

The *H19* long noncoding RNA gives rise to microRNAs miR-675-3p and miR-675-5p to promote skeletal muscle differentiation and regeneration

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Regulated expression of the *H19* long noncoding RNA gene has been well characterized as a paradigm for genomic imprinting, but the *H19* RNA's biological function remains largely unclear. *H19* is abundantly expressed maternally in embryonic tissues but is strongly repressed after birth, and significant transcription persists only in skeletal muscle. Thus, we examined the role of the *H19* RNA in skeletal muscle differentiation and regeneration. Knockdown of *H19* RNA in myoblast cells and *H19* knockout mouse satellite cells decreases differentiation. *H19* exon1 encodes two conserved microRNAs, miR-675-3p and miR-675-5p, both of which are induced during skeletal muscle differentiation. The inhibition of myogenesis by *H19* depletion during myoblast differentiation is rescued by exogenous expression of miR-675-3p and miR-675-5p. *H19*-deficient mice display abnormal skeletal muscle regeneration after injury, which is rectified by reintroduction of miR-675-3p and miR-675-5p. miR-675-3p and miR-675-5p function by directly targeting and down-regulating the anti-differentiation Smad transcription factors critical for the bone morphogenetic protein (BMP) pathway and the DNA replication initiation factor Cdc6. Therefore, the *H19* long noncoding RNA has a critical *trans*-regulatory function in skeletal muscle differentiation and regeneration that is mediated by the microRNAs encoded within *H19*.

[*Keywords:* *H19*; miR-675; long noncoding RNA; skeletal muscle; differentiation; regeneration]

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H19 is one of the best known imprinted genes that was discovered from several genetic screens (Pachnis et al. 1984; Davis et al. 1987; Poirier et al. 1991). It was first isolated in a screen for genes that were up-regulated by α -fetoprotein in the liver (Pachnis et al. 1984). Concurrently, *H19* was identified in the same genetic screen for myogenic differentiation that identified MyoD and was called MyoH (Davis et al. 1987). *H19* was also found to be up-regulated in a screen during embryonic stem cell differentiation (Poirier et al. 1991). These findings indicate that *H19* may have a role in cellular differentiation.

The *H19* gene is located on chromosome 7 in mice and chromosome 11 in humans and is expressed only from the maternal allele in both species (Bartolomei et al. 1991; Zhang and Tycko 1992). Although the *H19* gene is imprinted paternally, the *H19* RNA itself does not participate in the imprinting mechanism (Brannan et al. 1990).

The locus has been intensively analyzed as a model system for genomic imprinting; however, the biological functions of the *H19* gene product are only now being elucidated. The *H19* RNA does not contain any conserved ORFs between mice and humans, and evolutionarily conserved structure prediction studies suggest that *H19* is a noncoding RNA (Brannan et al. 1990; Juan et al. 2000). It has recently been established that *H19* exon1 encodes two conserved microRNAs: miR-675-3p and miR-675-5p (Cai and Cullen 2007).

H19 is dysregulated in many cancers, and various studies have suggested both tumorigenic and anti-tumorigenic roles for the *H19* RNA (Moulton et al. 1994; Adriaenssens et al. 1998; Yoshimizu et al. 2008). Especially, loss of *H19* expression is associated with

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Wilms' tumor and rhabdomyosarcoma (Chung et al. 1996; Lynch et al. 2002; Rump et al. 2005; Ecke et al. 2009), and, in fact, restoration of *H19* expression can mitigate the tumor phenotypes (Hao et al. 1993). More recently, mouse genetic studies indicate that *H19* RNA represses embryonic placental growth (Keniry et al. 2012) and *trans*-regulates a network of imprinted genes (Gabory et al. 2009) during fetal development.

H19 is a highly abundant transcript in almost all embryonic and neonatal tissues, especially in skeletal muscle, where expression is mediated by a downstream skeletal muscle-specific enhancer, and represents ~1% of all cellular mRNA (Eun et al. 2013a,b). Shortly after birth, *H19* RNA is dramatically down-regulated in all tissues. However, skeletal muscle is unusual in that *H19* repression is only partial so that significant amounts of *H19* RNA are detected in adult animals. Interestingly, *H19* expression is induced by MyoD (Borensztein et al. 2013). However, we do not know whether *H19* is important for skeletal muscle differentiation and, if so, what its mechanism of action is.

Here we establish a definitive role of *H19* in skeletal muscle differentiation and regeneration. *H19* is essential and required for proper muscle differentiation in vitro and muscle regeneration in vivo. Two conserved microRNAs, miR-675-3p and miR-675-5p, encoded by the exon1 of *H19* are responsible for this biological function of *H19*. miR-675-3p represses the bone morphogenetic protein (BMP) pathway by targeting anti-differentiation Smad transcription factors, *smad1* and *Smad5*, and miR-675-5p represses *Cdc6*, a DNA replication initiation factor. Thus, *H19* long noncoding RNA has an essential function in skeletal muscle differentiation and regeneration that is mediated by the microRNAs embedded within it.

Results

H19 long noncoding RNA and its encoded microRNAs, miR-675-3p and miR-675-5p, are expressed in the skeletal muscles and up-regulated during myoblast differentiation and muscle regeneration

To gain an understanding of *H19* function, we first looked at the expression pattern of *H19* in a panel of adult mouse tissues and embryonic samples using quantitative RT-PCR (qRT-PCR). After expression in embryos, *H19* RNA was dramatically down-regulated in most tissues except skeletal muscle (Fig. 1A; Supplemental Fig. 1A). The C2C12 myoblast cells serve as an excellent model system for studying muscle cell differentiation in vitro. Differentiation of myoblast cells into myocytes or myotubes can be accomplished by reducing serum supplements. *H19* was gradually up-regulated during differentiation of C2C12 myoblast cells, with a slight decrease after differentiation day 4 (DM4), when myotubes were completely formed (Fig. 1B; Supplemental Fig. 1B). Similarly, *H19* was also up-regulated during both mouse and human skeletal muscle satellite cell differentiation (Fig. 1C,D; Supplemental Fig. 1C).

