miR-26a is required for skeletal muscle differentiation and regeneration in mice

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Multiple microRNAs are known to be induced during the differentiation of myoblasts to myotubes. Yet, experiments in animals have not provided clear evidence for the requirement of most of these microRNAs in myogenic differentiation *in vivo*. miR-26a is induced during skeletal muscle differentiation and is predicted to target a well-known inhibitor of differentiation, the transforming growth factor β /bone morphogenetic protein (TGF- β /BMP) signaling pathway. Here we show that exogenous miR-26a promotes differentiation of myoblasts, while inhibition of miR-26a by antisense oligonucleotides or by Tough-Decoys delays differentiation. miR-26a targets the transcription factors Smad1 and Smad4, critical for the TGF- β /BMP pathway, and expression of microRNA-resistant forms of these transcription factors inhibits differentiation. Injection of antagomirs specific to miR-26a into neonatal mice derepressed both Smad expression and activity and consequently inhibited skeletal muscle differentiation. In addition, miR-26a is induced during skeletal muscle regeneration after injury. Inhibiting miR-26a in the tibialis anterior muscles through the injection of adeno-associated virus expressing a Tough-Decoy targeting miR-26a prevents Smad down-regulation and delays regeneration. These findings provide evidence for the requirement of miR-26a for skeletal muscle differentiation and regeneration *in vivo*.

[**Keywords:** miR-26a; TGF- β /BMP; myoblast; skeletal muscle stem cells; differentiation; regeneration]

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Skeletal muscle is mainly composed of multinucleated muscle fibers. However, postnatal growth and the regenerative ability of adult skeletal muscle are dependent on adult muscle stem cells known as satellite cells that reside beneath the basal lamina of the mature fibers (Dhawan and Rando 2005; Rudnicki et al. 2008). Activated satellite cells give rise to myoblast cells that undergo several rounds of division before terminal differentiation. Skeletal muscle development during embryogenesis as well as during regeneration after muscle damage in adults requires a fine balance between myogenic differentiation and self-renewal (Buckingham et al. 2006).

Transforming growth factor β /bone morphogenetic protein (TGF- β /BMP) signaling helps to maintain a pool of muscle stem cells by preventing myogenic differentiation (H Wang et al. 2010). This family is generally divided into two branches on the basis of their downstream Smad transcription factors. The TGF- β branch signals through Smad2 and Smad3, while the BMPs signal through Smad1, Smad5, and Smad8 (Schmierer and Hill 2007). Activated TGF- β /BMP receptors phosphorylate Smad transcription

factors (Smad1, Smad2, Smad3, or Smad5). Phosphorylated Smads form a complex with the common mediator Smad, Smad4, and translocate to the nucleus to regulate gene expression by directly binding to the regulatory elements of their target genes. TGF- β 1 inhibits myogenesis by repressing the transcriptional activity of muscle regulatory factors (MRFs) (Martin et al. 1992; Liu et al. 2001, 2004), while BMP-2/4 inhibit this process at least in part by up-regulating the inhibitors of differentiation (Id1–Id3) that also repress myogenesis (Hollnagel et al. 1999; Katagiri et al. 2002; Kowanetz et al. 2004). This signaling pathway is required for myoblast proliferation but is down-regulated during differentiation *in vitro* and *in vivo*. The mechanism by which the TGF- β /BMP pathway is down-regulated during differentiation is not well understood.

Most of the current understanding of muscle differentiation is based on the regulation of muscle-specific transcription factors; e.g., MyoD and Mef2 (Berkes and Tapscott 2005; Potthoff and Olson 2007). More recently, we have begun to appreciate that microRNAs play critical roles during muscle proliferation and differentiation (Chen et al. 2009; Williams et al. 2009a; Ge and Chen 2011; Gagan et al. 2012). MicroRNAs are a class of small, noncoding RNAs of 18–25 nucleotides that regulate gene expression by inhibiting translation or inducing mRNA degradation (Bartel 2004; Wu et al. 2006). The

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specific expression of certain microRNAs in muscles suggests that microRNAs may have a specific role in muscle function. In mammals, miR-1 and miR-133 are expressed in both skeletal and cardiac muscles, and miR-206 is specifically expressed in skeletal muscles (Chen et al. 2006; Kim et al. 2006). Overexpression and knock-down experiments elucidated the function of these microRNAs in muscle differentiation in C2C12 myoblast cell lines (Chen et al. 2006; Kim et al. 2006). Transfection of these microRNAs into undifferentiated cells changed the gene expression profile to that in muscle tissue (Lim et al. 2005; Kim et al. 2006). Overexpression of miR-1 in mice resulted in defects in cardiac development (Zhao et al. 2005). Similarly, miR-26a, miR-206, miR-322/424, miR-378, miR-486, and miR-503 are induced during differentiation of C2C12 cells from myoblasts to myotubes, and transfection of these microRNAs into C2C12 myoblasts accelerates myotube formation and expression of myogenic markers (Kim et al. 2006; Wong and Tellam 2008; Sarkar et al. 2010; Dey et al. 2011; Gagan et al. 2011). Targeted deletion of Dicer, an enzyme critical for microRNA biogenesis, in the myogenic compartments caused perinatal lethality with reduced skeletal muscle mass and abnormalities in muscle fiber morphology, suggesting the role of muscle differentiation-induced (MDI) microRNAs for proper skeletal muscle differentiation in animals (O'Rourke et al. 2007).

Despite these disparate lines of evidence implicating microRNA function in skeletal muscle differentiation, the knockout of specific MDI microRNAs in mice shows very little effect on skeletal muscle differentiation. Haploinsufficiency of only one out of two miR-1 genes resulted in profound cardiac defects but showed no effect in skeletal muscles (Zhao et al. 2007). On the other hand, germline deletion of miR-206, which is exclusively expressed in skeletal muscle, did not show any gross defects in skeletal muscle development (Williams et al. 2009b). Thus, there is a great need to develop new approaches for evaluating the functions of MDI microRNAs in animals. For this study, we developed a new system based on Tough-Decoy (TuD) that sponges microRNAs to knock down miR-26a and reveals its role in mice skeletal muscles (Haraguchi et al. 2009).

