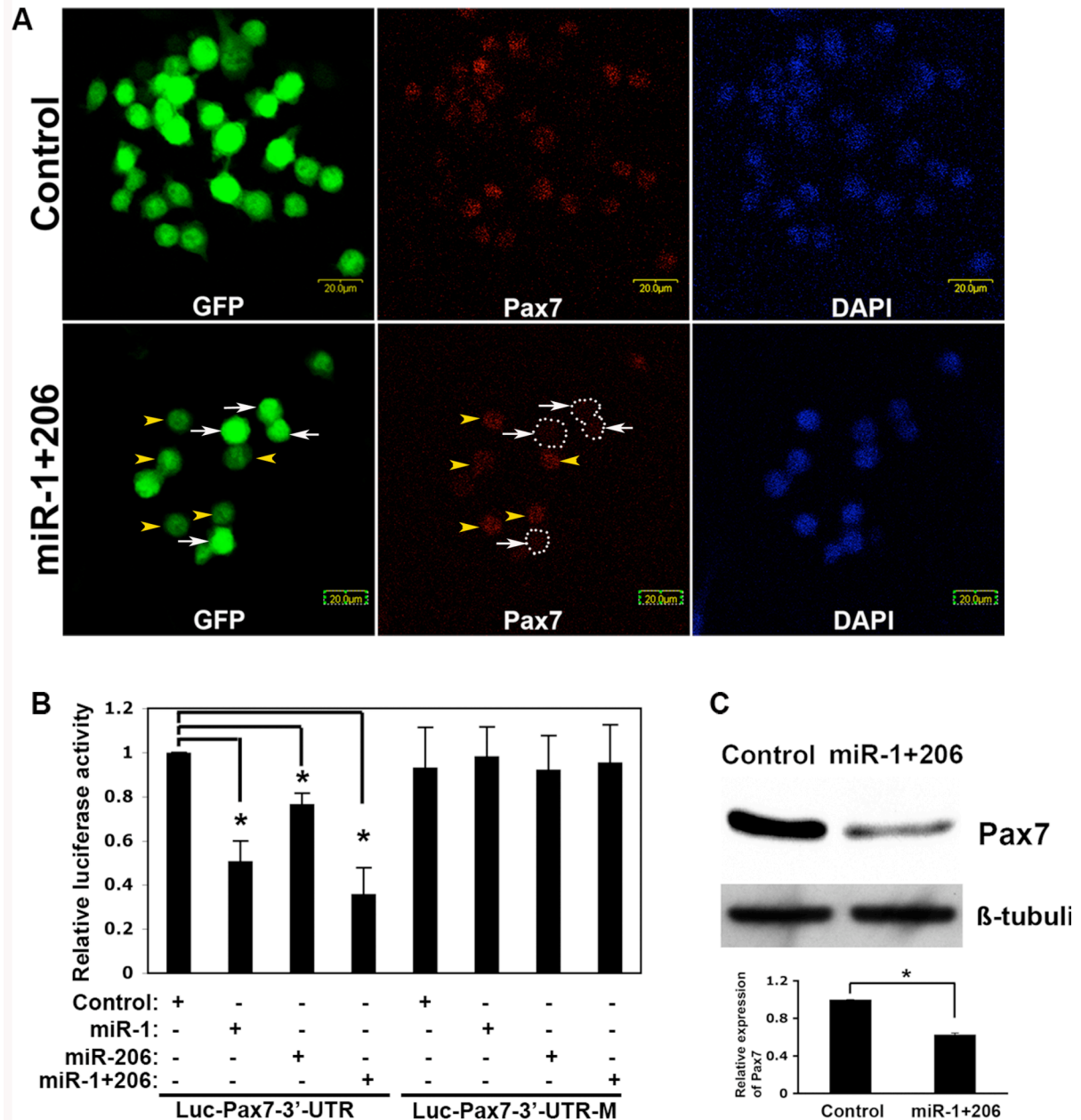
Figure 26 Pax7 is a direct regulatory target of miR-1 and miR-206
(A) Confocal microscopic images of satellite cell colonies infected with retroviral vector expressing miR-1 and miR-206 or mutated miR-1 and miR-206 (control). Note the mutual exclusive expression level of Pax7 (red, arrow heads) and miR1+206 (green, arrows). DAPI counterstained nuclei. (B) Repression of Pax7 3'UTR by miR-1 and miR-206. Luciferase reporters were linked with Pax7 3'-UTRs containing either putative miR-1/miR-206 binding sites (Luc-Pax7-3'-UTR) or mutated miR-1 and miR-206 binding sites (Luc-Pax7-3'-UTR-M). miR-1, miR-206 or miR-1+206 plasmids were co-transfected with luciferase-UTR constructs and luciferase activity determined. miR-208 (control) was used to serve as a control for the specificity of miRNA. Data represent the mean ± s.d. from three independent experiments (*P < 0.05). (C) Western blot analysis (upper) and quantification (lower) of Pax7 expression in satellite cells infected with retroviral vectors expressing miR-1+206 or a control GFP. Data represent the mean ± s.d. from three independent experiments (*P < 0.05).