As differentiation is an important event during adult skeletal muscle regeneration, we investigated *H19* RNA levels after cardiotoxin (CTX)-mediated injury of mouse skeletal muscle, tibialis anterior (TA). The injury activates quiescent muscle satellite cells, which re-enter the cell cycle and, after proliferation, differentiate to replenish the degenerated muscle fibers (Yan et al. 2003; Dey et al. 2012). *H19* RNA decreased rapidly on days 1 and 3 after injury, during the phase of muscle degeneration, and then increased through days 5–7, during muscle regeneration (Fig. 1E). Similar to what we observed after terminal myoblast differentiation in vitro, *H19* levels were slightly decreased at day 14, a stage when new myofibers were already formed (Fig. 1E). We then sought to determine the expression pattern of two conserved microRNAs, miR-675-3p and miR-675-5p, encoded by exon1 of *H19*. These microRNAs displayed an expression pattern similar to *H19* in mouse tissues and during both myoblast differentiation in vitro and skeletal muscle regeneration in vivo (Fig. 1A–E; Supplemental Figs. 1A–C, 2A,B).

H19 promotes skeletal muscle differentiation, and its trans-regulatory function is mediated by miR-675-3p and miR-675-5p

To determine the role of *H19* long noncoding RNA in skeletal muscle differentiation, we knocked down *H19* in C2C12 myoblast cells and human skeletal muscle satellite cells. Although the conventional view is that siRNA cannot target nuclear RNA, several nuclear long noncoding RNAs have been knocked down by many groups using siRNAs (Clemson et al. 2009; Matoba et al. 2011; Tripathi et al. 2013). Therefore, we transfected siRNAs specific to mouse *H19* in C2C12 myoblast cells in growth medium (GM) and changed to differentiation medium (DM). The next day (DM1), siRNAs were again transfected, and the cells were harvested on DM3. *H19* siRNAs reduced *H19* RNA level by 43% (Fig. 2A; Supplemental Fig. 3) and decreased myogenesis, as shown by significant (41% and 58%, respectively) reduction of two established myogenic markers, myogenin and MHC (Dey et al. 2011), and morphology (Fig. 2B; Supplemental Fig. 4). Genome-wide microarray analyses revealed that a large number of differentiation-specific genes, including *MyoD*, *myogenin*, *myosin* family, *Mef2c*, *IGF2*, *troponin*, *titin*, and *creatin kinase*, were significantly down-regulated, whereas cell cycle and replication factors such as *Cyclin E1*, *Cyclin E2*, *Cyclin D1*, *Cyclin D2*, *Brca1*, *Mcm4*, *Mcm6*, *Mcm7*, and *Cdc6* were up-regulated after knockdown of *H19* (Supplemental Table 1). We performed similar experiments in human skeletal muscle satellite cells using siRNAs against human *H19*. The siRNAs against human *H19* reduced *H19* RNA level by 62% (Fig. 2C), and this knockdown was associated with decrease in differentiation, as measured by reduction of myogenin and MHC mRNA level by 41% and 72%, respectively (Fig. 2D). A similar result was obtained from mouse primary satellite cells (Supplemental Fig. 5A,B). Finally, a similar decrease in differentiation was seen in primary myoblast cells obtained from *H19*-deficient mice (Δ Muscle Enhancer/+),

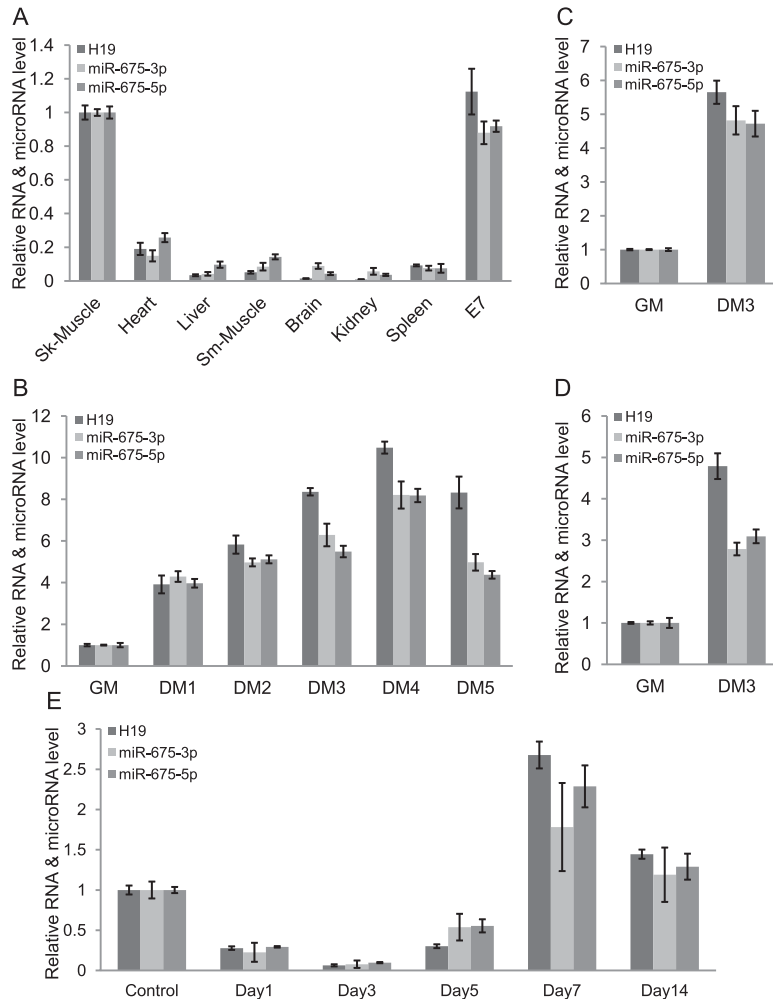


Figure 1. *H19* long noncoding RNA and its encoded microRNAs, miR-675-3p and miR-675-5p, are expressed in the adult skeletal muscles and up-regulated during myoblast differentiation and muscle regeneration. (A) *H19*, miR-675-3p, and miR-675-5p are abundantly expressed in the adult skeletal muscles and whole embryos. (E7) Embryonic day 7. *H19*, miR-675-3p, and miR-675-5p are up-regulated during differentiation of C2C12 myoblast cells (B), mouse satellite cells (C), and human satellite cells (D). qRT-PCR of *H19*, miR-675-3p, and miR-675-5p after the indicated days in differentiation medium (DM) is shown. (GM) Growth medium. Each value is normalized to GAPDH (for *H19*) or U6sn RNA (for microRNAs) and expressed relative to skeletal muscle (A) or cells in GM (B–D). (E) Expression of *H19*, miR-675-3p, and miR-675-5p is down-regulated on days 1–3, is up-regulated on days 5–7, and decreases slightly on day 14 after TA muscle injury induced by injection of CTX. Mean \pm standard error of the mean (SEM) of three biological replicates.

where the + allele is paternal and so is repressed by imprinting) (Fig. 2E,F). Altogether, these results strongly demonstrate that *H19* is a critical factor for skeletal muscle differentiation.