We found that miR-26a is highly abundant in the skeletal muscles and up-regulated during satellite cell differentiation and muscle regeneration. An earlier report also suggests that miR-26a is up-regulated during myoblast differentiation and promotes differentiation in vitro (Wong and Tellam 2008). However, the in vivo role of this microRNA in skeletal muscle development remains unknown. To study the developmental role of miR-26a, we knocked down miR-26a in neonatal mice using an antagomir specific to this microRNA. We used an adeno-associated virus (AAV) expressing a TuD against miR-26a to evaluate the role of miR-26a in adult skeletal muscle regeneration. In vitro cell culture and animal studies establish that miR-26a plays an important role in promoting muscle differentiation and regeneration by down-regulating the critical transcription factors of the TGF- β /BMP signaling pathway, Smad1 and Smad4, by directly

targeting their 3' untranslated regions (UTRs). Thus, our study not only establishes the role of an MDI microRNA in skeletal muscle development in vivo, it elucidates the mechanism by which the MDI microRNA regulates muscle differentiation and regeneration by inhibiting well-established repressors of myogenesis.

Results

miR-26a is up-regulated during myoblast differentiation and skeletal muscle regeneration and promotes myoblast differentiation

Mouse C2C12 is an immortalized myoblast cell line derived from adult skeletal muscles and serves as an excellent in vitro system to study the molecular mechanisms underlying skeletal muscle differentiation (Yaffe and Saxel 1977). These cells proliferate in the presence of high serum and differentiate in low serum. We previously reported that a number of microRNAs were induced during C2C12 myoblast differentiation (Dey et al. 2011). Of these, miR-26a has been shown to promote C2C12 myoblast differentiation in vitro (Wong and Tellam 2008), and we focused on miR-26a to elucidate its function in skeletal muscle differentiation and regeneration in mice.

First, we confirmed and extended the role of miR-26a in muscle differentiation in vitro. Besides being induced during C2C12 myoblast differentiation (Fig. 1A), miR-26a was also up-regulated during both mouse and human primary myoblast differentiation (Fig. 1B,C). miR-26a expression pattern showed high specificity in skeletal muscle (Fig. 1D), where its level of expression was about twofold that in C2C12 on day 5 in differentiation medium (DM5). Fluorescent in situ hybridization (FISH) showed that miR-26a is abundantly expressed in the mature fibers but is also seen in a subset of nuclei at the periphery (Fig. 1F, top right panels). Following cardiotoxin (CTX) injury, adult skeletal muscle activates quiescent satellite cells, which re-enter the cell cycle and, after a few days, differentiate to replenish the degenerated muscle fibers (Supplemental Fig. 1A). When we injected CTX in adult skeletal muscle to induce injury and regeneration, miR-26a expression decreased rapidly on days 1 and 3, while the muscle degenerated and increased through days 5–14 as the skeletal muscle regenerated (Fig. 1E). To determine the cell types where miR-26a was up-regulated during regeneration phase, we carried out FISH for miR-26a and immunostaining for MyoD or Pax7. miR-26a was up-regulated in the MyoD- and Pax7-positive satellite cells (Fig. 1F; Supplemental Fig. 1B). These findings suggest that miR-26a may have a role in muscle physiology, particularly at the level of myoblast differentiation.

To assess the function of miR-26a in myogenic differentiation, we transfected C2C12 cells with RNA duplexes encoding miR-26a or GL2 (22 bases from luciferase gene) as a negative control in growth medium (GM) and then transferred them to differentiation medium (DM). The exogenous miR-26a was ~1.2-fold compared with the level in C2C12 cells on DM5. The exogenous miR-26a increased differentiation, as seen by the morphology and

Figure 1. miR-26a is abundantly expressed in skeletal muscle and up-regulated during muscle differentiation and regeneration. miR-26a is up-regulated during differentiation of C2C12 myoblast (A), mouse primary myoblast (B), and human primary myoblast (C). Quantitative RT-PCR (qRT-PCR) of miR-26a after indicated days in differentiation medium (DM). Fold change of miR-26a, normalized to U6sn RNA, is expressed relative to cells in growth medium (GM). Means $\pm$ standard deviation of three replicates. (D) miR-26a is abundantly expressed in mouse and human skeletal muscles as compared with other tissue types. qRT-PCR for miR-26a, which is normalized to U6sn RNA. Mean $\pm$ standard deviation of three replicates. (E) miR-26a is down-regulated on days 1–3 after injury and up-regulated on days 5–14 after injury by injection of CTX. The rest is as in D. (F) FISH of control adult skeletal muscles using locked nucleic acid (LNA)-based scrambled (top left two panels) or anti-miR-26a (top right two panels) probes. FISH of miR-26a and immunostaining for MyoD are shown for regenerating muscle on day 5 (middle) and on day 7 (bottom) after CTX injection. Yellow or white arrows indicate miR-26a- and MyoD-positive or -negative cells, respectively, located outside the skeletal muscle fibers. Bars, 20 μ m.

the number of Myogenin- and MHC-positive cells (Fig. 2A,B; Supplemental Table 1). Both Myogenin and myosin heavy chain (MHC) mRNA were up-regulated in miR-26a transfected cells even when the culture was maintained in GM (Fig. 2C). The level of MHC mRNA reached in miR-26a-transfected cells in Figure 2C was $\sim$ 0.6-fold the level in C2C12 cells on DM3. Cell cycle arrest in the G1 phase is a hallmark of cellular differentiation. Indeed, transfection of miR-26a in GM caused an increase in the G1 population of cells by 25%, and a decrease in S- and G2/M-phase cells by 15% and 6%, respectively (Fig. 2D; Supplemental Fig. 2). Similar to C2C12 cells, transfection of miR-26a in mouse primary myoblast cells also increased Myogenin and MHC mRNA levels (Supplemental Fig. 3A). These results demonstrate that miR-26a is required for differentiation of skeletal muscle myoblasts in vitro.

