

RESEARCH ARTICLE

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Transgenic overexpression of miR-133a in skeletal muscle

Zhongliang Deng^{1,2*}, Jian-Fu Chen² and Da-Zhi Wang^{2,3*}**Abstract**

Background: MicroRNAs (miRNAs) are a class of non-coding regulatory RNAs of ~22 nucleotides in length. miRNAs regulate gene expression post-transcriptionally, primarily by associating with the 3' untranslated region (UTR) of their regulatory target mRNAs. Recent work has begun to reveal roles for miRNAs in a wide range of biological processes, including cell proliferation, differentiation and apoptosis. Many miRNAs are expressed in cardiac and skeletal muscle, and dysregulated miRNA expression has been correlated with muscle-related disorders. We have previously reported that the expression of muscle-specific miR-1 and miR-133 is induced during skeletal muscle differentiation and miR-1 and miR-133 play central regulatory roles in myoblast proliferation and differentiation in vitro.

Methods: In this study, we measured the expression of miRNAs in the skeletal muscle of *mdx* mice, an animal model for human muscular dystrophy. We also generated transgenic mice to overexpress miR-133a in skeletal muscle.

Results: We examined the expression of miRNAs in the skeletal muscle of *mdx* mice. We found that the expression of muscle miRNAs, including miR-1a, miR-133a and miR-206, was up-regulated in the skeletal muscle of *mdx* mice. In order to further investigate the function of miR-133a in skeletal muscle in vivo, we have created several independent transgenic founder lines. Surprisingly, skeletal muscle development and function appear to be unaffected in miR-133a transgenic mice.

Conclusions: Our results indicate that miR-133a is dispensable for the normal development and function of skeletal muscle.

Keywords: microRNA-133a, skeletal muscle, transgenic, differentiation

Background

MicroRNAs (miRNAs) are a class of ~ 22 nt non-coding RNAs that regulate gene expression post-transcriptionally [1-3]. The involvement of miRNAs in muscle biology has recently been reported [4-11]. miRNAs regulate the expression of transcription factors and signaling mediators important for cardiac and skeletal muscle development and function [7,12-14]. Aberrant miRNA expression has been observed in muscle diseases, including cardiac and skeletal muscle hypertrophy, heart failure and muscular dystrophy [13,15-17].

A subset of miRNAs, miR-1, miR-133, miR-206 and miR-208, are either specifically or highly expressed in cardiac and skeletal muscle and are called myomiRs [6,7,13]. Among them, miR-133 was shown to promote the proliferation of myoblasts and inhibits their differentiation in cultured skeletal muscle myoblasts. miR-133 enhances myocyte proliferation, at least in part, by reducing protein levels of SRF, a crucial regulator for muscle differentiation [18,19]. miR-133 also inhibits the translation of polypyrimidine tract-binding protein (nPTB), which controls differential transcript splicing during skeletal-muscle differentiation [20]. Paradoxically, miR-1 and miR-133 exert opposing effects to skeletal-muscle development despite originating from the same miRNA polycistronic transcript. Interestingly, miR-1 and miR-133 also produce opposing effects on apoptosis [21].

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Additionally, embryonic stem (ES) cell differentiation towards cardiomyocytes is promoted by miR-1 and inhibited by miR-133 [22]. Furthermore, miR-1 and miR-133 are also important regulators of cardiomyocyte differentiation and heart development [22-24].