miR-1 and miR-206-Mediated Repression of Pax7 is Essential for Myogenic Progenitor Cell Differentiation

Pax7 plays a central role in satellite cell fate maintenance and the initiation of myogenic program (Oustanina et al., 2004; Relaix et al., 2004). Intriguingly, it has also been observed that sustained expression of Pax7 in myogenic progenitor cells significantly represses myogenic terminal differentiation (McFarlane et al., 2007; Olguin and Olwin, 2004). We hypothesized that miR-1 and miR-206-mediated repression of Pax7 protein level through its 3'-UTR might be essential for the transition of muscle progenitor cells from proliferation to differentiation. To test this hypothesis, we built two Pax7 expression constructs by which the 3'-UTR is either included or excluded (referred as Pax7-UTR and Pax7, respectively); we also built another Pax7 expression construct in which both miR-1 and miR-206 targeting sites at its 3'-UTR were mutated (referred as Pax7-UTR-M) (Figure 27A). We then examined the effects of these Pax7 expression constructs on Pax7 protein expression and myoblast differentiation. Under growth condition where miR-1 and miR-206 expression is barely detected in myoblasts, the expression levels of Pax7 mRNAs and proteins were similar for all three Pax7 constructs (Figure 27B). Under differentiation condition in which miR-1 and miR-206 are rapidly induced, however, Pax7 protein expression level is significantly lower in Pax7-UTR transfected cells compared to Pax7 or Pax7-UTR-M transfected