H19 exon1 encodes two conserved microRNAs (miR-675-3p and miR-675-5p) (Cai and Cullen 2007), and knockdown in *H19* RNA by siRNA treatment or genetic ablation results in the expected concomitant reduction in these microRNAs (Fig. 2A,C,E; Supplemental Fig. 3). To examine whether the prodifferentiation function of the *H19* noncoding RNA is mediated by these microRNAs, we cotransfected mature miR-675-3p and miR-675-5p when *H19* was down-regulated in the myoblast cells. Exogenous miR-675-3p and miR-675-5p together rescued the reduction in differentiation almost entirely in all three cell types examined (Fig. 2B,D,F). Thus, miR-675-3p and miR-675-5p appear to be the major contributors to the *trans*-regulatory prodifferentiation function of *H19*.

miR-675-3p and miR-675-5p promote muscle differentiation

We next determined whether miR-675-3p and miR-675-5p can promote myogenic differentiation in C2C12 myoblast

cells. C2C12 myoblast cells were transfected with RNA duplexes encoding miR-675-3p and miR-675-5p (miR-675) or GL2 control microRNA (22 bases from luciferase gene) (Dey et al. 2011) in GM and then transferred to DM. The exogenous miR-675-3p and miR-675-5p increased differentiation, as seen by the morphology and the number of myogenin- and MHC-positive cells (Fig. 3A,B; Supplemental Table 2). Myogenin and MHC mRNAs were up-regulated in miR-675-3p- and miR-675-5p-transfected myoblasts even when the cells were transfected and continuously grown in GM (Fig. 3C), suggesting that miR-675-3p and miR-675-5p are sufficient to induce myogenic differentiation. Consistent with this, ectopic expression of the fragment of *H19* (mid) containing the pre-microRNA specifically increased myogenic differentiation, and this ability was lost when this fragment was mutated in the sequences of both microRNAs (mid-Mut) (Supplemental Fig. 6A,B).

In a reciprocal experiment, we knocked down miR-675-3p and miR-675-5p levels in the C2C12 cells by transfecting 2'-O-methyl antisense oligonucleotides against both of these microRNAs (anti-675) or a control oligonucleotide (anti-GL2) in GM and then transferring the cells to DM. We show later that both antisense oligonucleotides

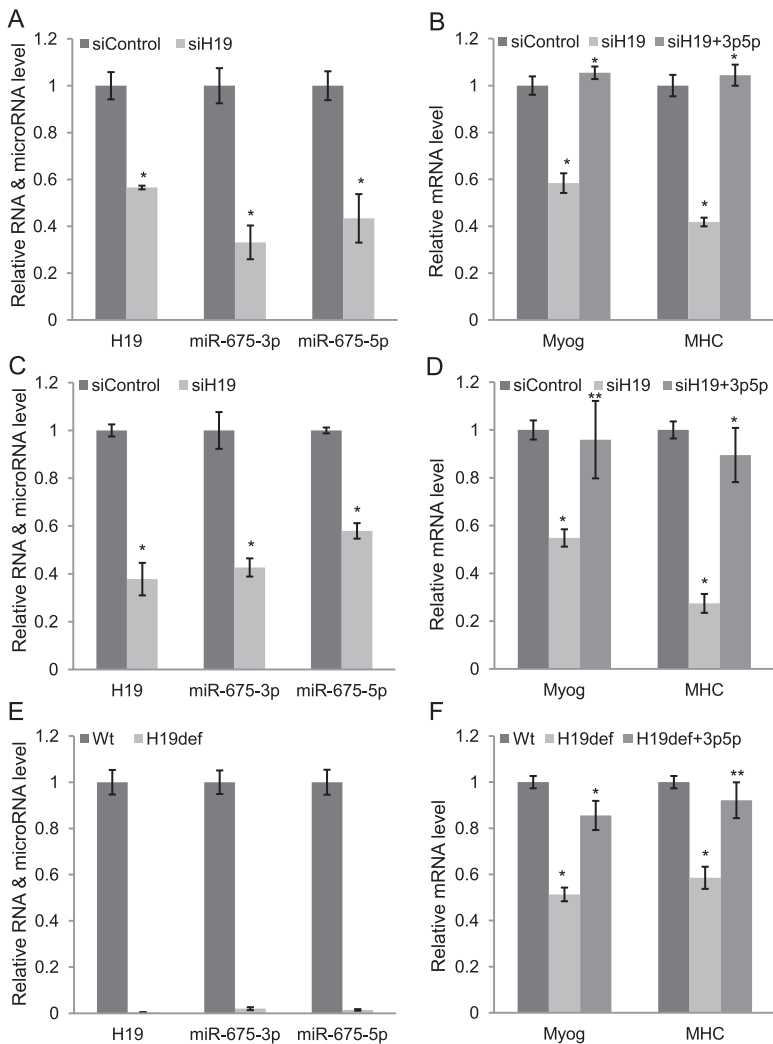


Figure 2. *H19* promotes muscle differentiation in C2C12 cells, primary human satellite cells, and primary mouse myoblasts; its *trans*-regulatory function is mediated by miR-675-3p and miR-675-5p. We transfected siRNAs specific to mouse or human *H19* in C2C12 myoblast cells or primary human satellite cells in GM and changed to DM. The next day (DM1), siRNAs were again transfected, and the cells were harvested for RNA analysis on DM3. Alternatively, we transfected cells with GL2 (22 bases from luciferase gene) as negative controls. Knockdown of *H19* decreases *H19*, miR-675-3p, and miR-675-5p levels in C2C12 myoblast cells (A) and human satellite cells (C) and results in decreased expression of the differentiation markers myogenin and MHC mRNAs (B,D). (B,D) Exogenous miR-675-3p and miR-675-5p rescue the differentiation in both cell types. (E,F) Satellite cells derived from *H19*-deficient mice (*H19* Δ ME/+) lack *H19*, miR-675-3p, and miR-675-5p (E) and show decreased expression of myogenin and MHC mRNAs at DM1 (F). (F) Exogenous miR-675-3p and miR-675-5p restore expression of the differentiation markers. The normalization for *H19*, myogenin, MHC mRNAs, and microRNAs was as described in Figure 1. Mean \pm SEM of three biological replicates are shown. (*) $P < 0.001$; (**) $P < 0.005$.

effectively neutralize the actions of the microRNAs on two specific targets (Supplemental Fig. 7A,B). Anti-675-transfected cells reduced differentiation, as measured by myogenin and MHC staining and their respective RNA levels (Fig. 3D–F). Therefore, miR-675-3p and miR-675-5p are prodifferentiation factors for C2C12 differentiation.