To test the role of miR-26a in vivo, we needed reagents to inhibit the microRNA in cells. A 2'-O-methyl antisense miR-26a oligonucleotide (anti-26a) decreased the appearance of myogenic differentiation markers Myogenin and

MHC in both C2C12 (Fig. 2E–G; Supplemental Table 1) and primary mouse myoblast cells (Supplemental Fig. 3B). In addition, we adopted a new system based on TuD that inhibits microRNA function by acting as a sponge for microRNAs (Haraguchi et al. 2009). An AAV vector was modified to express either AAV-miR-26aTuD (hereafter termed 26a-TuD) or negative control TuD (NC-TuD) (Supplemental Fig. 8) and was used to infect C2C12 cells in vitro. These cells were then transfected with a luciferase reporter driven by the muscle creatine kinase promoter (MCK-luciferase) to measure the extent of myoblast differentiation. 26a-TuD decreased the MCK-luciferase activity $>60\%$ as compared with NC-TuD, suggesting that lack of miR-26a delays differentiation of myoblast cells (Fig. 2H). Therefore, the reagents described here inhibit miR-26a in cells and delay differentiation.

Later on, we used an antisense miR-26a RNA, antagomir-26a (Ant26a), that was chemically modified with proprietary technology for improving entry into cells in vivo (Fig. 5, below; Krutzfeldt et al. 2005). We also used the AAV expressing 26a-TuD in adult animals to study

Figure 2. miR-26a promotes and is required for myoblast differentiation. (A) C2C12 cells were transfected three times at 24-h intervals with GL2 or miR-26a in GM, and the cells were transferred to DM and stained for Myogenin at 32 h or for MHC at 60 h. (Green) Myogenin or MHC; (blue) nuclei stained by DAPI. (B) Fractions of Myogenin- and MHC-positive cells are presented relative to the GL2 control (100%). Mean $\pm$ standard deviation of 10 measurements (Supplemental Table 1). (C) C2C12 cells transfected with GL2 or miR-26a as above and held in GM for an extra 24 h; qRT-PCR was performed for Myogenin and MHC. The results were normalized to GAPDH in the same sample and then again to the level in GL2 transfected cells. Mean $\pm$ standard deviation of three replicates. (D) Increase in G1-phase cells after transfection of miR-26a in C2C12 cells. Propidium iodide staining for DNA content and FACS analysis (Supplemental Fig. 1). The results are expressed as the percentage change of cells in a given phase of the cell cycle in the microRNA transfected cells relative to that in the GL2 control. Mean $\pm$ standard deviation of three measurements. (E,F) 2'-O-methyl antisense oligonucleotides against GL2 or miR-26a were transfected as in A, and C2C12 cells were stained for Myogenin at 32 h or for MHC at 60 h. Data are presented as in A and B. (G) qRT-PCR performed for Myogenin and MHC mRNA from cells in E, and data are presented as in C. (H) TuD for miR-26a inhibits myoblast differentiation as indicated by MCK-luciferase activity at 48 h. A *Renilla* (rr) luciferase construct was cotransfected with MCK-firefly (pp) luciferase as a transfection control. The result is expressed as pp/rr relative to the control TuD-infected samples. Mean $\pm$ standard deviation of three replicates.

the requirement of miR-26a in skeletal muscle regeneration (Fig. 6, below).

Transcription factors critical for the TGF- β /BMP pathway, Smad1 and Smad4, are directly targeted by miR-26a

To measure the effect of microRNA depletion in vivo, we looked for specific targets of miR-26a that can be measured in vivo after the experimental manipulations. miR-26a is predicted to target Smad1 and Smad4, the downstream transcription factors of a well-known inhibitor of skeletal muscle differentiation, the TGF- β /BMP signaling pathway. In addition, Smad1 and Smad4 were down-regulated by miR-26a in vascular smooth muscle cells (Leeper et al. 2011), but no evidence was presented that these were direct targets of the microRNA, that they were targeted also in skeletal muscle, or that their down-regulation was important for differentiation.

During C2C12 myoblast differentiation, Smad1 and Smad4 proteins decreased starting from day 1, with the level reduced below 60% by day 3 (Fig. 3A,B). Over this time course, *Smad1* and *Smad4* mRNA declined in a more gradual fashion, with a 40% reduction seen only after day 5 of serum withdrawal (Fig. 3C,D). miR-26a, on the other hand, was gradually up-regulated as differentiation progressed (Fig. 1A). The faster kinetics of re-

pression of the Smad1 and Smad4 proteins relative to that of their mRNAs is consistent with MDI microRNAs like miR-26a repressing Smad1 and Smad4 expression post-transcriptionally.

MicroRNA target prediction algorithms RNAhybrid, miRanda, and TargetScan predicted four target sites of miR-26a in the 3' UTR of *Smad1* and two target sites in the 3' UTR of *Smad4* (Supplemental Fig. 4). To determine whether these sites in the 3' UTRs of *Smad1* and *Smad4* were true targets for miR-26a, their 3' UTRs were separately fused to a luciferase reporter gene driven by the cytomegalovirus (CMV) promoter. Cotransfection of miR-26a with either the *Smad1* or *Smad4* luciferase construct repressed the luciferase activity by 50% and 33%, respectively (Fig. 3E,F). Mutation of each of the target sites in *Smad1* partially relieved the repression, and mutations of all of the sites together relieved the repression entirely (Fig. 3E). In similar experiments, both of the predicted target sites in the *Smad4* 3' UTR were also found to be true target sites for miR-26a (Fig. 3F).

miR-26a negatively regulates Smad1 and Smad4 through their 3' UTRs

Since the 3' UTRs of *Smad1* and *Smad4* were direct targets of miR-26a (Fig. 3E,F), we tested whether Smad1 and Smad4 were down-regulated by miR-26a during skeletal