Primary skeletal-muscle disorders involve different groups of diseases, including muscular dystrophies, inflammatory myopathies and congenital myopathies. Although the number of genes that are involved in muscle disorders increases every year and histological pathology of disease tissue is well documented, the underlying molecular pathways remain poorly understood [25]. Recent studies have begun to link miRNAs to certain muscle-related diseases [6,13,15,26,27]. In a recent report, comprehensive miRNA expression profiling revealed that a total of 185 miRNAs were dysregulated in samples of diseased muscle tissue from 10 different muscle disorders. Five miRNAs (miR-146b, miR-221, miR-155, miR-214 and miR-222) were consistently regulated in almost all samples that were examined [15], suggesting a possible involvement of common miRNA-mediated regulatory mechanisms in muscle disorders. In addition to those studies of miRNA expression on muscle disorders, a direct genetic link has connected miRNA function to muscular hypertrophy [28]. A mutation that is responsible for the exceptional muscularity of Texel sheep has been mapped to a single G-to-A mutation within the 3' UTR of the mRNA encoding myostatin, a member of the transforming growth factor- β (TGF β) family, which functions to repress muscle growth. This mutation creates a binding site for miR-1 and miR-206, leading to the translational repression of myostatin, which phenocopies the "muscle doubling" that results from the loss of myostatin in mice, cattle, and humans [29,30]. These findings underscore the importance of miRNA-mediated regulation in diverse muscle biological processes and disease status.

In this study, we attempted to determine the function of miR-133 in skeletal muscle. We employed a gain-of-function approach and generated transgenic mice to overexpress miR-133a-1 in skeletal muscle, using the well-characterized muscle creatine kinase (MCK) promoter. Surprisingly, we found that miR-133a-1 transgenic mice appear to be normal. Additional analyses indicated that skeletal muscle development and function were not altered in miR-133a-1 transgenic mice. Our study therefore suggests that miR-133a is dispensable for skeletal muscle development.

Methods

Mice

The *mdx* mice which carry a point mutation in the dystrophin gene were obtained from the Jackson Lab [31]. Skeletal muscle was collected from the hind legs of 1

month old *mdx* and control mice for RNA extraction. In order to generate miR-133a-1 transgenic mice, a genomic fragment encoding the precursor and flanking sequences of the miR-133a-1 gene, which is located on mouse chromosome 18, was amplified by PCR using mouse genomic DNA as a template. The primers used for amplification are: miR-133a-1F: 5' AAGCTAGC-GAATTCATGTGACCCCTCACACACA 3'; miR-133a-1R: 5' TTCTCGAGACAAGGGGAGCCTG-GATCCC 3'. Underlined nucleotide sequences are added adaptors for restriction enzyme digestion. The same primers were used for genotyping of transgenic mice.

The DNA fragment was cloned into a transgenic vector plasmid driving by a muscle-specific muscle creatine kinase (MCK) promoter [32]. The miR-133a-1 transgenic construct was injected into the pronuclei of C57/Bl6 X C3H hybrid embryos and implanted into pseudo-pregnant recipient females by the University of North Carolina Animal Models Core. Five positive founder lines were obtained.

Ethics Statement

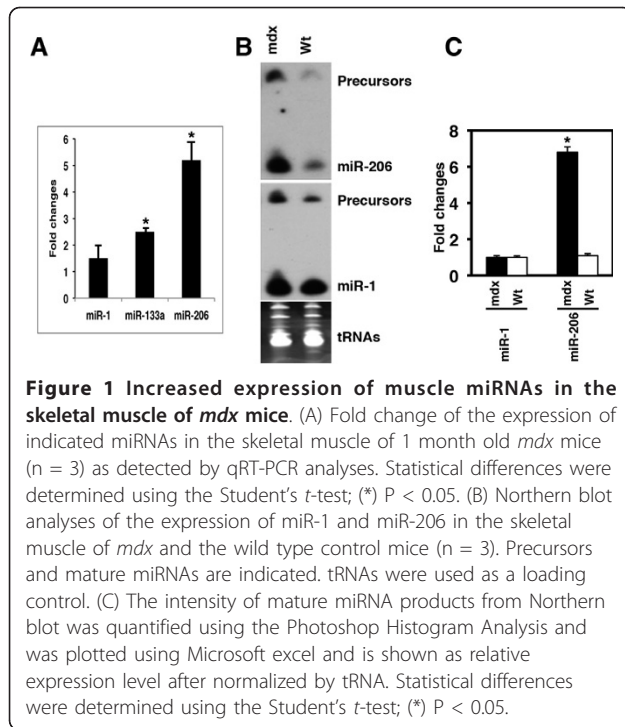
All animal procedures were approved by and performed in accordance with the University of North Carolina Institutional Animal Care and Use Committee under the protocol 08-227.0.