H19 mutant mice impair skeletal muscle regeneration that can be rescued by reintroduction of miR-675-3p and miR-675-5p

To test whether *H19* plays a role in skeletal muscle regeneration *in vivo*, we employed a mouse model for skeletal muscle regeneration (Yan et al. 2003; Dey et al. 2012). Specifically, we examined mice carrying a 1-kb deletion of *H19* (*H19* Δ Exon1/+) that removes almost the entire exon1, including the region encoding the microRNAs, and reduces levels of even the partial *H19* transcript by >100-fold (Srivastava et al. 2003; C Gebert and K Pfeifer, unpubl.). During the first 3 d following CTX injury, wild-type mouse TA muscles display extensive myofiber degeneration, but muscle regeneration is nearly

complete on day 14 after injury (Dey et al. 2012). *H19* Δ Exon1/+ mice showed impaired regeneration on day 14 after CTX injury, as marked by the persistence of inflammatory cells and smaller myofibers (Fig. 4A, first two panels, cf. the GL2 control duplex-injected wild type and *H19* Δ Exon1/+). We immunostained the muscle sections for desmin and laminin. Desmin is an intermediate filament protein that is abundantly expressed in newly made muscle fibers during muscle regeneration (Kuisk et al. 1996), whereas laminin demarcates the muscle fiber boundary. The newly made muscle fibers were significantly smaller in *H19* Δ Exon1/+ muscle as compared with the wild-type muscle (Fig. 4B). Thus, we conclude that lack of *H19* significantly impairs the regeneration of adult skeletal muscle.

We next examined whether introduction of mature miR-675-3p and miR-675-5p can rescue the defects in regeneration in the *H19*-deficient muscles. Mature miR-675-3p and miR-675-5p were injected in the TA muscle of one leg, and GL2 control microRNA was injected in the contralateral leg as described (Ge et al. 2011; Eulalio et al. 2012). Injection of mature miR-675-3p and miR-675-5p in

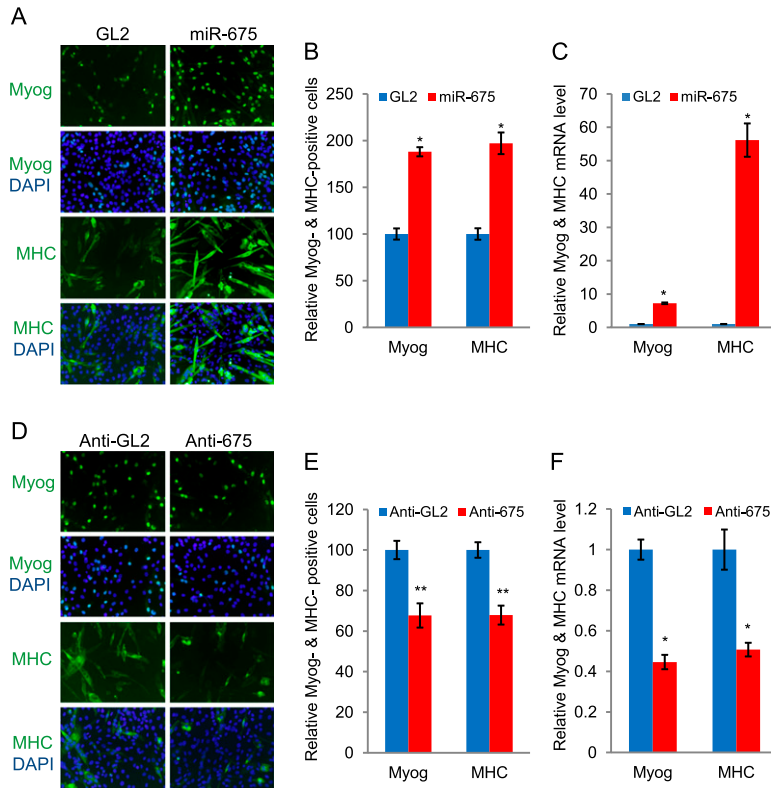


Figure 3. miR-675-3p and miR-675-5p promote muscle differentiation. (A) C2C12 myoblast cells in GM were transfected twice at 24-h intervals with GL2 control microRNA or miR-675-3p and miR-675-5p (miR-675). The cells were then transferred to DM and stained for myogenin at 32 h or MHC at 60 h. (Green) Myogenin or MHC; (blue) nuclei stained by DAPI. (B) Numbers of myogenin- and MHC-positive cells are presented relative to the GL2 control, which is set as 100%. Mean \pm SEM of 10 random fields (see Supplemental Table 2 for details). (*) $P < 0.001$. (C) C2C12 myoblast cells were transfected as in A and kept in GM for an extra 24 h before harvesting to measure myogenin and MHC mRNA by qRT-PCR. Each value was normalized to GAPDH in the same sample and then again to the value in GL2-transfected cells. Mean \pm SEM of three biological replicates. (*) $P < 0.001$. (D,E) 2'-O-methyl antisense oligonucleotides against GL2 (anti-GL2) or miR-675-3p and miR-675-5p (anti-675) were transfected as in A, and the cells were stained for myogenin at 32 h or MHC at 60 h in DM. Data are presented as in B. (F) Measurement of myogenin and MHC mRNAs by qRT-PCR as in C. Mean \pm SEM of three biological replicates are shown. (*) $P < 0.001$; (**) $P < 0.005$.

the TA muscle deficient in *H19* restored miR-675-3p and miR-675-5p levels (Fig. 4C) and improved the regeneration at day 14, whereas control GL2-injected samples failed to regenerate completely (Fig. 4A,B).

We repeated these analyses in mice with an alternative mutation called *H19R*. The *H19R* chromosome carries an insertion of a transcriptional insulator that prevents interactions between the *H19* promoter and the downstream skeletal muscle enhancer (ME) and thus results in a dramatic reduction of *H19* specifically in muscle cells while leaving expression in endodermal cells unaffected (Yoon et al. 2007). As with *H19* Δ Exon1/+ mice, *H19R*/+ mice showed defects in regeneration that was rescued by injection of miR-675-3p and miR-675-5p (Fig. 4D–F). These results confirm that *H19* is required for normal muscle regeneration in vivo. Moreover, the cell type specificity of the mutation confirms that *H19*-dependent regeneration is cell-autonomous: The muscle cells themselves require *H19*.