Figure 3. miR-26a targets Smad1 and Smad4. (A,B) Smad1 and Smad4 proteins are down-regulated during differentiation. (A) Western blot of indicated proteins in C2C12 cells in GM or on various days following transfer to DM. GAPDH served as loading control. (B) Quantification of expression of Smad1 and Smad4 protein normalized to GAPDH. (C,D) Relative *Smad1* and *Smad4* mRNA levels normalized to GAPDH mRNA in C2C12 cells as in A. (E,F) Luciferase assays were performed to measure the effect of transfected miR-26a on a *Renilla* luciferase reporter fused to *Smad1* or *Smad4* 3' UTRs. Mutations at target sites indicated in Supplemental Figure 4. A firefly (pp) luciferase plasmid was cotransfected with the *Renilla* (rr) luciferase construct as a transfection control. The rr/pp was normalized to that for a control *Renilla* luciferase plasmid without a *Smad1* or *Smad4* 3' UTR segment and is expressed relative to the normalized rr/pp in cells transfected with the GL2 control. Mean $\pm$ SD of three measurements.

muscle differentiation and whether this down-regulation was important for the differentiation. Transfection of C2C12 cells with miR-26a in GM down-regulated both the Smad1 and Smad4 proteins (Fig. 4A). In a reciprocal experiment, inhibition of the endogenous miR-26a using 2'-O-methyl antisense inhibitors to miR-26a caused a longer persistence of endogenous Smad1 and Smad4 proteins during differentiation (Fig. 4B). The Smad activity as measured by phosphoSmad1/5 was also increased in these samples (Fig. 4B). Smad1 and Smad4 down-regulation by siRNA in C2C12 cells promoted differentiation, as measured by morphology and MHC expression, demonstrating that Smad1 and Smad4 down-regulation is rate-limiting for

skeletal muscle differentiation (Fig. 4C). These results are consistent with the hypothesis that miR-26a can repress Smad1 and Smad4 proteins and thus can promote myogenic differentiation.

Next, we tested whether the 3' UTR of *Smad1* or *Smad4* containing the miR-26a target sites was important for the down-regulation of the Smad1 or Smad4 protein and for differentiation of myoblasts to myotubes. Retroviral vectors expressing the *Smad1/4* ORF devoid of its 3' UTR (*Smad1/4* ORF) and *Smad1/4* 3' UTR still attached (*Smad1/4* ORF+3' UTR) were used to create C2C12 myoblast derivatives. The basal expression levels of exogenous Smad1 (or Smad4) protein in GM was similar regardless of whether the vector contained a *Smad1* (or *Smad4*) 3' UTR (Fig. 4D), and this level was about three times the level of endogenous Smad1 (Supplemental Fig. 5). In DM, however, Smad1 and Smad4 proteins persisted up to day 3 in the cells expressing *Smad1/4* without their cognate 3' UTRs, in contrast to the cells expressing *Smad1/4* with corresponding 3' UTRs (Fig. 4E; Supplemental Fig. 5). Thus, the 3' UTRs of both *Smad* genes were essential for the down-regulation of the proteins during differentiation.

C2C12 cells that could down-regulate the exogenous *Smad1* or *Smad4* (+3' UTR) differentiated well, as detected by immunoblotting or immunofluorescence for MHC (Fig. 4E,F). In contrast, the cells that expressed Smad genes without their 3' UTRs failed to differentiate well (Fig. 4E,F). These results demonstrate that 3' UTRs containing the miR-26a target sites are essential for the down-regulation of Smad1 and Smad4 proteins and for optimal differentiation of myoblasts. These findings also establish these two *Smad* mRNAs and proteins as good biomarkers for assaying the effect of miR-26a down-regulation in vivo.

Knockdown of miR-26a increases the Pax7-positive proliferating cells in vivo

We knocked down miR-26a in the skeletal muscle of neonatal mice using antagomirs (Krutzfeldt et al. 2005). An antagomir is a chemically modified synthetic RNA complementary to a specific microRNA that inhibits and degrades the target microRNA irreversibly (Krutzfeldt et al. 2005). Injection of Ant26a knocked down miR-26a but not a control microRNA, miR-192, in neonatal skeletal muscle (Fig. 5A; Supplemental Fig. 9A). The targets of miR-26a, *Smad1* and *Smad4* mRNAs, were derepressed in the skeletal muscle (Fig. 5B). *Ezh2*, another target of miR-26a (Wong and Tellam 2008), was also induced by Ant26a (Supplemental Fig. 6A). Smad activity was up-regulated in these muscles, as indicated by the increase in the mRNA of *Id3*, a gene activated by the Smads (Fig. 5B). Activation of the TGF- β /BMP pathway results in phosphorylated Smad1 and Smad5 in the nucleus. The number of nuclei containing phosphoSmad1/5 was indeed increased in the animals injected with Ant26a (Fig. 5C). Collectively, these results suggest that miR-26a is decreased and its targets are elevated in the developing mouse muscle upon injection of Ant26a.

Figure 4. miR-26a down-regulates Smad1 and Smad4 in differentiating myoblasts by directly targeting their 3' UTRs. (A) Transfection of miR-26a in C2C12 cells down-regulates Smad1 and Smad4 protein levels as detected by Western blotting on day 4 of transfection. GAPDH served as a loading control. (B) 2'-O-methyl antisense oligonucleotide against miR-26a (Anti-26a) causes longer persistence Smad1, Smad4, and pSmad1/5 protein levels in C2C12 cells held in DM for 2 d compared with cells transfected with the anti-GL2 oligonucleotides. GAPDH served as a loading control. (C) siRNA of *Smad1* and *Smad4* causes differentiation of C2C12 in GM as detected by MHC immunostaining. (Green) MHC; (blue) DAPI. (D, left) Flag Western blot of C2C12 cells stably expressing empty retroviral vector, the Flag-tagged *Smad1* ORF, or the Flag-tagged *Smad1* ORF+3' UTR. (Right) Similarly, for Flag-Smad4. GAPDH represents the loading control. (E) Level of Flag-Smad1 or Smad4 (top panel) and MHC (middle panel) proteins after transfer to DM3 of C2C12 cells expressing constructs indicated at the top. GAPDH served as the loading control. (F) C2C12 cells stably expressing various constructs were induced to differentiate by transfer to DM. Overexpression of the *Smad1* or *Smad4* ORF inhibited differentiation in DM3, whereas overexpression of *Smad1* ORF+3' UTR or *Smad4* ORF+3' UTR permitted cells to differentiate to a degree similar to cells infected with empty vector. (Green) MHC; (blue) DAPI staining of nuclei.

