MicroRNA-24-3p promotes skeletal muscle differentiation and regeneration by regulating high mobility group AT-hook 1

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Running title: miR-24-3p/HMGA1 axis regulates skeletal muscle differentiation and regeneration.

Keywords: myoblast, skeletal muscle, differentiation, regeneration, microRNA, miR-24-3p, HMGA1.

Abstract

Skeletal muscle regenerates throughout the lifetime to maintain normal development, growth, and physiological function. Skeletal muscle regeneration occurs in a coordinated fashion and requires strict regulation of myogenic gene expression during the process. Numerous studies have established the critical role of microRNAs in regulating post-transcriptional gene expression in diverse biological processes including differentiation, development, and regeneration. We have revealed in an earlier study that a large number of microRNAs were differentially expressed during myoblast differentiation. Here, we report the role of one such microRNA, the miR-24-3p, in skeletal muscle differentiation and regeneration. miR-24-3p is induced during myoblast differentiation and skeletal muscle regeneration. Exogenous miR-24-3p promotes while inhibition of miR-24-3p represses myoblast differentiation. miR-24-3p promotes myoblast differentiation by directly targeting and regulating the high mobility group AT-hook 1 (HMGA1). Consistent with the finding that HMGA1 is a repressor of myogenic differentiation, the miR-24-3p-resistant form of HMGA1 devoid of 3'untranslated region, inhibits myoblast differentiation. Intramuscular injection of antagomirs specific to miR-24-3p into the tibialis anterior muscle prevents HMGA1 down-regulation and impairs regeneration. These findings provide evidence for the requirement of the miR-24-3p/HMGA1 axis for skeletal muscle differentiation and regeneration.

Introduction

During postnatal skeletal muscle development and regeneration, the quiescent muscle stem cells (MuSCs) are activated to reenter the cell cycle, followed by proliferation to form a pool of myoblasts, which then differentiate and fuse into newly formed or existing myofibers[1,2]. This extensive process of making new muscle is known as myogenesis and is quintessential for the maintenance of normal physiological function. Dysregulation of the critical molecular and cellular events linked to skeletal myogenesis is associated with numerous muscle degenerative diseases. Most of the current understanding of the key myogenic processes including myoblast differentiation and skeletal muscle regeneration is mainly based on the regulation of myogenic transcription factors and signaling molecules[3-6]. However, it's not well understood how these factors of the myogenic network are regulated to maintain normal myogenesis and skeletal muscle function. In this context, an emerging area is the role of microRNAs in post-transcriptional gene regulation during skeletal myogenesis.

microRNAs are initially transcribed as a long primary transcript by RNA Polymerase II or III and then processed to pre-microRNAs by the microprocessor complex composed of the double-stranded RNA-binding protein DGCR8, the type III RNase DROSHA, and the DEAD-box RNA helicases p68 and p72[7-9]. The pre-microRNAs are then exported to the cytoplasm and further processed by a type III RNase Dicer, resulting in 22-27 nucleotides double-stranded mature microRNA duplexes[10,11]. The double-stranded microRNA is loaded to the RNA-induced silencing complex (RISC) containing the Argonaute (AGO) family of proteins where one strand becomes the mature microRNA[12]. The mature microRNA guides the RISC to the 3' untranslated region (3'UTR) of its target mRNAs and causes degradation of mRNAs and/or repression of translation[13].

Not until recently, we have begun to elucidate the role of microRNAs in skeletal muscle differentiation, development, and muscle degenerative diseases[14-25]. Many studies including our own have revealed that a large number of microRNAs are differentially expressed during myoblast differentiation[15-22,24,25]. The majority of the studies has demonstrated the role of a subset of these microRNAs in muscle differentiation by overexpression and knockdown experiments in C2C12 myoblast cell lines. The earlier studies have shown that miR-1 and miR-206 sharing the identical target binding seed sequences promote myoblast differentiation by regulating histone deacetylase 4 (HDAC4) and DNA polymerase 1 (Pola1)[24,25]. Consistent with the fact that a single microRNA can target multiple target mRNAs, miR-206 has been shown to regulate myoblast differentiation by regulating additional direct targets including paired box 7 transcription factor (Pax7), connexin 43 (cx43), follistatinlike 1(Fstl1), utrophin (Utrn), estrogen receptor alpha (ERa), butyrate-induced transcript 1 (Bind1), monocyte-to-macrophage differentiation-associated protein (Mmd), and cMET. miR-26a promotes myoblast differentiation by regulating TGFb/BMP pathway

downstream Smad1 and Smad4 transcription factors[16] and a histone methyltransferase called enhancer of zeste homologue 2 (EZH2)[26]. miR-181 was shown to induce muscle differentiation by repressing HoxA11[27]. miR-27b induce muscle differentiation by represses Pax3 and myostatin[22,28]. In addition to targeting the well-known myogenic repressors, cell cycle regulators, and histone modifying enzymes, microRNAs have been shown to regulate the critical myogenic transcription factors. These includes muscle stem cell factor Pax7, myogenin, MHC, and MRFs. On the other hand, certain microRNAs are downregulated during myogenesis and repress differentiation. miR-125b is one such microRNAs that negatively regulate myoblast differentiation by targeting insulin-like growth factor 2 (IGF-II)[29]. Despite miR-133 is upregulated during myoblast differentiation, it was paradoxically shown to promote promotes myoblast proliferation by repressing serum-response-factor[24]. miR-133 also regulates splicing patterns during myoblast differentiation by an alternative splicing factor, n-polypyrimidine tract-binding[30]. Many of the microRNA seed sequences are conserved among species and are also important for muscle differentiation in nonmammalian organisms including Drosophila[31,32] and Caenorhabditis elegans[33]. microRNAs involved in muscle differentiation has been recently reviewed[34].

We have shown in our earlier studies that a number of microRNAs including miR-26a, miR-206, miR-322/424, miR-378, miR-486, and miR-503 are induced during myoblast differentiation and promotes myoblast differentiation by regulating critical myogenic regulatory factors[15-21,25,26]. Intriguingly, we have found that H19 non-coding RNA generates miR-675-3p and miR-675-5p and promotes muscle differentiation[17]. Through ChIP-seq analysis we have demonstrated that important myogenic transcription factors including MyoD and Mef2c repress their repressors by inducing myogenic microRNAs[15,20]. These examples suggest that microRNAs tightly regulate many critical factors of myogenic network and maintain the balance between proliferation and differentiation and perhaps this tightly regulation is required for normal muscle physiology.