Northern Blot and RT-PCR Analysis

Taqman-based miRNA quantitative RT-PCR (Applied Biosystems) was performed as described [33] using total RNAs isolated from skeletal muscle of 1 month old *mdx* and the control mice (n = 4). MicroRNA Northern blot analyses were performed as described previously [8,18]. Briefly, 20 μ g of total RNAs isolated from skeletal muscle of 1 month old *mdx* and the control mice (Figure 1), or from the heart, skeletal muscle and liver tissues of miR-133a-1 transgenic and the control mice (Figure 2), were used and miRNA oligonucleotides with corresponding miRNAs (miR-1a, miR-133a and miR-206) sequences were used as probes. qRT-PCR was repeated three times and Northern blots were performed two times on skeletal muscle of four mice per genotype tested.

Histological and immunohistochemistry analyses

Histological processing and immunohistochemistry staining of skeletal muscle tissues were performed as described previously [8,34]. Samples were stained with Hematoxylin and Eosin (H&E) for routine examination. Laminin conjugated staining was applied to identify sarcolemmal membranes so that myofiber diameter could be directly visualized. Histological and immunohistochemistry analyses were performed on least 4 animals

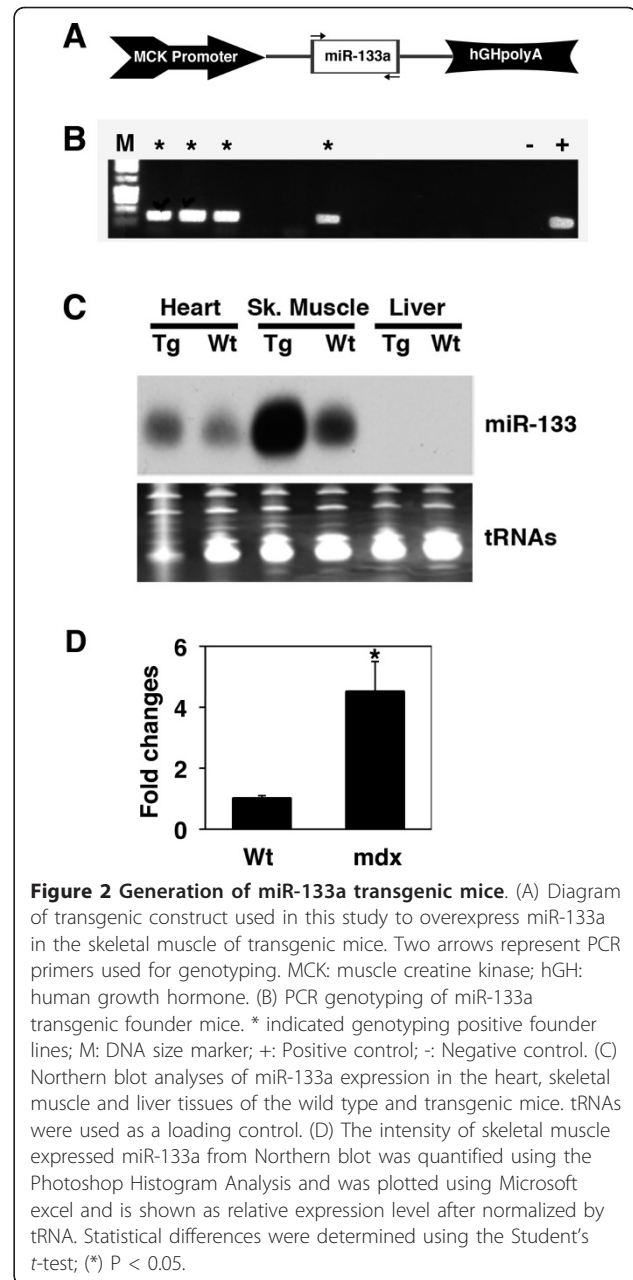


per genotype and repeated independently. All images were acquired by a camera (UFX-DX; Nikon) mounted on an inverted (TE2000, Nikon) or an upright fluorescence microscope (Microphot-SA; Nikon). Digital fluorescent images were captured at room temperature and the images were processed using SPOT (version 3.5.4 for MacOS; Diagnostic Instruments) software and were scaled down and cropped in Photoshop (Adobe) to prepare the final figures. Gel images were quantified using the Photoshop Histogram Analysis and was plotted using Microsoft excel and is shown as relative expression level after normalized by controls.