Ki67 staining and BrdU incorporation mark proliferating cells. There was a significant increase of Ki67-positive (Fig. 5D) and BrdU-positive (Fig. 5E) cells in Ant26a-injected mice. The majority of the BrdU-incorporating and Ki67-positive nuclei are at the periphery of the muscle fibers and coexpress Pax7, consistent with the proliferation occurring in the undifferentiated cells (Fig. 5C–F; Supplemental Fig. 7). Conversely, the mRNA levels of the myogenic markers Myogenin and MHC were decreased in the mice injected with Ant26a (Fig. 5G). Thus, our findings suggest that miR-26a is an important inhibitor of TGF- β /BMP signaling and cell proliferation during neonatal skeletal muscle development. Conversely, miR-26a appears to be an important inducer of differentiation in mice.

miR-26a promotes muscle regeneration after injury

We injected CTX in adult skeletal muscle to induce injury and regeneration (Supplemental Fig. 1A). As described already, miR-26a is down-regulated on days 1–3 after CTX-induced injury but then increases steadily as myotubes are formed (Fig. 1E,F). The mRNA level of the miR-26a targets *Smad1* and *Smad4*, on the other hand, demonstrated an anti-correlation to miR-26a, increasing on days 1–3 after injury and then decreasing steadily on days 5–14 after injury (Fig. 6A). This result is consistent with the miR-26a repressing *Smad1* and *Smad4* during regeneration.

The AAV9 serotype is known to infect nearly 100% of skeletal muscle cells (Inagaki et al. 2006). We therefore produced an AAV9 serotype of 26a-TuD (Supplemental Fig. 8) or NC-TuD, tested it in cell culture (Fig. 2H), and injected the viruses expressing 26a-TuD or NC-TuD before injuring the tibialis anterior (TA) muscle with CTX (Supplemental Fig. 9B). Infection by AAV was assessed by immunostaining for GFP, coexpressed by all of the AAV vectors in addition to their TuD payloads (Supplemental Fig. 10A). GFP was expressed in nearly all of the cells, including Pax7-positive satellite cells (Supplemental Fig. 10A,B). Ten days after 26a-TuD injection, the miR-26a level was down-regulated in the muscle more than fourfold (Fig. 6B). Concurrently, *Smad1*, *Smad4*, and *Ezh2* mRNA levels were up-regulated (Fig. 6C; Supplemental Fig. 6B). In addition, a target of Smads, *Id3*, and a satellite cell marker, *Pax7*, were also up-regulated upon 26a-TuD injection (Fig. 6C). miR-26a remained up-regulated in a subset of myoblasts that were not infected with AAV expressing 26a-TuD (GFP-negative), while it was decreased in cells that were infected with AAV expressing 26a-TuD and GFP (Supplemental Fig. 10C). Together, these results establish that TuD-26a knocked down miR-26a in adult skeletal muscle cells, including Pax7-positive satellite cells, and in turn derepressed the expression of *Smad1* and *Smad4* and their target genes and induced expression of *Pax7*, a satellite cell marker.

miR-26a was up-regulated during days 5–14 after CTX injury, the active phase of muscle regeneration (Fig. 1E;

Figure 5. Knockdown of miR-26a increases the Pax7-positive proliferating cells in vivo. (A) Injection of Ant26a knocks down endogenous miR-26a in neonatal skeletal muscle. qRT-PCR of microRNAs normalized to U6sn RNA are expressed relative to control antagomir-injected samples. Mean $\pm$ standard deviation of three mice. (B) Ant26a increases *Smad1*, *Smad4*, and *Id3* mRNA levels. qRT-PCR of *Smad1*, *Smad4*, and *Id3* on hind leg skeletal muscle, normalized to GAPDH in the same sample and then again to the level in controls. Mean $\pm$ standard deviation of the sample from three mice. (*) $P < 0.001$. (C, left) Immunostaining images of pSmad1/5 showing that Ant26a increases pSmad1/5-positive cells. (Right) Quantitation of ~ 2000 nuclei from random fields. Mean $\pm$ standard deviation of three different samples of Ant26a-injected or control-injected neonates. (*) $P < 0.001$. Bar, 50 μm . (D) As in C, immunostaining images of Ki67 (left) and quantitation (right). (*) $P < 0.001$. (E, left) Confocal microscopy images of skeletal muscle 2 h after BrdU labeling from Ant26a-injected or control antagomir-injected neonates. Cell proliferation was determined by anti-BrdU antibody (green), cell surface was marked by laminin (red), and nuclei were counterstained with DAPI (blue). (Right) The percentage of BrdU-positive nuclei was quantitated. Mean $\pm$ standard deviation of 10 different random fields from three different samples. (*) $P < 0.001$. Bar, 50 μm . (F) Confocal microscopy images of Ant26a-injected neonatal skeletal muscles from E immunostained for Pax7 and BrdU. (Yellow arrows) Satellite cells doubly stained for BrdU and Pax7. Bar, 25 μm . (G) Ant26a decreases differentiation marker Myogenin and MHC. qRT-PCR performed for Myogenin and MHC mRNAs. The results were normalized to GAPDH and expressed relative to control. Mean $\pm$ standard deviation of the sample from three mice. (*) $P < 0.001$.

Supplemental Fig. 1A). Therefore, we next asked whether inhibition of miR-26a would delay the normal kinetics of muscle regeneration. The TA muscle in one leg was injected with 26a-TuD, the contralateral leg was injected with NC-TuD, and 10 d later, CTX was injected in both legs (day 0 in Fig. 6D–J). During the first 3 d following CTX injury, both groups of samples displayed extensive myofiber degeneration and accumulation of neutrophils, macrophages, and proliferating myoblasts (Supplemental Fig. 1A). At day 5 after injury, the regeneration process was at the very early stage, where new myofiber formation had just begun (Supplemental Fig. 1A). Therefore, we assessed both groups on days 7–14 after injury (Fig. 6D–J; Supplemental Figs. 6B–D, 11A–F). The miR-26a level was low all through the regeneration process in the muscles injected with 26a-TuD (Fig. 6D). As expected, the targets of miR-26a, *Smad1*, *Smad4*, and *Ezh2*, and the Smad target *Id3* had higher expression in the 26a-TuD-infected muscles (Fig. 6E–G; Supplemental Fig. 6B–D). In the control muscles, most of the damaged myofibers were cleared and replaced by newly formed myofibers containing central nuclei on day 7 after injury, and normal muscle morphology was mostly restored by day 14 (Fig. 6H, top row). In contrast, the regeneration kinetics were delayed with numerous inflammatory cells and damaged myofibers persisting in the 26a-TuD-injected muscles on days 7–14 after injury (Fig. 6H, bottom row).