Despite many studies have demonstrated the role of microRNAs in myoblast differentiation, the role of these myogenic microRNAs in skeletal muscle development and regeneration in animals remains limited. Skeletal muscle-specific deletion of a microRNA biogenesis enzyme Dicer caused perinatal lethality associated with reduced skeletal muscle mass and defective muscle fiber morphology suggesting an important role of myogenic microRNAs during skeletal muscle development in animals[35]. Surprisingly, germline deletion of muscle-specific miR-206 did not show any significant defects in skeletal muscle development[36]. However, the deletion of miR-206 in the amyotrophic lateral sclerosis (ALS) mouse model accelerates the disease progression[36]. We have shown that miR-26a is essential for skeletal muscle development and regeneration in mice. We have further shown that H19 long non-coding RNA-derived miR-675-3p and miR-675-5p were critical for skeletal muscle regeneration after injury[17]. These findings suggest that there

is a great need to determine the role of microRNAs in myogenesis *in vivo* to expand our knowledge for the development of new approaches for muscle degenerative diseases.

Here we report that miR-24-3p is highly abundant in the skeletal muscles and up-regulated during myoblast differentiation and muscle regeneration. An earlier study also suggests that this microRNA is up-regulated during myoblast differentiation and promotes differentiation *in vitro*. However, it remains unknown how miR-24-3p promotes myoblast differentiation and whether it has any role during myogenesis *in vivo*. Here we show that miR-24-3p promotes myoblast differentiation by directly targeting and regulating the high mobility group AT-hook 1 (HMGA1). More importantly, intramuscular injection of antagomirs specific to miR-24-3p into the tibialis anterior muscles prevents HMGA1 down-regulation and impairs regeneration. Thus, our study not only establishes the role of a myogenic microRNA in skeletal muscle function *in vivo*, but it also elucidates the mechanism by which this myogenic microRNA regulates muscle differentiation and regeneration by inhibiting a well-established repressor of myogenesis.

Materials and Methods

Cell culture

Mouse C2C12 myoblast cell line was purchased from American Type Culture Collection (ATCC)[37]. These cells were cultured at subconfluent densities in a growth medium (GM) made up of DMEM (ATCC) supplemented with 10% heat-inactivated FBS (ATCC) and 1X Antibiotic-Antimycotic (Thermo Scientific). These cells were differentiated into myocytes or myotubes in differentiation medium (DM)[38]. The DM was made up of DMEM containing 2% heat-inactivated horse serum (Hyclone) and 1X Antibiotic-Antimycotic (Thermo Scientific). Mouse primary myoblast cells were isolated from C57/BL6 mice following our standard protocol as described earlier. Mouse primary myoblast cells were differentiated as described[15]. U2OS cells were cultured in DMEM (ATCC) supplemented with 10% heat-inactivated FBS (ATCC) and 1X Antibiotic-Antimycotic (Thermo Scientific).

Isolation of total RNA and performance of qRT–PCR

We isolation of total RNAs using Trizol reagent (Invitrogen) or RNeasy minikit (Qiagen) following the manufacturer's instructions. We carried out cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad) as instructed. We performed qRT-PCR using the Sybr green PCR master mix (Bio-Rad) in a Bio-Rad thermal cycler using specific primers for Myogenin, Myosin Heavy Chain (MHC), and HMGA1. We used RSP13 or GAPDH primer pairs as a housekeeping gene for normalizing the values of Myogenin, MHC, and HMGA1. We carried out qRT-PCR for miR-24-3p using the miRCURRY LNA kit as described (Qiagen).

Luciferase assays

We first transfected U2OS osteosarcoma cells with miR-24-3p or GL2 and after 24 hours we transfected the same cells with luciferase plasmids. pGL3 (Promega) was co-transfected as an internal transfection control. We harvest the cells after another 48 hours and performed luciferase assays with a dual-luciferase reporter assay system using a GloMax luminometer (Promega) following our established protocol[15,16]. We first normalized Renilla luciferase (rr) values to the co-transfected pGL3 control firefly Photinus pyralis (pp) luciferase values. Then we normalized each rr/pp value in the miR-24-3p-transfected samples with the rr/pp values obtained from GL2-transfected samples.

Western blotting and antibodies

We harvested the cells, washed with 1X PBS, and lysed in NP40 lysis buffer (50mMTris-HCl, 150mMNaCl, 0.1% NP-40, 5mM EDTA, 10% glycerol) with protease inhibitors cocktail (Sigma). We separated proteins in SDS-PAGE, transferred, and immunoblotted with various antibodies.

The antibodies used were anti-HMGA1 (dilution 1:500; Cell Signaling), anti-Myogenin (dilution 1:500; Santa Cruz) and anti-MHC (dilution 1:3000; Sigma), and anti-GAPDH (dilution 1:10000; Sigma).

Immunocytochemistry and immunohistochemistry

We carried out immunocytochemistry following our standard protocol as described previously[16,39]. Briefly, we grew the cells on sterile glass coverslips and fixed with 2% formaldehyde in PBS for 15 min. Next, we permeabilized the cells with 0.2% Triton X-100 and 1% normal goat serum in ice-cold PBS for 5 min and blocked with 1% NGS in PBS twice for 15 min. We incubated the fixed and blocked cells with primary antibody (myogenin 1:50; Santa Cruz Biotechnology and MHC 1:400; Sigma, in 1% NGS) for 1 hour. We washed the cells twice with 1X PBS and incubated with FITC-conjugated anti-mouse IgG (dilution 1:500; Dako Cytomation) for 1 hour. We washed the cells again twice and counterstained the nuclei with DAPI while mounting the coverslips on a glass slide (H-1200, Vector Laboratories). Images were captured with an Olympus Hi-Mag microscope. H&E staining was carried out using a standard protocol and bright-field images were captured using an Olympus microscope.