cells (Figure 27D). Importantly, we didn't detect significant difference in Pax7 mRNA level when individual Pax7 expression constructs were transfected under the same condition (Figure 27C), indicating that miR-1 and miR-206 contributed to the inhibition of Pax7 protein expression post-transcriptionally via Pax7 3'-UTR. As expected, Pax7 and Pax7-UTR-M
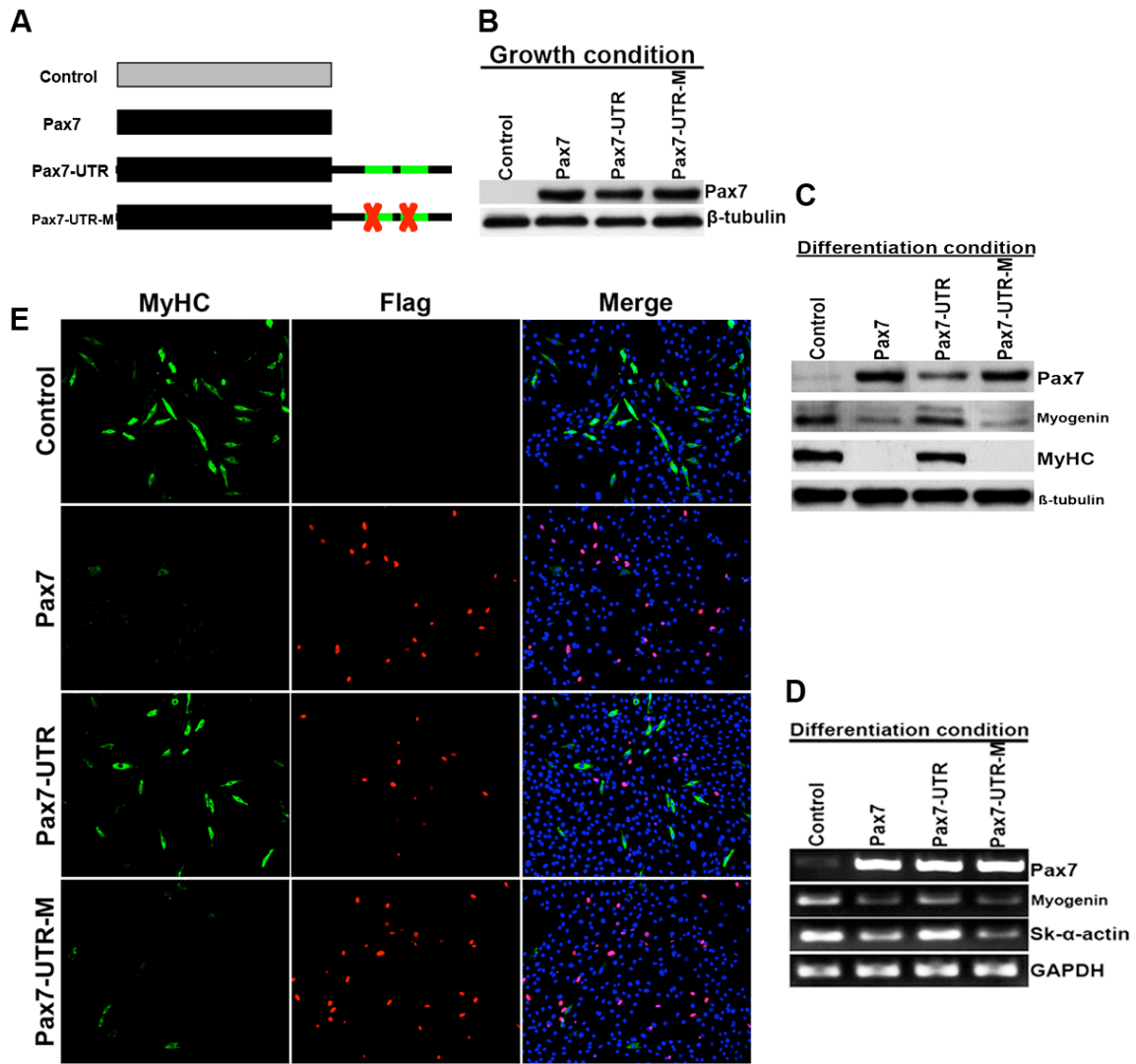
Figure 27 Functional significance of miR-1 and miR-206 mediated repression of Pax7.
(A) Scheme of expression constructs including a control plasmid, Pax7 ORF only (Pax7), Pax7 with
its UTR containing two miR-1 and miR-206 binding sites (Pax7-UTR), or with the two miR-1 and
miR-206 binding site mutated (Pax7-UTR-M). (B) Western-blot analyses of Pax7 expression in
C2C12 myoblasts transfected with control, Pax7, Pax7-UTR or Pax7-UTR-M expression constructs
under growth condition (GM). β-tubulin serves as a loading control. (C) Expression of Pax7 and
other myogenic markers in C2C12 myoblasts transfected with control, Pax7, Pax7-UTR or Pax7-
UTR-M expression constructs under differentiation condition. (upper panels) RT-PCR analysis using
indicated primers. (lower panels) Western-blot analyses using antibodies for Pax7, myogenin and
MyHC. (D) Immunofluorescence of C2C12 myoblasts transfected with indicated expression
constructs for 12 h and then switched to differentiation medium for additional 36 h. The cells were
stained with antibodies against MyHC or anti-Flag antibody for Pax7 expression, DAPI stained
nuclei.