Immunostaining of the regenerating muscle on day 7 and day 14 after injury for desmin further confirmed the poor regeneration in the presence of 26a-TuD. Desmin is an intermediate filament protein that is abundantly expressed in newly made muscle fibers during embryogenesis and muscle regeneration (Kuisk et al. 1996). Desmin was expressed in the myofibers of regenerating muscles expressing both types of TuD (Fig. 6I; additional panels in Supplemental Fig. 11A–F). However, the muscle fibers were significantly smaller in 26a-TuD-infected muscle as compared with the NC-TuD muscle (Fig. 6I; Supplemental Fig. 11A–F), which is evident by the quantitation of a cross-sectional area of newly regenerated fibers (Fig. 6J). In addition, interfiber spaces were larger and filled with nuclei, further supporting delayed regeneration in the 26a-TuD muscle (Fig. 6I; Supplemental Fig. 11A–F). Thus, inhibition of miR-26a significantly delays the regeneration of adult skeletal muscle by allowing the persistence of miR-26 targets.

Discussion

In this study, we demonstrated a role of miR-26a in muscle differentiation and regeneration in vivo. To date, several studies have implicated MDI microRNAs in myoblast differentiation, mostly in cell culture. Studies in animals have not precisely demonstrated an essential role for a specific MDI microRNA in muscle differentiation in vivo. Here we focused on miR-26a, as we found that this microRNA was abundantly expressed in both mouse and human skeletal muscles and promoted muscle differentiation in vitro (Figs. 1, 2). We demonstrated that miR-26a regulates a well-known repressor of myogenesis,

Figure 6. TuD-26a delays muscle regeneration after injury. (A) *Smad1* and *Smad4* are up-regulated in TA muscles on days 1–3 after injury with CTX, and the opposite pattern was seen on days 5–14 after injury. qRT-PCR of *Smad1* and *Smad4* normalized to GAPDH. Mean $\pm$ standard deviation of the samples from three mice. (B,C) miR-26a level was decreased, and *Smad1*, *Smad4*, *Id3*, and *Pax7* mRNA was increased after 10 d of injection of TuD-26a into the TA muscles as compared with NC-TuD. 26a-TuD qRT-PCR values were normalized to GAPDH in the same sample and then again to the level in NC-TuD. Mean $\pm$ standard deviation of the samples from three mice. (*) $P < 0.001$. (D–G) Injection of TuD-26a before CTX injury disrupts the expression patterns of miR-26a, Smads, and their targets during regeneration (days 7, 10, and 14). qRT-PCR was performed and data are represented as in B and C. (H) H&E staining of representative images showing that the TuD-26a-treated sample delays regeneration. Bar, 100 μ m. (I,J) Desmin and laminin staining and cross-section area of regenerating fibers on day 7 using ImageJ software. More than 200 fibers were counted in each group. NC-TuD or 26a-TuD and CTX were injected as in H. Bar, 100 μ m.

the TGF- β /BMP signaling pathway, by directly targeting the 3' UTRs of *Smad1* and *Smad4*, the genes encoding critical transcription factors of this pathway (Figs. 3, 4). Inhibiting miR-26a in cell culture or in neonatal mice derepressed *Smad1* and *Smad4* expression and activity and increased the proliferation of satellite cells in vivo (Figs. 4, 5). Importantly, we developed an AAV-based TuD expression system, which appears to be highly effective in specifically knocking down miR-26a in skeletal muscle. This method allowed us to experimentally show that inhibition of miR-26a stimulates the levels and activities of the Smads and delays muscle regeneration in vivo (Fig. 6). Taken together, our data provide strong evidence for the requirement of miR-26a in muscle differentiation and regeneration in vivo.

TGF- β /BMP are well-known inhibitors of muscle differentiation, and natural mutation of a member of this family, myostatin, results in animals with excess skeletal

muscle mass (McPherron and Lee 1997). Several studies have shown that myogenic differentiation is also regulated by BMP proteins (H Wang et al. 2010; Ono et al. 2011). The activity of BMPs, such as BMP-2 or BMP-4, in myoblast cell cultures inhibits myogenesis and induces osteoblastic differentiation (Katagiri et al. 1994). TGF- β -type ligands interacting with TGF- β receptors and activating *Smad2* and *Smad3* maintain myoblasts in their proliferative stage and inhibit differentiation (Massague et al. 1986; Massague 1998; Kowanz et al. 2004). *Smad2* and *Smad3*, like *Smad1* and *Smad5*, interact with the common *Smad*, *Smad4*, and translocate to the nucleus and activate gene expression. However, the TGF- β /BMP pathway is down-regulated in normal muscle differentiation and regeneration. A BMP-inhibiting molecule, noggin, and the inhibitory Smads *Smad6* and *Smad7* have been reported as negative regulators of the BMP pathway (Re'em-Kalma et al. 1995; Hayashi et al. 1997; Hata et al.

1998). Additionally, E3 ubiquitin ligases that target specific Smads have been reported in germ layer specification and bone formation, but their role in regulating skeletal muscle differentiation is not known (Dupont et al. 2005; Yamashita et al. 2005). Here we showed that miR-26a plays an important role in down-regulating the TGF- β /BMP pathways during skeletal muscle differentiation and regeneration by repressing their downstream Smads.

In support of our findings, Smad1 and Smad4 were also shown to be down-regulated by miR-26a in vascular smooth muscle cells (Leeper et al. 2011). Our results illustrate that Smad1 and Smad4 are direct targets of miR-26a and that miR-26a-induced down-regulation of these two Smads promotes and is essential for muscle differentiation.