Skeletal muscle regeneration model and TA muscle injection

We developed an injury-mediated mouse model of skeletal muscle regeneration by intramuscular injection of cardiotoxin (CTX) from Naja nigricollis (EMD Millipore) following our established procedure as described earlier[16,17]. Briefly, about 10-week-old male C57/BL6 mice were injected on TA muscles with 100 ul of 10 uM CTX. This high volume of injection materials, the pressure during injection, and post-injection massages spread the injected material throughout the TA compartment. However, we performed our analysis on the middle two-thirds of the TA muscle that was closest to the injection site. We injected 100 ul of Ant-miR-24-3p into the TA muscle of one leg, and Ant-NC into the contralateral leg 3 d after the CTX injection. Mice were anesthetized and sacrificed by cervical dislocation to harvest muscle samples at different time intervals.

Statistical analyses

Data are presented as the mean+/- standard deviation of three or more replicates. Two-tailed Student's t-test was employed to determine P-values.

Results

miR-24-3p is abundantly expressed in adult skeletal muscles and regulated during myoblast differentiation and skeletal muscle regeneration

Using a transcription-wide screening for microRNAs we identified a number of microRNAs, which were differentially expressed during C2C12 myoblast differentiation[15]. One of these upregulated microRNAs, the miR-24-3p, had been shown to induce C2C12 myoblast differentiation *in vitro*[39]. However, it remained undetermined how miR-24-3p promotes myoblast differentiation and whether it plays any role in skeletal myogenesis *in vivo*. We focused on miR-24-3p to reveal its function and molecular mechanism in skeletal muscle differentiation and regeneration *in vivo* using a mouse model. We first confirmed that miR-24-3p was indeed upregulated during C2C12 myoblast differentiation (Suppl Fig 1). We also determined that miR-24-3p was also upregulated during physiologically more relevant mouse primary myoblast differentiation (Fig 1A). We then compared the expression level of miR-24-3p in skeletal muscle with various mouse tissues. We found that miR-24-3p is abundantly expressed in skeletal muscles as compared to the other mouse tissues analyzed (Fig 1B). We then examined the expression of miR-24-3p during skeletal muscle regeneration. We generated a well-accepted cardiotoxin (CTX)-mediated mouse regeneration model[16,17,40] (Fig 1C). We observed a degeneration phase when quiescent muscle stem cells (MuSCs) are activated, re-enter the cell cycle, and increase myoblast numbers after the injury (Fig 1C). This phase is followed by a regeneration phase when myoblasts differentiate, fuse to make new myofibers (Fig 1C). miR-24-3p decreased rapidly on days 1 through 3 (degeneration phase) and increased on days 5 through 14 (regeneration phase) (Fig 1D). These findings suggest that miR-24-3p plays a role in myoblast differentiation and skeletal muscle regeneration.

miR-24-3p promotes myoblast differentiation

Since MuSC-derived myoblast differentiation is a key step in postnatal muscle development and regeneration, we assessed the function of miR-24-3p in primary myoblast differentiation. We first confirmed an earlier report that miR-24-3p promotes C2C12 myoblast differentiation (Suppl Fig 2). Then we extend the role of miR-24-3p during mouse primary myoblast differentiation. We transfected primary myoblast cells with mature miR-24-3p duplexes or GL2, an identical length of RNA duplexes from the luciferase gene, as a negative control in growth medium (GM). Myoblast cells were transfected twice at 24-hour intervals in GM, and the cells were then transferred to differentiation medium (DM). Immunostaining was carried out in these cells for Myogenin at 24 hours and for MHC at 48hours. The transfection of exogenous miR-24-3p increased differentiation, as seen by the morphology and the number of Myogenin- and MHC-positive cells

(Fig. 2A, B). We also transfected primary myoblast cells with GL2 or miR-24-3p as above, held the cells in GM for an extra 24 hours, and carried out qRT-PCR for Myogenin and myosin heavy chain (MHC). Both Myogenin and MHC mRNA were up-regulated in miR-24-3p-transfected cells even when the culture was maintained in GM (Fig. 2C). These results demonstrate that miR-24-3p promotes primary myoblast differentiation.

We then asked whether miR-24-3p is required for primary myoblast differentiation. We transfected primary myoblast cells twice at 24-hour intervals with 2'-O-methyl antisense oligonucleotides specific to GL2 (Anti-GL2) or miR-24-3p (Anti- miR-24-3p) and then transferred the cells into DM. We carried out immunostaining using these cells for Myogenin at 24 hours and for MHC at 48 hours. Anti-miR-24-3p decreased the appearance of myogenic differentiation markers Myogenin and MHC in primary myoblast cells (Fig 3A, B). We also quantitated Myogenin and MHC mRNA levels in these cells by qRT-PCR. Both Myogenin and MHC mRNA levels were significantly decreased in the anti-miR-24-3p-transfected primary myoblast cells. These findings suggest that miR-24-3p is essential for myoblast differentiation.

miR-24-3p promotes muscle differentiation by directly targeting and regulating HMGA1

To determine how miR-24-3p promotes myoblast differentiation, we revealed its direct target relevant to myogenesis. The microRNA target prediction algorithm miRanda revealed several genes including HMGA1 as potential targets for miR-24-3p (Fig 4A). We chose HMGA1 to investigate further its important role in myogenesis[41]. To determine whether HMGA1 3'UTR has true target sites for miR-24-3p, we fused HMGA1 3'UTR to a luciferase reporter gene driven by the cytomegalovirus (CMV) promoter. The co-transfection of miR-24-3p with HMGA1 3'UTR luciferase construct repressed the luciferase activity two-fold (Fig. 4B). Mutation of the predicted target site in HMGA1 3'UTR relieved the repression very mildly (Fig 4B), suggesting an unconventional (non-seed-match) target site/s for miR-24-3p in the HMGA1 3'UTR. The non-seed-match target sites have been reported by us and others earlier[39,42]. To confirm this notion, we co-transfected HMGA1 3'UTR with a mutated form of miR-24-3p duplexes. Our results revealed that repression of luciferase activity by miR-24-3p was entirely rescued with this mutant form of miR-24-3p (Fig 4C), suggesting that miR-24-3p targets HMGA1 3'UTR through an unusual non-seed-match target site/s.

To further confirm that HMGA1 is indeed a true target of miR-24-3p, we transfected primary myoblast with GL2 or miR-24-3p twice at 24-hour intervals in GM and harvested the cells for Western blotting for HMGA1. We found that HMGA1 protein level was drastically downregulated by miR-24-3p in these samples (Fig 4D). In a reciprocal experiment, inhibition of miR-24-3p using 2'-O-methyl antisense inhibitors of miR-24-3p in DM caused longer persistence of endogenous

HMGA1 protein during primary myoblast differentiation (Fig 4E). These findings demonstrate that miR-24-3p is indeed responsible for the repression of HMGA1 protein during myoblast differentiation.