Results

Changed expression of muscle miRNAs in the skeletal muscle of *mdx* mice

Mdx mice harbor a spontaneous mutation in the dystrophin gene [31]. The skeletal muscle of the *mdx* mice is histologically normal early in postnatal development, but starting around 3 weeks muscle necrosis develops with some visible muscle weakness. Therefore, *mdx* mice have been widely used as an animal model to study the skeletal muscle degeneration/regeneration process [35-37]. We examined miRNA expression in the skeletal muscle of *mdx* mice. We found that the expression levels of miR-1, miR-133 and miR-206 were higher in the skeletal muscle of one month-old *mdx* mice (Figure 1A). Northern blot analyses further confirmed a significantly increased expression of miR-206, whereas the



expression of miR-1 modestly increased in the muscle of *mdx* mice (Figure 1B, C). These data indicate that these muscle miRNAs might be involved in the regulation of skeletal muscle degeneration and/or regeneration.

Transgenic overexpression of miR-133a-1 in the skeletal muscle

Recently, miR-133 genes (miR-133a-1 and miR-133a-2) were knocked out from the mouse genome. Analysis of mice that lost either miR-133a-1 or miR-133a-2 revealed that both miRNAs are dispensable for development or

viability under normal physiological conditions. However, double knockout mice display defects in the heart [24]. In order to further investigate the function of miR-133 in vivo, we took a gain-of-function approach and generated transgenic mice to overexpress miR-133a-1 in skeletal muscle. We used the well-characterized muscle creatine kinase (MCK) promoter to drive miR-133a-1 expression in skeletal muscle (and to a less extent, cardiac muscle). Genomic DNA from mouse chromosome 18 encoding the miR-133a-1 gene was inserted into an expression vector (Figure 2A). The MCK-miR-133a-1 transgenic construct was injected into fertilized mouse eggs and multiple transgenic founder lines were obtained, as verified by PCR genotyping (Figure 2B). The overexpression of miR-133a-1 in germline-transmitted stable transgenic mice was confirmed by Northern blot analyses. Total RNAs were isolated from indicated tissues, and the overexpression of miR-133a-1 was clearly detected in the skeletal muscle, and to a much less extent, the cardiac muscle, but not in the liver of transgenic mice (Figure 2C, D).

Normal skeletal muscle development in miR-133 transgenic mice

All miR-133a-1 transgenic mice were viable and fertile without overt abnormality (Figure 3A, B). The body weight and size between wild type and miR-133a-1 transgenic adult mice (ages from 2 to 12 months) were indistinguishable ($n = 40$, data not shown). There was not difference in skeletal muscle formation or body fat deposit between miR-133a-1 transgenic mice and their littermate controls (Figure 3C, D). Because the MCK promoter also directed miR-133a-1 overexpression in the heart, albeit at lower level, we determined whether heart development was affected in the transgenic mice.

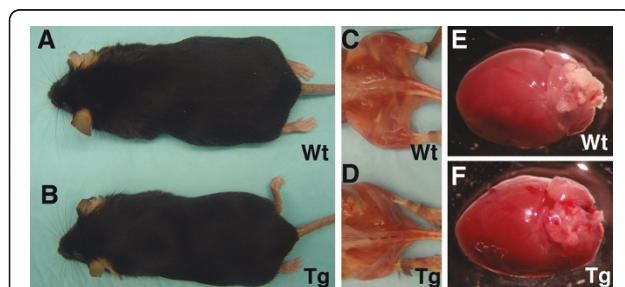


Figure 3 Normal cardiac and skeletal muscle development in miR-133a transgenic mice. (A-B) The gross morphology of a 6-month old miR-133a transgenic (Tg) mouse is normal and indistinguishable from its wild type (Wt) littermate ($n = 4$). (C-D) Representative de-skinned mice show normal skeletal muscle morphology in miR-133a transgenic mice (Tg) when compared with the wild type littermate (Wt). (E-F) The heart of miR-133a transgenic mouse (Tg) is indistinguishable from that of the wild type (Wt) littermate.