Mice with knockout of an MDI microRNA, miR-1-2, show defects in cardiac development and lethality but no phenotype in skeletal muscles (Zhao et al. 2007). Knockout of miR-206 shows no gross defect in skeletal muscle development and exerts only a mild effect on skeletal muscle innervation following barium chloride-induced injury, most likely due to the functional redundancy with miR-1 (Williams et al. 2009b). In a recent study, however, the same group showed that the mice with miR-206 knockout delays skeletal muscle regeneration following CTX injury (Liu et al. 2012). Thus, along with our results, evidence suggests that specific microRNAs play an essential role in skeletal muscle development and regeneration.

Redundancy between microRNAs with similar seed sequences may obscure the role of a microRNA in mice with complete knockout of a specific microRNA gene. Such knockouts are also difficult because several microRNAs, like miR-1 or miR-26, are expressed from multiple genomic loci, necessitating multiple independent gene knockouts and extensive breeding to knock out all copies of a microRNA gene. Clustering of microRNAs or star microRNA in a single primary microRNA locus also complicates efforts to cleanly knock out one microRNA gene while sparing others. Finally, many microRNAs may be important in multiple tissues and organs (e.g., miR-1 in the heart muscles), making it difficult to study the role of a specific microRNA in another tissue of interest by simple knockout strategies. These problems encouraged us to take different approaches to study the *in vivo* function of miR-26a in skeletal muscle development and regeneration. We used chemical Ant26a to inhibit miR-26a in neonatal skeletal muscle, allowing us to study the role of miR-26a early in development. The use of AAV to express 26a-TuD allowed us to elucidate the role of miR-26a in skeletal muscle regeneration *in vivo*. As different AAV serotypes have different tissue specificity (Michelfelder and Trepel 2009), AAV-based TuD will be suitable for targeted knockdown of other microRNAs *in vivo*. It will be a particularly attractive method for knocking down a class of microRNAs with a common seed sequence in a specific tissue.

miR-26a appears to be important for several human skeletal muscle diseases. Human Smad1 and Smad4 are predicted to have the miR-26a target sites in their 3' UTR

(Supplemental Fig. 12). Rhabdomyosarcoma is the most common soft-tissue tumor in children, and the hallmark of this disease is poor terminal differentiation of skeletal muscles. Consistent with the role of miR-26a in Smad down-regulation during skeletal muscle differentiation, one study showed that molecules downstream from TGF- β 1, including Smad4, were significantly up-regulated, and another study showed that miR-26a was down-regulated in rhabdomyosarcomas (Ciarapica et al. 2009; S Wang et al. 2010). miR-26a is down-regulated in patients with Duchene muscular dystrophy, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis reveals that mRNAs of the TGF- β signaling pathway are enriched in the same tissues (Eisenberg et al. 2007). These findings suggest that manipulating miR-26a levels by approaches similar to those described here may be useful for treating skeletal muscle diseases like rhabdomyosarcoma and Duchene muscular dystrophy.

Materials and methods

Cell culture

Mouse myoblast cell line C2C12 was obtained from American Type Culture Collection (Yaffe and Saxel 1977), maintained in GM, and differentiated in DM (Andres and Walsh 1996) as described (Dey et al. 2011). Briefly, C2C12 cells were cultured at subconfluent densities in DMEM supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin, and for differentiation of myoblasts into myotubes, DMEM containing 2% heat-inactivated horse serum and 1% penicillin/streptomycin was used. U2OS cells were cultured in DMEM supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin. Mouse primary myoblasts and differentiated myotubes were kind gifts from Denis Guttridge (Ohio State University). Mouse primary myoblast culture protocol was adapted as described earlier (Springer et al. 2002). Human primary myoblast cells were purchased from Lonza, cultured, and differentiated according to the supplier's instruction.

RNA extraction and RT-PCR

Cells were harvested and washed twice with 1 $\times$ PBS, and total RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's instructions. Total RNA from human skeletal muscle, heart, liver, brain, lung, kidney, and small intestine were purchased from Clontech Laboratories, Inc. cDNA synthesis for mRNA and microRNA detection were carried out using SuperScript III first strand synthesis system for RT-PCR and Ncode microRNA first strand cDNA synthesis and quantitative RT-PCR (qRT-PCR) kit, respectively (Invitrogen). qPCR was carried out in an ABI cycler using SYBR Green PCR master mix, and quantification was done using ABI 7300 software (Applied Biosystems).

Plasmid construction

Mouse *Smad1* 3' UTR of 1397-base-pair (bp) and *Smad4* 3' UTR of 1655 bp were amplified by PCR from C2C12 myoblast genomic DNA and cloned into pRL-CMV as described earlier (Kim et al. 2006). Specific point mutations in *Smad1* and *Smad4* 3' UTRs (shown in Supplemental Fig. 4) cloned into pRL-CMV vector were generated using site-directed mutagenesis kit (Stratagene). *Smad1* and *Smad4* ORFs and *Smad1* and *Smad4*

ORFs with wild-type 3' UTRs were subcloned into pMSCV retroviral expression vector as described (Dey et al. 2011). Retroviruses from these constructs were made in HEK-293T cells by cotransfecting helper plasmids using a standard protocol. As listed in Supplemental Figure 8, a miR-26a TuD forward and reverse template pair was annealed and cloned into pmU6-TuD shuttle vector digested with BsmBI (Haraguchi et al. 2009). A pmU6-TuD vector containing a U6 promoter upstream of and a terminator downstream from a 26a-TuD cassette was obtained by digesting with BamHI and EcoRI, and this cassette was then subcloned into AAV basic vector (Vector Biolabs) to make 26a-TuD (Supplemental Fig. 8). Similarly, an AAV vector was made expressing NC-TuD. The NC-TuD template sequences used were as follows: forward, CATCAACTATCGCGAGTATCGACGTCGAGGCCAAGTATTCTGGTCCACAGAATACAACATATCGCGAGTATCGACGTCGAGGCCCAAG; and reverse, TCATCTTGGGCTCGACGTCGATACTCGCGATAGTTGTATTCTGTGACCAGAATACTTGGGCTCGACGTCGATACTCGCGATAGTT.