Having demonstrated that HMGA1 is an important target of miR-24-3p, we next asked whether transfection of the HMGA1 devoid of its 3'UTR (HMGA1 ORF) is resistant to this microRNA and suppresses miR-24-3p-mediated myoblast differentiation. We first confirmed that the exogenous HMGA1 persisted in DM and was resistant to miR-24-3p. As expected, we found that the primary myoblast cells containing an empty vector differentiate following normal kinetics but HMGA1 devoid of its 3'UTR represses myoblast differentiation as shown by decreased level of MHC expression (Fig 4F). These findings together confirm that HMGA1 is a bonafide target for miR-24-3p and miR-24-3p promotes myoblast differentiation by directly targeting and regulating HMGA1.

miR-24-3p is essential for adult muscle regeneration

We developed a well-established injury-mediated mouse model of skeletal muscle regeneration by intramuscular injection of CTX into TA muscles. This model first induces injury and later regeneration and thus allows us to study adult muscle regeneration (Fig 1C). As described earlier, miR-24-3p is down-regulated on days 1-3 after CTX-mediated injury (degeneration phase) and then gradually increases as new muscles are formed (Fig 1D). We examined HMGA1 levels in these samples and found an expression pattern anti-correlated to miR-24-3p, increasing on days 1-3 after injury and then decreasing steadily on days 5-14 after injury (Fig 5A). These findings suggest that miR-24-3p regulates HMGA1 during regeneration.

Next, we determine the role of miR-24-3p during skeletal muscle regeneration. Since miR-24-3p was up-regulated on days 5-14 after CTX injury, we injected antagomiR inhibitors of miR-24-3p (Ant- miR-24-3p) into the TA muscles of one leg on day 3. Similarly, we injected Ant-NC into TA muscles of the contralateral legs. On day 5 after injury, the regeneration process was at the very early stage, where new myofiber formation had just begun and therefore we examined the impact of Ant-miR-24-3p injection on 7 and 14 days after injury. We first confirmed that the miR-24-3p level remained repressed during the regeneration phase (Fig 5B). In the Ant-NC injected TA muscles, most of the damaged myofibers regenerate following normal kinetics and we observed normal muscle morphology was mostly restored by day 14 (Fig 5D). In contrast, the regeneration kinetics were delayed with numerous inflammatory cells and damaged myofibers persisting in the Ant-miR-24-3p-injected muscles on days 7-14 after injury (Fig 5E, F). These findings suggest that miR-24-3p plays a critical role during skeletal muscle regeneration *in vivo*.

Discussions

Most of the current understanding of skeletal muscle development and regeneration is based on the regulation of the myogenic transcription factors and signaling molecules[3,43-50]. However, it remains elusive how these critical factors of the fundamental myogenic processes are themselves regulated. Molecular mechanisms underlying the regulation of gene expression during skeletal muscle development and regeneration are not completely understood, hindering the development of therapeutic interventions for muscle degenerative diseases. To fill this knowledge gap, we have examined the role of a myogenic microRNA in myoblast differentiation and skeletal muscle regeneration. Thus far, the majority of the studies have demonstrated the role of microRNAs in C2C12 myoblast differentiation *in vitro*[15,21,22,25,27,51-58]. Only a limited number of studies including ours have revealed the precise function of microRNAs using animal models of muscle development and regeneration[16,17,23]. In this study, we demonstrated the role of miR-24-3p in muscle differentiation and regeneration in mice. We revealed that miR-24-3p is highly abundant in the skeletal muscles and up-regulated during myoblast differentiation and muscle regeneration. An earlier study also suggests that this microRNA is up-regulated during myoblast differentiation and promotes differentiation *in vitro*[59]. However, it remains unknown how miR-24-3p promotes myoblast differentiation and whether it has any role in myogenesis *in vivo*. Here we demonstrated that miR-24-3p promotes myoblast differentiation by directly targeting and regulating HMGA1, a well-known repressor of myogenesis[41]. More importantly, we have shown that miR-24-3p is essential for skeletal muscle regeneration. Our study establishes the role of a myogenic microRNA in skeletal muscle function *in vivo* and also provides mechanistic insights into how a myogenic microRNA sculpts myogenesis by regulating a well-established repressor of myogenesis.

HMGA1 protein is a prominent member of the high mobility group A (HMGA) proteins that are preferentially expressed in the proliferating embryonic tissues but usually are absent in differentiated cells[60,61]. An earlier study has shown that HMGA1 down-regulation is an essential step for C2C12 myoblast differentiation[41]. Interestingly, the exogenous expression of HMGA1 prevents myoblast differentiation. Mechanistically, HMGA proteins regulate gene transcription through the architectural modulation of chromatin and the formation of multi-protein complexes on promoter or enhancer regions of specific target genes. However, further studies are required to determine whether HMGA1 takes part in specific gene regulation locally and/or affecting chromatin structures globally during myogenesis. Nevertheless, the sustained expression of HMGA1 in these cells caused down-regulation of promyogenic genes including MyoD, myogenin, Igf1, Igf2, Igfbp1-3, and up-regulation of the myogenic repressor Msx1[41]. Consistent with this, HMGA1 is involved in the differentiation of several cell types including embryonic lympho-

hematopoietic differentiation[62], sperm cell differentiation[63], and adipogenic differentiation[64]. These findings suggest that a strictly regulated expression of HMGA1 is critical for optimal cellular differentiation and function including myogenic differentiation. Our study revealed how this important factor is regulated *in vitro* and *in vivo* by a myogenic microRNA. Our studies suggest that miR-24-3p/HMGA1 axis is essential for normal myogenesis. Since the loss of Hmga1 gene function affects specific differentiation processes[61] and Hmga1 knockout mice develop diseases including type 2 diabetes[65], cardiac hypertrophy[66] and myelo-lymphoproliferative disorders[62], we expect that dysregulation of miR-24-3p/HMGA1 axis may lead to various muscle degenerative diseases including Duchenne muscular dystrophy (DMD).