Smad1, Smad5, and Cdc6 are the important targets for miR-675-3p and miR-675-5p

Because miR-675-3p and miR-675-5p promote skeletal muscle differentiation and regeneration, we anticipated that their direct targets would include anti-differentiation or proliferation genes. Target prediction algorithms predicted a large number (>100) of targets for miR-675-3p and miR-675-5p (Supplemental Fig. 8; Supplemental Table 3; data not shown). Of the predicted targets, the transcription factors that mediate anti-differentiation

effects of the BMP pathway, *Smad1* and *Smad5*, and a DNA replication factor, *Cdc6*, attracted our attention because BMP pathway and DNA replication factors need to be down-regulated during myoblast differentiation (Dey et al. 2012; data not shown). To demonstrate whether these are the direct targets, the 3' untranslated regions (UTRs) of *Smad1*, *Smad5*, and *Cdc6* were separately fused to a luciferase reporter gene. *Smad1* and *Smad5* reporters were repressed by miR-675-3p by 47% and 48%, respectively (Fig. 5A,B). Similarly miR-675-5p repressed a luciferase reporter fused to the 3' UTR of *Cdc6* by 46% (Fig. 5C). Mutation of the microRNA target sites relieved the repression in all three cases (Fig. 5A–C). Not only were the luciferase reporters containing 3' UTRs of *Smad1* or *Cdc6* repressed by miR-675-3p or miR-675-5p, respectively, but the repression was relieved by the 2'-O-methyl inhibitor of miR-675-3p or miR-675-5p (Supplemental Fig. 7A,B). Consistent with *Smad1*, *Smad5*, and *Cdc6* being direct targets of these microRNAs, all three proteins were down-regulated by transfection of C2C12 cells in GM with the cognate RNAs (Fig. 5D–F). In a reciprocal experiment, we inhibited the endogenous miR-675-3p or miR-675-5p using 2'-O-methyl or LNA antisense inhibitor and observed increased levels of endogenous *Smad1*, *Smad5*, and *Cdc6* proteins during C2C12 differentiation (Fig. 5G–I). These results are consistent with the hypothesis that miR-675-3p and miR-675-5p can repress *Smad1*, *Smad5*, and *Cdc6* proteins and thus promote myogenic differentiation.

As shown in Figure 1, *H19* or its encoded miR-675-3p and miR-675-5p are down-regulated on days 1–3 after CTX-induced injury but then increases steadily on days

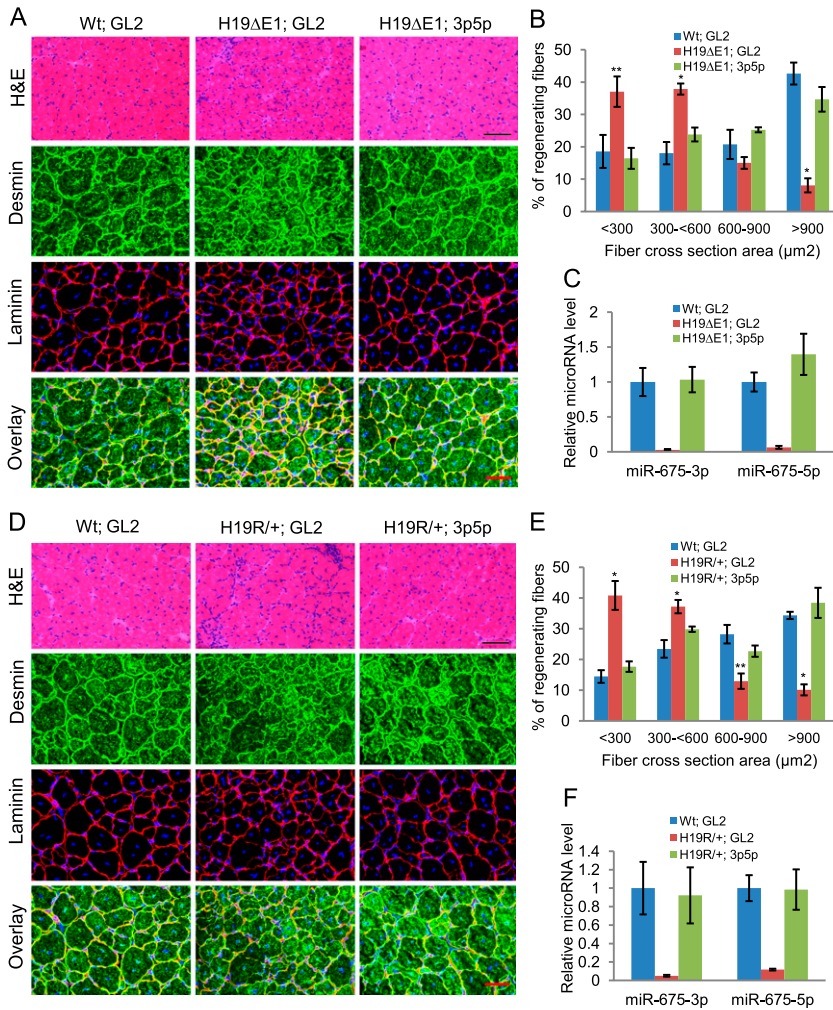


Figure 4. *H19* mutant mice show defects in muscle regeneration that can be rescued by exogenous miR-675-3p and miR-675-5p. (A) TA muscles in wild-type (Wt) or *H19*-deficient (*H19* Δ E1) littermates were injured by CTX injection and, after 3 d, were injected with miR-675-3p and miR-675-5p or with GL2 control microRNA in the contralateral leg. (Top panel) Representative H&E-stained images are from TA muscles harvested 14 d after CTX injury. Bar, 100 μ m. (Bottom three panels) TA muscles from the same specimens were stained with desmin and laminin. Bar, 50 μ m. Five mice were used in each group. (B) Cross-section areas of regenerating fibers of these samples were quantitated using Image J software. More than 200 individual fibers were counted in each group. (C) miR-675-3p and miR-675-5p levels were measured in these samples by qRT-PCR. (D–F) Data were obtained as in A–C from *H19*R/+ and control littermates. Bars: H&E, 100 μ m; desmin and laminin, 50 μ m. Mean \pm SEM ($n = 5$). (*) $P < 0.001$; (**) $P < 0.005$. Note that the *H19* mutations used in this study were specifically chosen because they do not disrupt imprinting of the adjoining *Igf2* gene.