Plasmid, siRNA, microRNA mimic, and 2'-O-methyl antisense microRNA transfection

Plasmid DNA was transfected using a Lipofectamine 2000 transfection reagent (Invitrogen), and siRNA, microRNA mimics, and 2'-O-methyl antisense microRNA were transfected into C2C12, primary myoblasts, or U2OS cells using RNAiMAX (Invitrogen) following the manufacturer's instruction.

Luciferase assays

U2OS cells were transfected with microRNAs twice at 24-h intervals. Six hours after the last transfection, luciferase plasmids were transfected. As an internal control, pGL3 (Promega) was cotransfected. At 32–48 h after plasmid transfection, luciferase assays were performed with dual-luciferase reporter assay system (Promega) using a luminometer (Monolight 3020, BD Biosciences). Renilla luciferase values (rr) were first normalized to the cotransfected pGL3-control firefly (*Photinus pyralis*) luciferase values (pp). Then, each rr/pp value in the microRNA transfected samples was again normalized to the rr/pp values obtained in the control GL2 transfected samples.

Western blotting and antibodies

Cells were harvested, washed with 1× PBS, and lysed in NP40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, 10% glycerol) with protease inhibitor cocktail (Sigma). Proteins were separated in SDS-PAGE, transferred, and immunoblotted with various antibodies. The antibodies used were mouse mAb, anti-MyoD (Santa Cruz Biotechnology), anti-Pax7 (DSH, R&D Systems), anti-Smad4 (Santa Cruz Biotechnology), anti-pSmad1/5 (Cell Signaling), anti-MHC, anti-β-actin (Santa Cruz Biotechnology), anti-GAPDH (Sigma), anti-Myogenin (Santa Cruz Biotechnology), and rabbit anti-Smad1 (Invitrogen).

Antagomir synthesis and neonatal injection

The use of animals in all of the studies was done following protocols approved by the Animal Care and Use Committee (ACUC) of University of Virginia. An antagomir specific to miR-26a (hereafter termed Ant26a) and a control antagomir were designed following the procedure, as described previously (Krutzfeldt et al. 2005), and were synthesized by Thermo Fisher Scientific. The Ant26a sequence was as follows: mA.*.mG.*.mC.mC.mU.mA.mU.mC. mC.mU.mG.mG.mA. mU.mU.mA.mC.

mU.mU.*.mG.*. mA.*.mA.*.3'-Chl. The control Antagomir sequence was as follows: mU.*.mA.*.mU.mC.mG.mC.mG.mA.mC.mU.mA.mC. mG.mU.mC.mG. mA.mG.*.mG.*. mC.*.mC.*.3'-Chl. Here, "m" represents a 2'-O-methyl-modified nucleotide, "*" indicates a phosphorothioate linkage, and "Chl" indicates cholesterol that is linked through a hydroxypropylol linkage. On postnatal day 3, C57BL/6J pups were injected intraperitoneally (IP). Antagomirs or controls were administered three times at a dose of 100 mg/kg body weight in 10–15 μL per injection on every day for 3 d. Animals were killed 24 h after the last injection for experimental analyses. BrdU was injected 2 or 4 h before harvesting. Muscle tissues were harvested from the mouse hind legs for qRT-PCR, fresh-frozen (OCT), and paraffin-embedded samples.

Mouse regeneration model and AAV TuD injection

A mouse regeneration model was generated by injecting CTX from *Naja nigricollis* (EMD Millipore) essentially following the procedure described earlier (Yan et al. 2003). Briefly 8- to 10-wk-old male C57BL/6 mice (Harlan) were injected in their TA muscles with 100 μL of 5 μM CTX. AAV9 serotype of 26a-TuD or NC-TuD was produced by the University of Pennsylvania Vector Core facility. We similarly injected 1 × 10¹² GCs (genome copies) of AAV expressing either TuD-26a or NC-TuD 10 d earlier than CTX injection. Mice were anesthetized with isoflurane and killed by cervical dislocation to harvest muscle. Groups of five mice were killed at 1, 3, 5, 7, 10, and 14 d post-injection to collect the TA muscles.

FISH, immunocytochemistry, and immunohistochemistry

Locked nucleic acid (LNA)-based FISH probes for scramble control and miR-26a probes were purchased from Exiqon, and the experiments were performed following the protocol as described earlier (Silahtaroglu et al. 2007). Immunostaining was carried out as described previously (Chen et al. 2006; Kim et al. 2006). Cells grown on sterile glass coverslips were fixed with 2% formaldehyde in PBS for 15 min. Then, the cells were permeabilized with 0.2% Triton X-100 and 1% normal goat serum in ice-cold PBS for 5 min, blocked with 1% normal goat serum (NGS) in PBS twice for 15 min, and incubated with primary antibody (Myogenin [1:50] and MHC [1:400] in 1% NGS) for 1 h. After washing twice, FITC-conjugated anti-mouse IgG (dilution 1:500; Dako Cytomation) was incubated for 1 h. After two washes, nuclei were counterstained with DAPI, and the coverslips were mounted on a glass slide (H-1200, Vector Laboratories). The immunostaining from tissue sections was carried out with slight modifications of the protocols described by others: anti-BrdU and laminin (McCarthy et al. 2011), anti-Ki67, anti-pSmad1/5 (Jalava et al. 2006), and anti-desmin (Liu et al. 2012). The primary antibodies used were mouse BrdU (1:50; Roche); rabbit Ki67 (1:100; Epitomics), mouse pSmad1/5 (1:50; Cell Signaling), mouse anti-laminin (1:100; Millipore), and mouse anti-desmin (1:200; Dako). The secondary antibodies used were Alexa Fluor 594 goat anti-mouse IgG1 (1:400; Invitrogen) and Alexa Fluor 488 goat anti-rabbit IgG (1:400; Invitrogen). Images were taken with a Zeiss confocal microscope. H&E was done as previously described (Yan et al. 2003). Images were captured using an Olympus Hi-Mag microscope or LSM-700 confocal microscope.

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miR-26a is required for skeletal muscle differentiation and regeneration in mice

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