Only a limited number of studies implicating the role of microRNAs in skeletal muscle function in animal development. This prompted us to study the function of miR-24-3p in skeletal muscle differentiation and regeneration in mice. Although, the defective skeletal muscle development in the muscle-specific deletion of Dicer suggesting a critical role for microRNAs during skeletal muscle development in animals[35], there some inconsistencies in the published literature on whether specific microRNAs are essential for these processes. The germline deletion of muscle-specific miR-206 did not show any significant defects in skeletal muscle development but exerts only a mild effect on skeletal muscle innervation following injury[36]. Although miR-1 is expressed in the skeletal muscles, miR-1 knockout mice showed defects only in the cardiac muscle development but no visible phenotypes were observed in skeletal muscles[67]. These discrepancies may happen due to the functional redundancy between miR-1 and miR-206 as they share the seed sequences. In another study, miR-206 knockout delays skeletal muscle regeneration following CTX injury[68] . We have earlier demonstrated that miR-26a and H19-derived miR-675-3p and miR-675-5p play essential roles in skeletal muscle development and regeneration[16,17]. Now we have added another important myogenic microRNAs to the list. Further studies will be required to explore the changes in their expression levels and role in muscle degenerative diseases. Nonetheless, our findings elucidated an essential role of miR-24-3p in skeletal muscle differentiation and regeneration *in vivo*.

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Figure Legends

Figure1: miR-24-3p is abundantly expressed in the adult skeletal muscles and upregulated during myoblast differentiation and muscle regeneration. (A) miR-24-3p is upregulated during mouse primary myoblast differentiation. qRT-PCR of miR-24-3p was carried out from the samples isolated after grown in indicated days in differentiation medium (DM). miR-24-3p values were normalized to U6sn RNA values and fold change of miR-24-3p is expressed relative to the primary myoblast cells grown in growth medium (GM). (B) miR-24-3p is abundantly expressed in the adult skeletal muscle. qRT-PCR was performed with RNA from various mouse tissues to detect miR-24-3p. (C) H&E staining of tibialis anterior (TA) muscle cross-sections from resting control (non-injected) and days 1, 3, 5, 7, and 14 post-injury. (D) miR-24-3p is downregulated on days 1-3 post-injury and up-regulated on days 5-14 post-injury caused by intramuscular injection of CTX. The values are expressed as Mean+/-SD of three measurements.

Figure 2: miR-24-3p promotes primary myoblast differentiation. (A) Primary myoblast cells were transfected twice at 24-hour intervals with GL2 and miR-24-3p in GM, and the cells were then transferred to DM. Immunostaining was carried out in these cells for Myogenin at 24 hours and for MHC at 48 hours. Green indicates Myogenin or MHC and blue indicates nuclei stained by DAPI. (B) Fractions of Myogenin and MHC-positive cells are presented relative to the GL2 control as 100%. (C) Primary myoblast cells transfected with GL2 or miR-24-3p as above and held in GM for an extra 24 hours. qRT-PCR was carried out for Myogenin and MHC. Myogenin and MHC values were normalized to RSP13 in the same sample and then again to the level in GL2 transfected samples. The values are expressed as Mean+/-SD of three measurements.

Figure 3: miR-24-3p is required for primary myoblast differentiation. (A) Primary myoblast cells were transfected twice at 24-hour intervals with 2'-O-methyl antisense oligonucleotides specific to GL2 (Anti-GL2) or miR-24-3p (Anti- miR-24-3p) and the cells were then transferred to DM. Immunostaining was carried out using these cells for Myogenin at 24 hours and MHC at 48 hours. (B) Fractions of Myogenin and MHC-positive cells are presented relative to the Anti-GL2 control as 100%. (C) qRT–PCR was carried out for Myogenin and MHC in these samples. Myogenin and MHC values were normalized to GAPDH in the same sample and then again to the level in Anti-GL2 transfected samples. The values are expressed as Mean+/-SD of three measurements.

Figure 4: miR-24-3p promotes myoblast differentiation by directly targeting and regulating HMGA1. (A) The microRNA target prediction program miRanda predicts a miR-24-3p binding site on HMGA1 3'UTR. (B) Luciferase assays were performed to measure the effect of transfected miR-24-3p on a Renilla luciferase reporter fused to wild type and mutated HMGA1 3'UTR (PRL-

HMGA1 and PRL-HMGA1-Mut). A firefly (pp) luciferase plasmid was co-transfected with the Renilla (rr) luciferase construct as a transfection control. The rr/pp was normalized to that for a control Renilla luciferase plasmid without an HMGA1 3'UTR (PRL) and is expressed relative to the normalized rr/pp in cells transfected with the GL2 control. (C) PRL or PRL-HMGA1 was co-transfected with GL2 or a mutant form of miR-24-3p (miR-24-3pMut) and luciferase activity was determined similarly. (D) Exogenous expression of miR-24-3p down-regulated HMGA1 protein level. (E) 2'-O-Me antisense-miR-24-3p sustained the expression of HMGA1 protein. (F) C2C12 cells stably expressing empty vector or HMGA1 lacking its 3'UTR constructs were induced to differentiate by transferring to DM. Immunostaining for MHC was carried out after 48 hours. Green indicates MHC and blue indicates DAPI staining of nuclei. The values are expressed as Mean+/- SD of three measurements.

Figure 5: miR-24-3p is essential for adult muscle regeneration. (A) As determined by qRT-PCR, HMGA1 is gradually up-regulated in TA muscles on days 1-3 post-injury and then gradually down-regulated on days 5-14 post-injury. HMGA1 values were normalized to RSP13. (B) qRT-PCR analysis shows that intramuscular injection of Ant-miR-24-3p sustained its endogens level lower days 7 and 14 post-injury. (C-F) H&E staining of representative images showing that the Ant-miR-24-3p-injected sample delays regeneration on days 7 and 14 post-injury. Scale Bar: 100 mm. Mean +/- standard deviation of the samples from three mice.

Figure1: miR-24-3p is abundantly expressed in the adult skeletal muscles and upregulated during myoblast differentiation and muscle regeneration.