expressing cells show profound inhibition in myoblast differentiation compared to cells transfected with Pax7-UTR, as evidenced by significant decrease expression of skeletal muscle differentiation marker genes, including myogenin, skeletal muscle α-actin and MyHC (Figure 27C,D). Furthermore, we performed immunofluorescent staining to examine the effects of different forms of Pax7 expression constructs on myoblast differentiation. Consistently, myoblasts transfected with Pax7 or Pax7-UTR-M expression constructs displayed much less MyHC expression. In contrast, myoblasts transfected with the Pax7-UTR expression construct had comparable expression of MyHC with the control (Figure 27E), indicating that miR-1 and miR-206-mediated repression of Pax7 expression remarkably facilitates cell differentiation. Taken together, these data demonstrate that down-regulation of Pax7 by miR-1 and miR-206 is functional significant for the progression of skeletal muscle progenitor cell differentiation.

DISCUSSION AND SUMMARY

In this study, we reveal a critical role of miR-1 and miR-206 in repressing satellite cell "stemness". We found that miR-1 and miR-206 are sharply up-regulated during satellite cell differentiation while strikingly decreased during skeletal muscle regeneration. We further showed that premature expression of miR-1 and miR-206 restrict proliferative potential of satellite cells. Conversely, satellite cell proliferation is increased when miR-1 and miR-206 are knocked down *in vivo*. Most importantly, we identified Pax7 as one of the regulatory targets of miR-1 and miR-206 during satellite cell differentiation and show that down-regulation of Pax7 by these two miRNAs is crucial for proper muscle progenitor cell differentiation. To our knowledge, our work is the first to document that the differentiation of

muscle adult stem cells, satellite cells, is under miRNA-mediated regulation at post-transcriptional level. Our observation that miR-1 and miR-206 facilitate satellite cell differentiation by limiting their maintenance gene Pax7 expression implicates that miRNAs may play a key role in repressing "stemness" of stem cells.

Recently, it has been reported that myogenic regulatory factors MyoD and Myogenin inhibit the expression of Pax7 in skeletal muscle primary myoblasts during differentiation (Olguin et al., 2007). It is intriguing that down-regulation of Pax7 is needed for skeletal muscle progenitor cells differentiation. However, the molecular mechanisms underlying this observation were not defined. The down-regulation of Pax7 is needed for skeletal muscle progenitor cells differentiation (McFarlane et al., 2007; Olguin and Olwin, 2004; Zammit et al., 2006). Recently, it has been reported that myogenic regulatory factors MyoD and Myogenin inhibit the expression of Pax7 in skeletal primary myoblasts during differentiation (Olguin et al., 2007). However, the molecular mechanisms underlying this inhibition were not defined. Our studies in which miR-1 and miR-206 eliminate Pax7 protein expression in differentiating muscle progenitor cells offer an explanation. Pax7 is expressed in undifferentiated satellite cells and is required for the initial activation of Myf5 and MyoD when satellite cells are induced to differentiate. Activated MyoD in satellite cell-derived myogenic progenitor cells will then turn on miR-1 and miR-206 (Chen et al., 2006; Rao et al., 2006; Rosenberg et al., 2006). As a consequence, these two miRNAs will repress Pax7 post-transcriptionally. Given the essential dual roles of Pax7 in satellite cell fate maintenance and myogenic terminal differentiation inhibition, we suggest that elimination of Pax7 proteins in activated satellite cells by miR-1 and miR-206 could be one of the major

mechanisms that miRNAs participate the developmental decision of satellite cells to ensure a