5–14 as new myofibers are formed (Fig. 1E). Thus, we tested the mRNA level of targets of miR-675-3p and miR-675-5p, *Smad1*, *Smad5*, and *Cdc6*, in these samples during regeneration. The levels of these targets were anti-correlated to *H19* and miR-675-3p and miR-675-5p, increasing on days 1–3 after injury and then decreasing steadily on days 5–14 after injury (Fig. 5J–L). This result is consistent with the notion that miR-675-3p and miR-675-5p repress *Smad1*, *Smad5*, and *Cdc6* during regeneration.

We showed that *H19* generates miR-675-3p and miR-675-5p to down-regulate *Smad1*, *Smad5*, and *Cdc6* during differentiation. To test whether misregulation of these proteins is sufficient to explain the reduced differentiation due to *H19* deficiency, we performed knockdown of *Smad1*, *Smad5*, and *Cdc6* with si*H19* in C2C12 cells. The depletion of *Smad1*, *Smad5*, and *Cdc6* in *H19*-depleted cells was sufficient to overcome the decrease in differentiation as measured by the levels of myogenin and MHC mRNAs (Fig. 5M). Thus, *Smad1*, *Smad5*, and *Cdc6* are important targets for the pro-differentiation action of *H19*.

Discussions

Our findings reveal a new function of *H19* in promoting skeletal muscle differentiation and regeneration. Interestingly, *H19*, named MyoH, was identified in the same genetic screen for myogenic differentiation that identified MyoD (Davis et al. 1987). *H19* was also found to be up-regulated in a screen during embryonic stem cell differentiation (Poirier et al. 1991). Together, these findings suggest that *H19* plays a role in cellular differentiation. Our result is consistent with the earlier findings that inactivation of *H19* due to loss of heterozygosity or hypermethylation of maternal alleles is associated with Wilms' tumor and rhabdomyosarcoma (Chung et al. 1996; Lynch et al. 2002; Ecke et al. 2009). Rhabdomyosarcoma arises from defects in skeletal muscle differentiation, and treatment of DNA methylation inhibitor 5-AzaC-2'-deoxycytidine reactivated *H19* in rhabdomyosarcoma cell lines and prevented rhabdomyosarcoma formation in mice (Chung et al. 1996; Lynch et al. 2002; Ecke et al. 2009). As miR-675-3p and miR-675-5p can induce muscle cell differentiation, we are curious whether introduction of

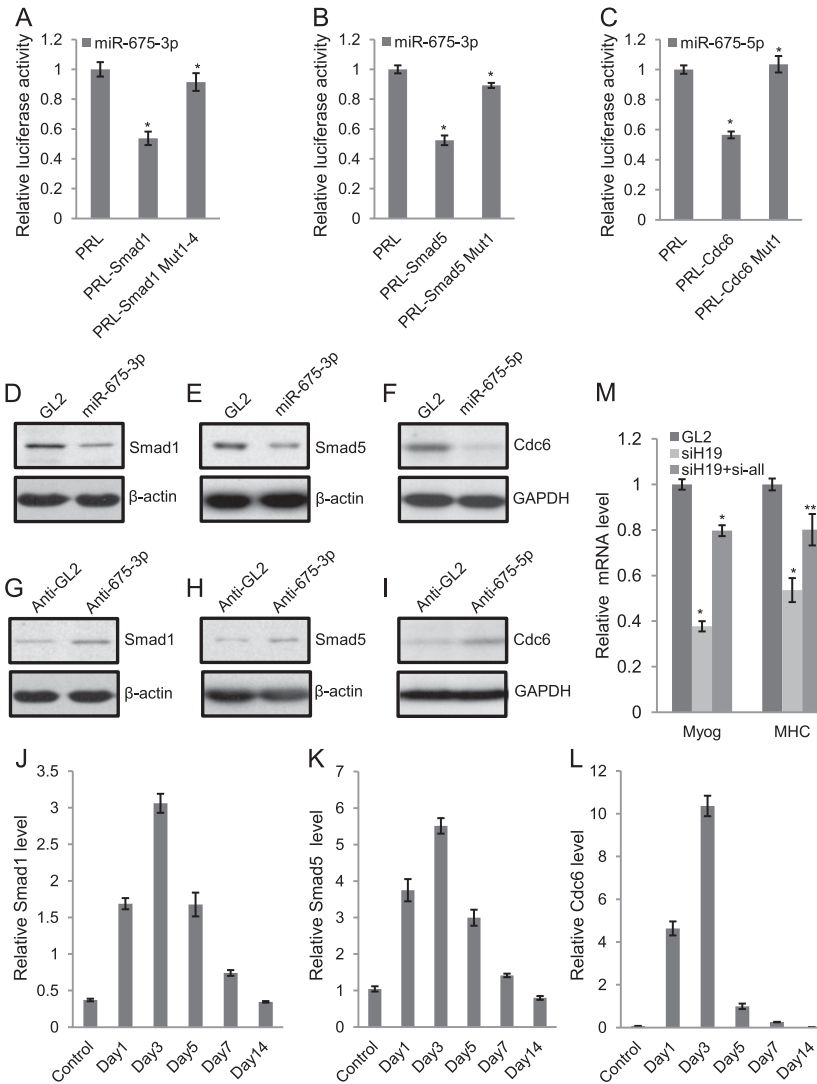


Figure 5. *Smad1*, *Smad5*, and *Cdc6* are important targets for miR-675-3p and miR-675-5p. (A–C) Cotransfection of miR-675-3p or miR-675-5p microRNA into U2OS osteosarcoma cells repressed *Renilla* (rr) luciferase activity when the rr reporter was fused to the target 3' UTR. Mutations in the target sites in the 3' UTR (Supplemental Fig. 7) relieved the repression. A firefly (pp) luciferase plasmid was cotransfected as a transfection control. The rr/pp was normalized to that for a control *Renilla* luciferase plasmid with a vector 3' UTR (PRL) and expressed relative to the normalized rr/pp in cells transfected with the GL2 RNA control. Mean \pm SEM of three individual experiments. (*) $P < 0.001$. (D–F) Transfection of miR-675-3p or miR-675-5p in C2C12 myoblast cells in GM decreased *Smad1*, *Smad5*, and *Cdc6* protein levels as detected by Western blotting 2 d after the second transfection. GL2 served as a control oligonucleotide. β -Actin or GAPDH was used as a loading control. (G–I) 2'-O-methyl oligonucleotide against miR-675-3p or LNA antisense oligonucleotide against miR-675-5p (anti-675-3p or anti-675-5p) increased endogenous *Smad1*, *Smad5*, and *Cdc6* protein levels in C2C12 cells cultured in DM for 2 d. 2'-O-methyl or LNA antisense GL2 (anti-GL2) served as a control oligonucleotide. β -Actin or GAPDH is used as a loading control. (J–L) As measured by qRT-PCR, miR-675-3p or miR-675-5p target genes display anti-correlation in their temporal expression pattern during regeneration of skeletal muscle after CTX injury. (M) C2C12 myoblast cells were transfected with GL2 control siRNA, *H19* siRNA, or *H19* siRNA combined with siRNAs specific to *Smad1*, *Smad5*, and *Cdc6* once in GM and then on DM1, and cells were harvested on DM3. qRT-PCR was performed for myogenin and MHC. The normalization for myogenin and MHC mRNAs was as described in Figure 1. Mean \pm SEM of three biological replicates are shown. (*) $P < 0.001$; (**) $P < 0.005$.