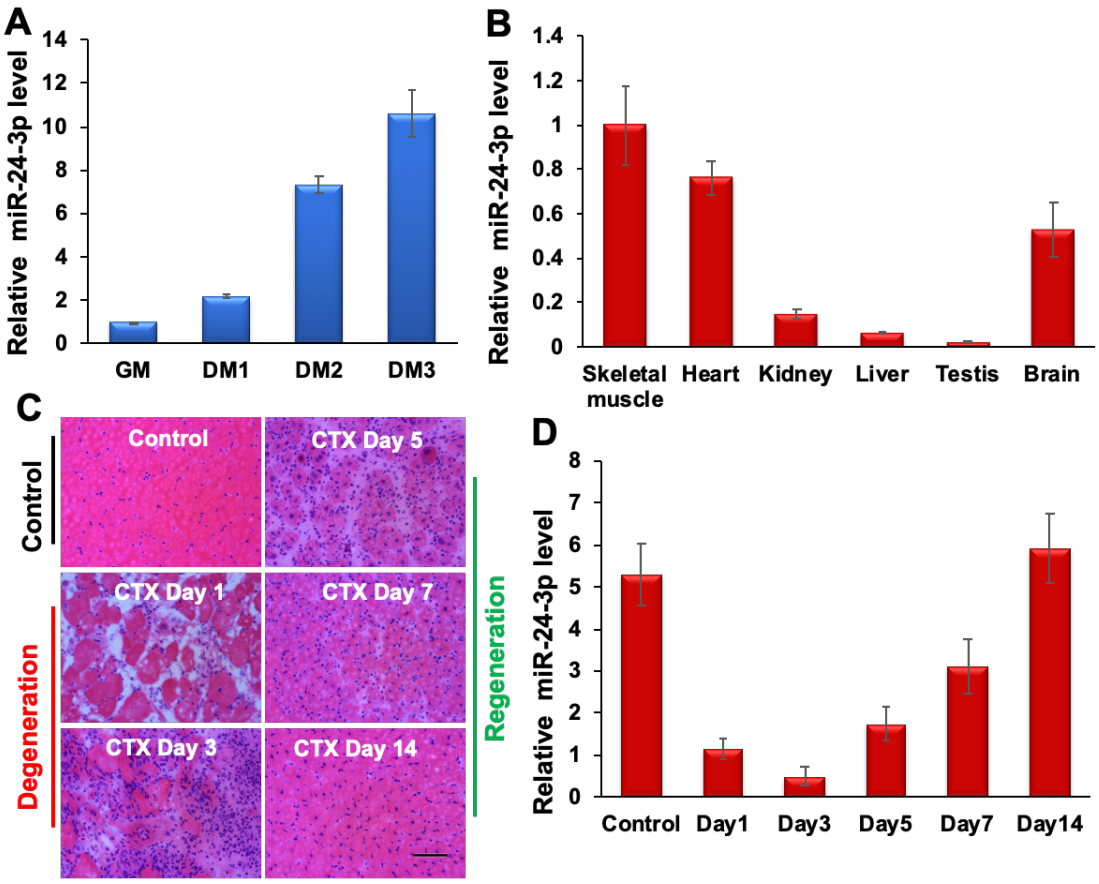


Figure 2: miR-24-3p promotes primary myoblast differentiation.

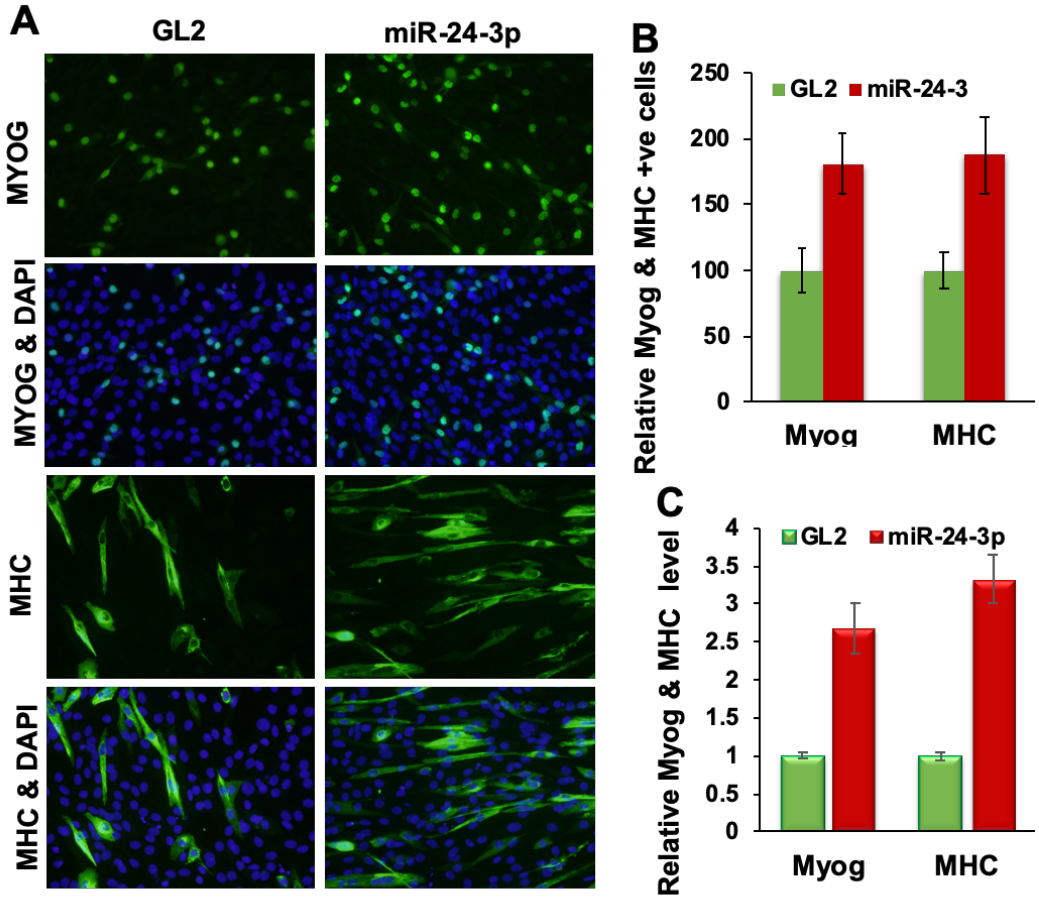


Figure 3: miR-24-3p is required for primary myoblast differentiation.