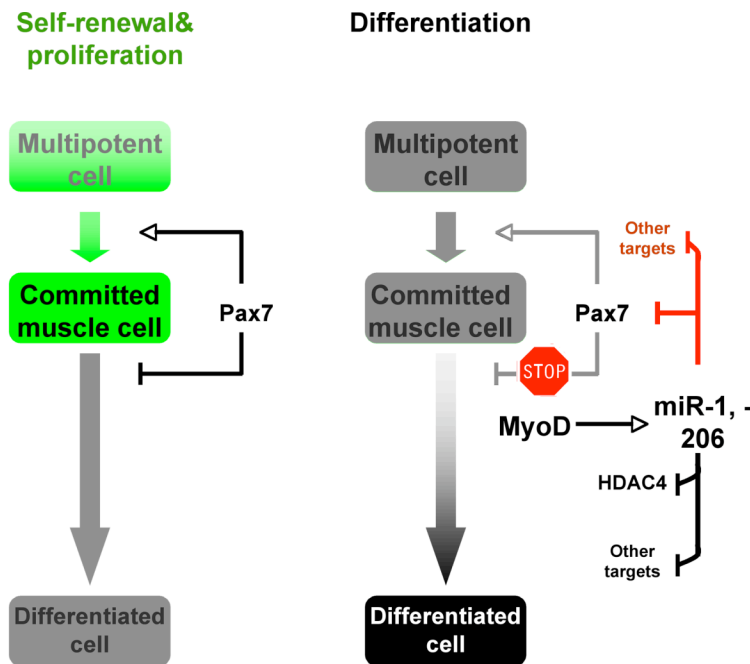transition from proliferation to differentiation (Figure 28).

Figure 28 Model of miR-1 and miR-206 mediated repression of Pax7 for satellite cell differentiation

Pax7 has multiple functions in satellite cell fate determination. One of such roles is to specify satellite cells into myogenic fate while prevents their precocious differentiation. Upon the initiation of myogenic differentiation, satellite cell derived myogenic progenitor cells will start to express myogenic transcription factors, including MyoD. MyoD, in turn, will activate the expression of miR-1 and miR-206. miR-1 and miR-206 potently enhance myogenic program by limiting and refining the expression of Pax7, in addition to repressing HDAC4, therefore confer the robustness of gene program switch from proliferation to differentiation during satellite cell differentiation.

We and others previously demonstrated that miR-1 and miR-206 promote skeletal

muscle terminal differentiation (Chen et al., 2006; Kim et al., 2006; Rosenberg et al., 2006).

One of the miR-1 regulatory targets which mediates miRNA function in myoblast is HDAC4,

an inhibitor for muscle differentiation (Chen et al., 2006; Lu et al., 2000; McKinsey et al.,

2000). In present study, we further show miR-1 and miR-206 repress stem cell maintenance

gene Pax7, therefore confer the robustness of gene program transition from proliferation to

differentiation in satellite cells. miR-1 and miR-206's role in restricting the proliferative

potential of satellite cells is remarkable, which is probably why the expression of miR-1 and

miR-206 rapidly increased when satellite cells are induced to differentiated. On the other

hand, both miR-1 and miR-206 was sharply down-regulated in injured skeletal muscle,

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presumably to allow active satellite cell proliferation and muscle regeneration. The current study, together with our previous report (Chen et al., 2006), support the view that miRNAs may regulate multiple mRNA targets that often encode proteins with related functions (Leung and Sharp, 2007). It will be important to identify and study additional miR-1 and miR-206 regulatory targets in skeletal muscle, which will be essential for our further understanding of the molecular mechanisms of miRNA function. In addition, it will be interesting to explore whether similar miR-1 regulatory targets and molecular mechanisms exist in cardiomyocytes, where miR-1 is also highly expressed. Intriguingly, the expression of miR-133 was also fund increased in differentiated satellite cells and decreased in injured skeletal muscle, along with the expression patterns of miR-1 and miR-206 (Figure 1). We and others have reported that miR-1 and miR-133 are encoded by polycistrons and they are co-transcribed together in cardiac and skeletal muscle. Unlike miR-1, which strongly promotes myoblast differentiation, miR-133 inhibits myoblast differentiation and enhances their proliferation (Chen et al., 2006). Similar observation was also reported in embryonic stem cells when they are induced to differentiate into cardiomyocyte (Ivey et al., 2008). It will be important to determine whether miR-133 affects satellite cell proliferation and differentiation processes, and if so, what are the downstream regulatory targets which mediates its function.