these microRNAs is sufficient to differentiate rhabdomyosarcoma cell lines or prevent rhabdomyosarcoma formation in vivo. Conversely, ectopic expression of *H19* transgene causes prenatal lethality (Brunkow and Tilghman 1991). However, other groups found that the *H19* transgenic mice are viable, and the transgene regulates the imprinted gene network (Pfeifer et al. 1996; Gabory et al. 2009). Thus, it will be interesting to test whether ectopic expression of miR-675-5p and miR-675-3p produces phenotypes similar to *H19* transgenic mice.

A genome-wide survey predicted that ~100 long noncoding RNAs encode microRNAs (He et al. 2008). Our findings demonstrate how one could experimentally validate whether the encoded microRNAs mediate specific functions of specific long noncoding RNAs.

miR-675-3p and miR-675-5p promote muscle differentiation and regeneration by negatively regulating two

important classes of targets: the BMP pathway transcription factors *Smad1* and *Smad5* and DNA replication initiation factor *Cdc6*. We showed earlier that miR-26a, another microRNA induced during muscle differentiation, promotes myogenesis by repressing signal transduction by other members of the BMP/TGF- β family (Dey et al. 2012). Recent studies identified *Igf2* and/or *Igf1r* as targets of miR-675-3p/miR-675-5p in embryonic placental growth and maintenance of hemopoietic stem cell quiescence (Keniry et al. 2012; Venkatraman et al. 2013). However, *Igf2* levels increase during differentiation (Ge et al. 2011), when the miR-675-3p and miR-675-5p increase (Fig. 1B), and we did not see any significant difference in the expression of *Igf2* and *Igf1r* in the muscles from mice that were wild type, *H19* mutant, or *H19* mutant restored with miR-675-3p and miR-675-5p (data not shown). Interestingly, *Igf2* is inhibited by miR-

125b, whose expression decreases during muscle differentiation and regeneration (Ge et al. 2011). Thus, it is likely that during skeletal muscle differentiation, *Igf2/Igf1r* genes are regulated by miR-125b or some other unknown mechanisms, while the proproliferative *Smad1*, *Smad5*, and *Cdc6* are the major targets of miR-675-3p and miR-675-5p during skeletal muscle differentiation and regeneration.

Other muscle differentiation-induced microRNAs inhibit other anti-differentiation factors: miR-206 inhibits Pax7 (Dey et al. 2011), HDAC4 (Chen et al. 2006), and Notch3 (Gagan et al. 2012), while miR-378 targets *MyoR* (Gagan et al. 2011). Similarly, we showed that three other microRNAs induced during muscle differentiation with promyogenic activity target cell cycle factors: miR-206 down-regulates DNA polymerase α (Kim et al. 2006), and miR-322 and miR-503 repress *Cdc25a*, the phosphatase that removes an inhibitory phosphate from CDK2 (Sarkar et al. 2010). These results indicate that the concerted actions of multiple microRNAs are important for muscle development by collectively down-regulating anti-differentiation and cell cycle factors. The redundancy of multiple prodifferentiation microRNAs is similar to the redundancy of myogenic transcription factors like MyoD and explains the normal skeletal muscle development following the knockout of miR-206 (Williams et al. 2009) or *H19* (Leighton et al. 1995). This bears a striking parallel with the normal development seen after knockout of one myogenic transcription factor like MyoD (Rudnicki et al. 1992). The requirement of these factors during differentiation becomes more evident under stressed conditions such as skeletal muscle regeneration; e.g., miR-206 (Liu et al. 2012), MyoD (White et al. 2000), and now *H19* in our study. As with the myogenic transcription factors, simultaneous knockout of multiple muscle differentiation-induced microRNAs will need to be tested to uncover the essential functions of microRNAs during skeletal muscle development. Consistent with the notion that the compensatory mechanisms are activated after chronic knockout of microRNA genes is the observation in Figure 2 that acute knockdown of *H19* by siRNA impairs differentiation to almost the same extent as in the *H19* knockout cells even though the former have more residual microRNAs.

A recent study reports that *H19* is a molecular sponge for let-7 in a human embryonic kidney (HEK293) cell line (Kallen et al. 2013). The investigators suggest that *H19* sponges let-7 in C2C12 cells to inhibit muscle differentiation. Our results are in stark contrast because we provide evidence from knockdown and genetic knockout of *H19* that it is a prodifferentiation factor: in vitro in C2C12 cells and primary myoblasts in culture and in vivo in regenerating skeletal muscle. Moreover, we show that the *H19*-encoded microRNAs miR-675-5p and miR-675-3p can execute the prodifferentiation function of *H19* in skeletal muscle lacking endogenous *H19*. A huge difference between the 90-fold induction of let-7 during C2C12 differentiation reported by Kallen et al. (2013) and the lack of any such induction of let-7 in our cells (Supplemental Table 4; Supplemental Fig. 9A–C) suggests that

there is a significant difference between the C2C12 cell lines and the in vitro differentiation conditions, which may account for the contradictory roles attributed to *H19*. In this context, it is particularly important that the prodifferentiation function of *H19* is confirmed in our experiments in primary myoblasts in vitro and in regenerating skeletal muscle in vivo. In addition, our studies provide important insight into how this critical *trans*-regulatory function of *H19* in skeletal muscle differentiation and regeneration is mediated by the microRNAs embedded within.