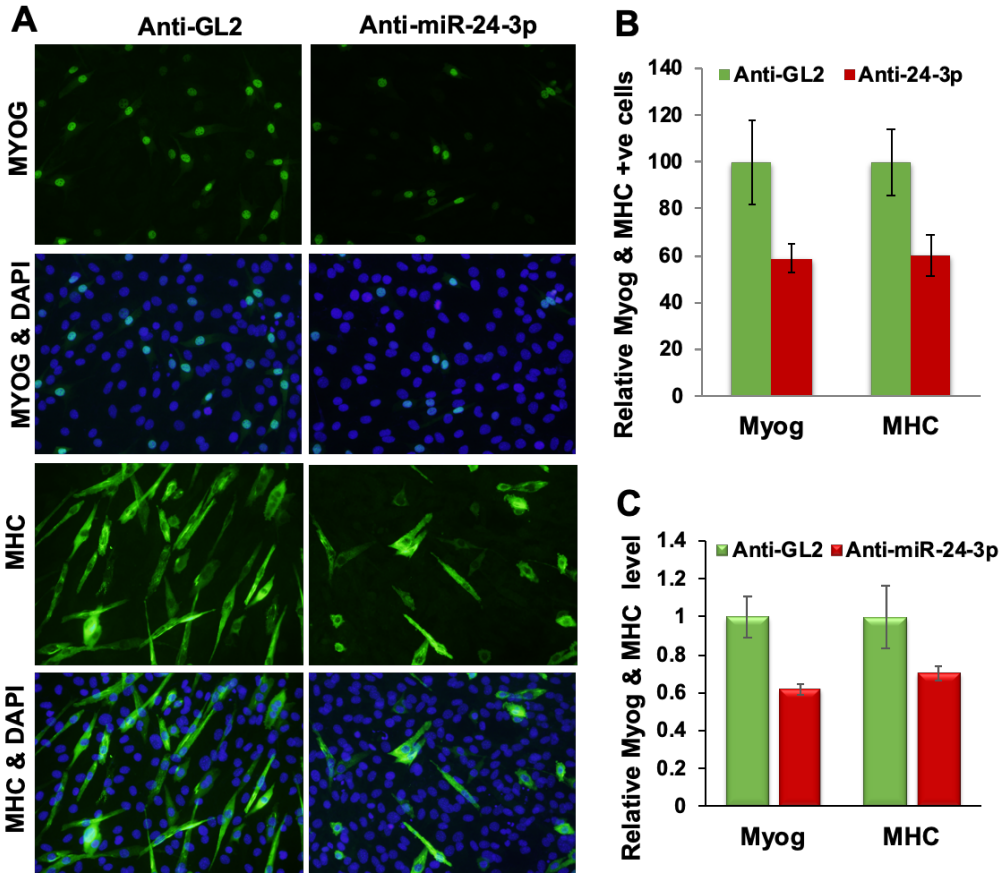


Figure 4: miR-24-3p promotes myoblast differentiation by directly targeting and regulating HMGA1.