However, our results showed that there was no difference in the gross morphology of the adult hearts of miR-133a-1 transgenic mice and wild type controls (Figure 3E, F).

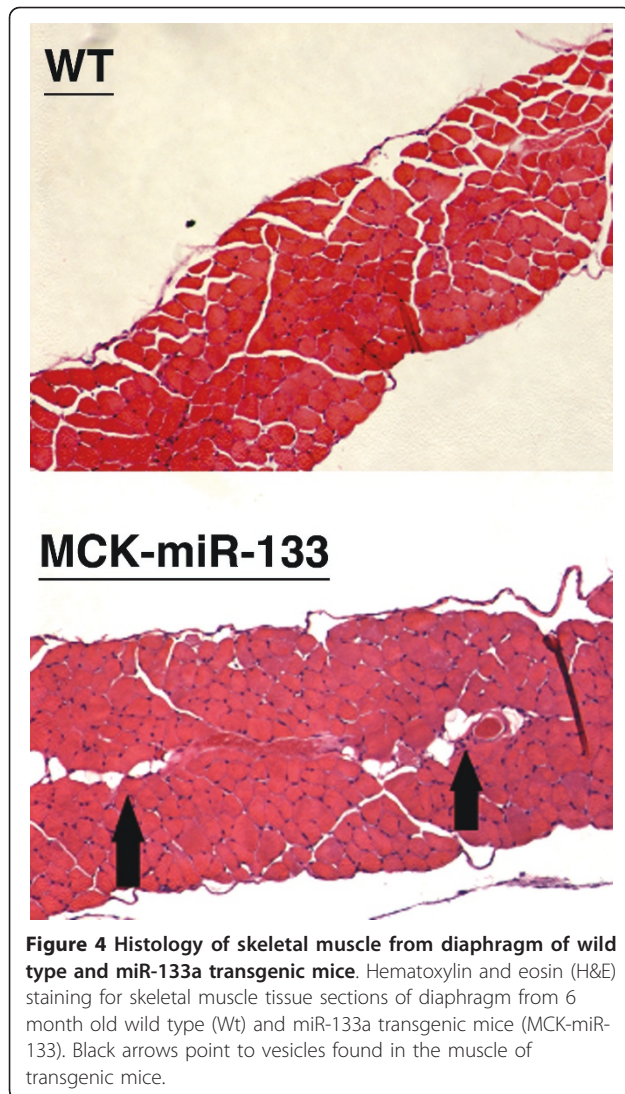
In order to further analyze muscle development, skeletal muscle from the diaphragms of six month old miR-133a-1 transgenic mice was collected and examined by tissue histology ($n = 4$). As shown in Figure 4, hematoxylin and eosin (H&E) staining of diaphragms indicated that the tissue thickness, muscle cell size and numbers were comparable between miR-133a-1 transgenic mice and the control wild type mice. It is noticeable that there is a slight increase in the vesicles in the transgenic diaphragm (Figure 4, arrows).

Similarly, we examined the skeletal muscle of the extensor digitorum longus (EDL) from six month old of both miR-133a-1 transgenic and wild type control mice ($n = 4$). H&E staining suggested that there was no difference in skeletal muscle morphology between miR-133a-1 transgenic mice and their control littermates (Figure 5A). Immunohistochemistry using an antibody that specifically recognizes laminin, a structural and biologically active component in basement membranes which labels the cell membrane, confirmed that the size and morphology of skeletal muscle in transgenic mice were indistinguishable from that of controls (Figure 5B). Together, these data suggest that miR-133a is dispensable for the normal development and function of skeletal muscle.