In summary, our findings demonstrate that miR-1 and miR-206 regulate the transition of satellite cells from proliferation to differentiation. They do so, at least partially, through limiting and refining the expression of important genes which are responsible for the maintenance of adult stem cells in an undifferentiated status. In this manner, miRNAs confer the robustness of satellite cells' decision from proliferation to differentiation.

CHAPTER 5

FUTURE DIRECTIONS

In the past years, we identified numerous miRNAs through different approaches and studied their functions in different biological systems *in vitro* and in *vivo*. Computational approaches gave us a broad picture of what genes miRNAs might be targeting. Muscle cells provide us with a powerful system to understand miRNAs function and mechanism involved during cell fate determination and morphogenesis in development. However, there are still questions to answer before we can therapeutically manipulate miRNAs for medical application in the muscular and cardiovascular field.

microRNA *in vivo* functions and their targets

The majority of studies on miRNAs are performed in the *in vitro* systems and/or low organisms. miRNAs exist in families with similar or even identical sequences and cluster together on different chromosomes, therefore creating substantial challenge to study their function using conventional loss-of-function approaches. As a result, we still know very little about miRNA function *in vivo*. Some miRNAs, including *lin-4* and *let-7*, function as a molecular switch to turn off a small number of key regulatory targets and their deletions result in obvious phenotypes at whole organ or organism level. However, the majority miRNAs are suggested to "fine-tune" gene expression and make their functional study unfeasible. Individual miRNAs have tens or even hundreds of regulatory targets according to

computational predictions while multiple miRNAs could target to one single mRNA, therefore creating a lot of complexity in miRNA-mediated gene regulation. Collectively, these unique characteristics of miRNAs pose a big challenge for the studies of miRNA function and the mechanisms involved.

miRNAs target identification remains a promising way to determine a given miRNA's function based on those known roles of regulatory targets. In fact, numerous approaches have been developed to identify miRNAs targets, including computational prediction (John et al., 2004; Kiriakidou et al., 2004; Krek et al., 2005; Lewis et al., 2003), proteomic and microarray methods (Lim et al., 2005; Nakahara et al., 2005), miRNP immunopurification coupled with microarray analyses (Beitzinger et al., 2007; Easow et al., 2007; Zhang et al., 2007). However, all these methods of miRNAs target identifiaction involve large-scale experiments and have intrinsic defects, including experimental variations, specificity and reliability. The validation of miRNAs targets from methods above mainly relys on luciferase reporter assay and Western-blot analyses. Given more than 1000 miRNAs have been found in the mammalian genome and many of them are identical or with high similarity (Griffiths-Jones, 2004), individual miRNAs can target hundreds of mRNA and many different miRNAs can regulate one mRNA, and most miRNAs fine-tune gene expression (Stark et al., 2005), it will take huge effort and a long time to identify those biologically significant miRNAs targets.

microRNAs as novel therapeutic targets

Distinct miRNAs expression profiles have been implicated in a variety of muscle diseases in mammalian (Tatsuguchi et al., 2007; Thum et al., 2007; van Rooij et al., 2006). The large

number of miRNAs and their broad functions suggest miRNAs could be explored for medical applications in the settings of muscular and cardiovascular diseases. miRNAs are quite stable compared to regular mRNAs and technologies exist to examine hundreds miRNAs expression profiles simultaneously, potentially making miRNAs useful biomarkers or diagnostic indicators for muscle disorders. miRNAs are also small molecules, making their *in vivo* delivery feasible compared with conventional drugs.