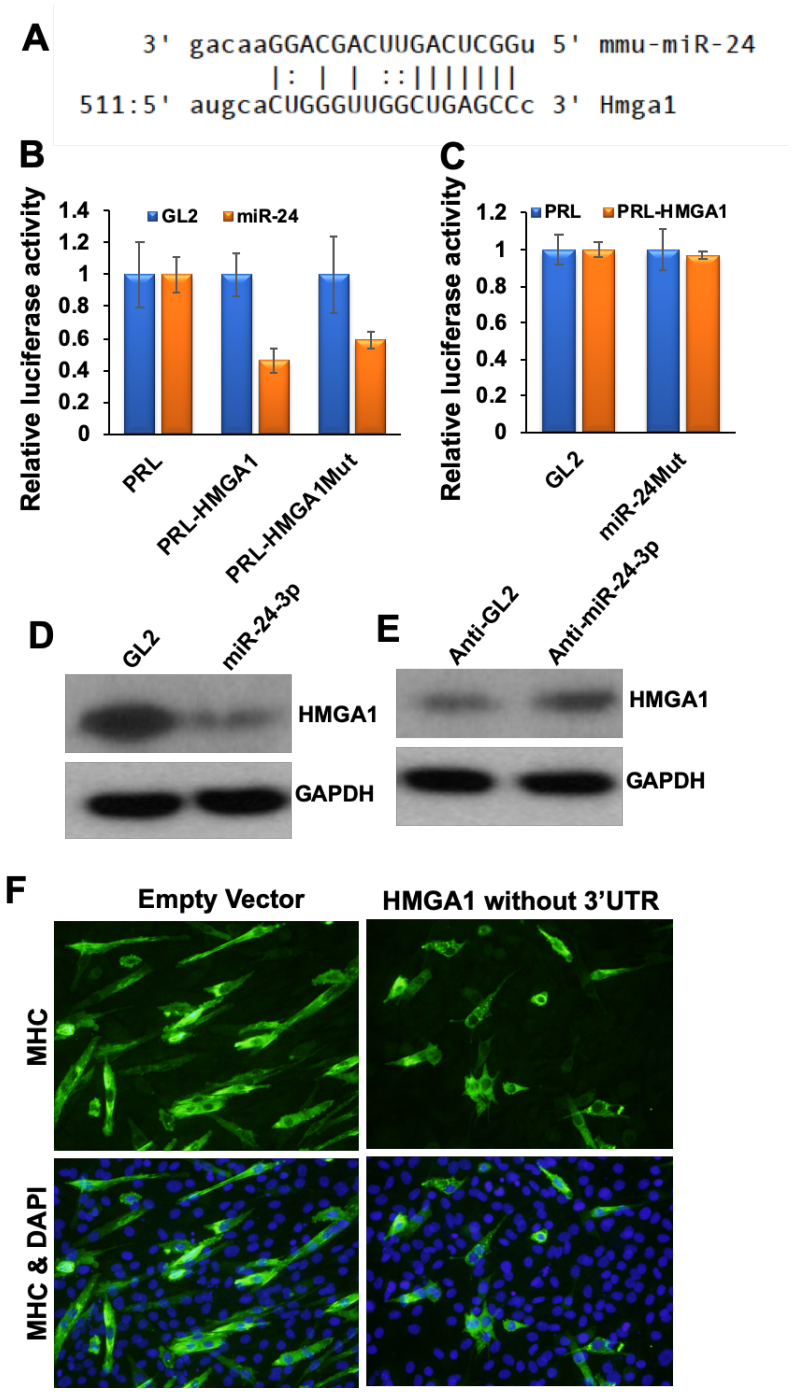
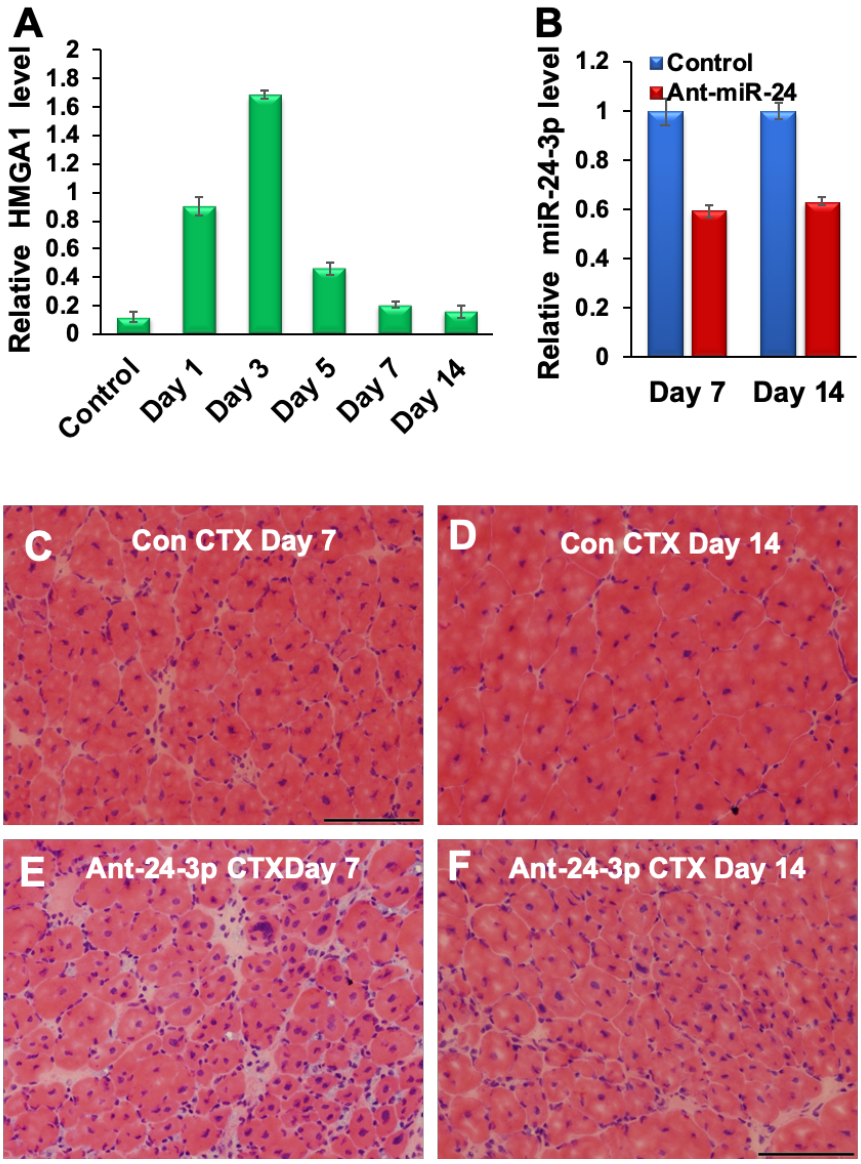
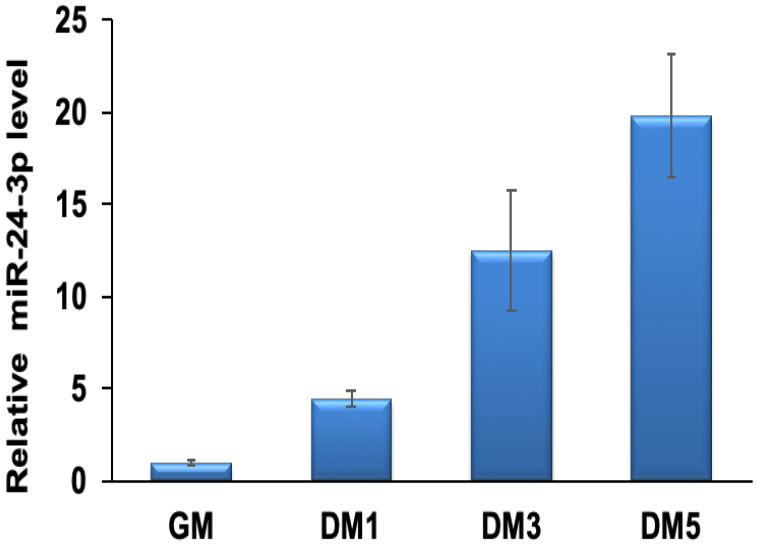
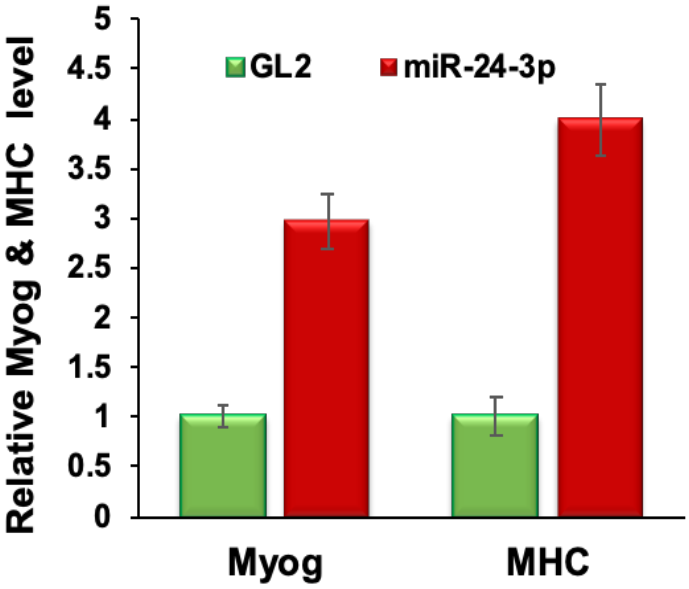


Figure 5: miR-24-3p is essential for adult muscle regeneration.



Supplemental Figure 1: miR-24-3p is upregulated during C2C12 myoblast differentiation.



Supplemental Figure 2: miR-24-3p promotes myoblast differentiation