Discussion

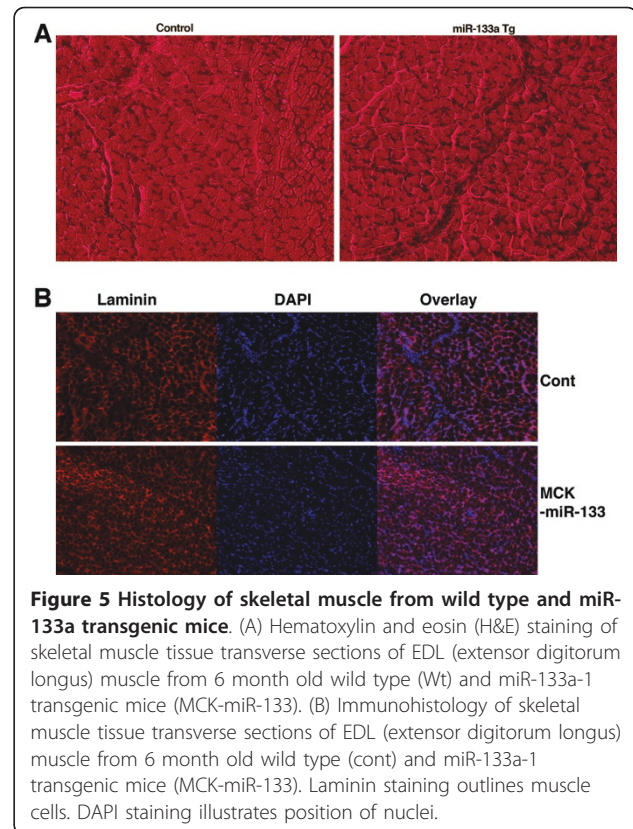
In this study, we investigated the function of miR-133a in skeletal muscle. We found that the expression miR-133a, together with that of miR-206 and miR-1a, was induced in the skeletal muscle of *mdx* mice. However, transgenic overexpression of miR-133a-1 in skeletal muscle did not result in a noticeable change in skeletal muscle development and morphogenesis. Our results are consistent with a recent report in which miR-133 loss-of-function mice did not induce overt defects in skeletal muscle [24].

It is known that many miRNAs are tissue-specifically expressed. Among them, miR-1, miR-133, miR-206, miR-208 and miR-499 have been described as muscle specific miRNAs, or myomiRs [6,13]. In addition to their muscle specific expression in normal physiological condition, the expression of some of those myomiRs was shown to be dynamically regulated in pathological and/or diseased muscles. For example, miR-206 levels are elevated in the diaphragm muscle of the *mdx* mouse, an animal model of muscular dystrophy [27]. Furthermore, miR-1 and miR-206 also participate the regulation of skeletal muscle satellite cell proliferation and differentiation [8]. Both gain- and loss-of-function



studies have started to uncover the *in vivo* function of those muscle miRNAs [10,23,34,38-41]. Interestingly, gene targeting of some of the myomiRs demonstrated that they are dispensable for the normal development and function of cardiac and skeletal muscles [10,40]. However, many of those miRNAs are indispensable for stress-responsive muscle remodeling. Recently, we reported that overexpression of miR-208a in the heart, which is normally restricted to cardiac tissue, was sufficient to induce cardiac hypertrophy in transgenic mice [34]. However, in the current study, we did not observe any overt muscle defect in mice expressing the MCK-miR-133a-1 transgene. This may suggest that cardiac and skeletal muscle have adapted distinct requirement for miRNAs for tissue homeostasis.

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 is apparent that many new and unanticipated functions of miRNAs in normal muscle development, function and disorders are waiting to be discovered. Considering that many miRNAs fine-tune gene-expression programs and the intrinsic complexity of miRNA functional models [42,43], it will be important in the future to systematically analyze the potential function of miRNAs in both gain- and loss-of-function animal models.

Conclusions

In this study, we demonstrate that miR-133a is dispensable for the normal development and function of skeletal muscle.

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Authors' contributions

ZD and DZW designed the experiment; ZD and JFC performed experiments; ZD and DZ. DZW analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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