The use of chemically modified oligonucletides to either inhibit miRNAs function or disrupt interaction between a miRNA and its regulatory targets is a straightforward approach to inactivate pathological miRNAs. Loss of miR-208 leads to the diminishment of cardiac hypertrophic response, fetal gene re-activation and fibrosis in response to cardiac stress, indicating miR-208 inhibition could enhance cardiac function after chronic or acute injury (van Rooij et al., 2007). A promising method to inactivate individual miRNAs has been developed using chemically modified oligonucletides, named antagomirs, based on complementary base pairing (Krutzfeldt et al., 2007; Krutzfeldt et al., 2005). In addition, elevation of miRNAs expression levels could also be used as a strategy to design drugs. As mentioned in previous sections, very few miRNAs are expressed in mouse and Zebrafish early development but large number of miRNAs are induced as development proceeds, indicating miRNAs favor differentiation process in total (Wienholds et al., 2005). miRNA expression profiles reveal that the majority of miRNAs are down-regulated to minimal levels in most cancers, indicating miRNAs expression is detrimental for cancer formation (Lu et al., 2005). It has been shown that the cellular origin of synovial sarcoma, a type of muscle cancer, is satellite cell-derived myogenic cells (Haldar et al., 2007). Our studies demonstrated that mir-1 and -206 predominantly repress satellite cell proliferation and restrict their

colongenic potential in muscle development, raising the possibility that elevated miR-1 and -206 expression could inhibit sarcoma development by restricting myogenic cells proliferation. Interestingly, the restoration of miR-206 expression in tumors has been shown to reduce tumor growth and metastasis (Tavazoie et al., 2008). Therefore, the ectopic introduction of miRNAs could restrict the cancer cell proliferation and growth by repressing multiple targets simultaneously.

However, the broad and as yet poorly understood consequences of manipulating miRNAs could create significant problems with respect to possible off-target effects. Our unpublished data shows that loss of miR-208 cause conduction defects in addition to those beneficial effects, including a diminishment of hypertrophy and fibrosis after cardiac stress. miR-133 has been implicated to block cardiac hypertrophy and elevated miR-133 expression is suggested to be beneficial to hearts during cardiac stress (Care et al., 2007). However, it's also noticed that the increased miR-133 expression causes cardiac arrhythmias, therefore bringing the concern about the application of miR-133 in cardiac hypertrophy (Xu et al., 2007). In order to minimize "off-target" effects, specifically modifying nucleotide sequences within the 3' UTR of target mRNAs could be an effective way to gain miRNA-mediated gene regulation in this particular target. The underlying molecular feature of Texel sheep massive meat has been mapped to a single G-to-A mutation within the 3' UTR of myostatin, a member of TGF-β growth factors family, which functions to repress muscle growth. This mutation creates a binding site for miR-1 and -206 and causes the translational repression of myostatin. This gain of miR-1/206 regulation in the myostatin mimics the loss-of-function mutation of myostatin in mice, cattle, and humans (Clop et al., 2006). Together, the

molecular natures and functional significance of the interactions between miRNAs and their targets will remain big task for future studies.

Future prospects for microRNAs in muscle biology and muscle disorders

miRNA biology is in its infancy and, as an emerging field, there are many more questions than answers. Current studies, especially computational analyses, give us a very big picture in terms of which genes miRNAs may be targeting. At least one third of human protein coding genes are predicted to be regulated by as many as one thousand miRNAs, many of which are totally unknown in terms of their expression pattern, function and regulatory targets. Given the vast number of miRNAs and the diverse functions in different biological processes observed in the relatively small number of miRNAs studied thus far, it's apparent that many new and unanticipated functions of miRNAs in normal muscle development, function and disorders are awaiting discovery. Considering that many miRNAs fine-tune gene expression program and the intrinsic complexity of miRNA functional models, it will take years and a huge effort to determine the specificity of miRNA-mediated gene regulation at whole organism levels. microRNAs certainly present a big challenge for the future.

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