Materials and methods

Cell culture

The C2C12 mouse myoblast cell line was supplied by American Type Culture Collection (ATCC) (Yaffe and Saxel 1977). Cells were cultured at subconfluent densities in GM, made up of DMEM supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin. C2C12 myoblast cells were differentiated into myocytes or myotubes in DM (Andres and Walsh 1996), consisting of DMEM containing 2% heat-inactivated horse serum and 1% penicillin/streptomycin. U2OS cells were cultured in DMEM supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin. Mouse primary myoblasts and differentiated myotubes were used as described (Dey et al. 2011). *H19*-deficient primary myoblast cells were obtained from a ME-deleted (*H19* Δ ME/+) mouse line (Kaffer et al. 2001). Δ ME removed a 10-kb region far downstream from *H19* that is essential for *H19* expression in skeletal muscle. Human satellite cells and *H19*-deficient primary myoblast cells were cultured as described (Bois and Grosveld 2003; Zhu et al. 2007).

Generation of constructs

Mouse *Smad1* 3' UTR of 1.4 kb, *Smad5* 3' UTR of 1.5, and *Cdc6* 3' UTR of 1.4 kb were amplified by PCR from C2C12 myoblast genomic DNA or cDNA and cloned into pRL-CMV vector. Specific point mutations in *Smad1*, *Smad5*, and *Cdc6* 3' UTRs cloned into pRL-CMV vector were created using a site-directed mutagenesis kit (Stratagene). Fragments of *H19* (5', bases 1–956; mid, bases 741–1407; 3', bases 1296–2068) were subcloned to the pMSCV retroviral vector. Mid-Mut carried mutations in the sequences of miR-675-5p and miR-675-3p as follows: TGG TGCGGAAAGGGCCACAGT was changed to CCACATTTCAAGGGCCACAGT, and CTGTATGCCCTAACCGCTCAGT was changed to CCACATCGCCATACCGCTCAGT. Retrovirus was made in HEK293T cells cotransfected with virus packaging plasmids using a standard protocol.

Transfection of plasmids, siRNAs, mature microRNAs, and 2'-O-methyl or LNA antisense microRNAs

We used Lipofectamine 2000 (Invitrogen) to transfect plasmid DNA and RNAiMAX (Invitrogen) to transfect siRNAs, microRNA mimics, and 2'-O-methyl or LNA antisense microRNAs following the manufacturer's instructions. The following siRNA sequences were used: mouse si*H19*, GGACUGGAGACUAGGG UAAAdTdT; human si*H19*, UAAGUCAUUGCACUGG UUUdTdT; GCUAGAGGAACCAGACCUdTdT; si-*Smad1*, GGGCGAU GAAGAAGAGAAAdTdT; si-*Smad5*, CCUGGGAUUGUUGU CAAAUGUUAUdTdT. A pool of three siRNAs against mouse *Cdc6* was purchased from Santa Cruz Biotechnology. We transfected 50 nM siRNAs specific to mouse or human *H19* (and

control siRNA) in C2C12 myoblast or human satellite cells in GM and changed to DM. The next day (DM1), siRNAs were again transfected, and the cells were harvested on DM3.

Isolation of total RNA and performance of qRT-PCR

Trizol reagent (Invitrogen) or RNeasy minikit (Qiagen) were used to extract total RNA from various cell lines and tissue samples following the manufacturer's instructions. Mouse tissue panels were purchased from Clontech Laboratories, Inc. cDNA synthesis for mRNA detection was carried out using SuperScript III first strand synthesis system for RT-PCR (Invitrogen). MicroRNA was detected using TaqMan microRNA assays (Applied Biosystems) or Ncode microRNA first strand cDNA synthesis kit (Invitrogen). qPCR for mRNA and microRNA detection was carried out in an ABI thermal cycler using SYBR Green according to the manufacturer's instructions. Quantification of amplicons was done using ABI 7300 software (Applied Biosystem).

Microarray analysis

si-H19 or si-control was transfected into C2C12 cells twice (once in GM and then on DM1), and cells were harvested on DM3. Total RNA was isolated, converted to cDNA probe, and hybridized to a mouse gene 1.0 ST array (Affymetrix). The signals for specific probes and fold change (si-H19/si-Control) were determined.

Luciferase assays

We transfected U2OS osteosarcoma cells with the desired microRNAs twice at 24-h intervals. We then transfected luciferase plasmids 6 h after the last transfection. pGL3 (Promega) was cotransfected as an internal control. Luciferase assays were performed with dual-luciferase reporter assay system (Promega) using a luminometer (Monolight 3020, BD Biosciences) or GlomaX microplate reader luminometer (Promega) at 32–48 h after plasmid transfection. We normalized Renilla luciferase values (rr) first to the cotransfected pGL3 control firefly *Photinus pyralis* luciferase values (pp), and then each rr/pp value in the microRNA-transfected samples was normalized with the rr/pp values obtained in 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 inhibitors cocktail (Sigma). Proteins were separated in SDS-PAGE, transferred, and immunoblotted with various antibodies. The antibodies used were anti-Smad1 (dilution 1:1500; Invitrogen), anti-Smad5 (dilution 1:500; Cell Signaling), anti-Cdc6 (dilution 1:500; Cell Signaling), anti- β -actin (dilution 1:3000; Santa Cruz Biotechnology), and anti-GAPDH (dilution 1:10000; Sigma).

Mouse strains

The following mouse strains were used: H19R/+ (Yoon et al. 2007), H19 Δ E1 (Srivastava et al. 2003), H19 Δ ME/+ (Kaffer et al. 2001), and C57BL/6 (Harlan). 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.

Skeletal muscle regeneration model and TA muscle injection

We injured mouse skeletal muscle by injecting CTX from *Naja nigricollis* (EMD Millipore) essentially following the procedure

described earlier (Yan et al. 2003). Briefly, ~10-wk-old male mice of the desired genotype were injected on TA muscles with 100 μ L of 10 μ M CTX. A high volume (100 μ L) and pressure of injection and post-injection massage spread the injected material throughout the TA compartment. We did all of our analysis on the middle two-thirds of the TA muscle that was closest to the injection site. We optimized the concentration and amount of microRNA injections to obtain "restored" microRNA levels that were close to wild-type levels. Many other groups have successfully injected microRNA and various forms of microRNA inhibitors in the TA muscle (Ge et al. 2011; Yin et al. 2013) and cardiac muscles (Eulalio et al. 2012). Finally, we made stock microRNA and lipid complexes with modifications as described earlier (Ge et al. 2011; Eulalio et al. 2012). One-hundred microliters each of 100 μ M miR-675-3p and miR-675-5p (Invitrogen) together with 100 μ L of RNase-free water were mixed with 300 μ L of RNAiMAX transfection reagent (Invitrogen) and incubated for 30 min at room temperature. For control, 200 μ L of 100 μ M GL2 (Invitrogen) and 100 μ L of RNase-free water was similarly mixed with RNAiMAX transfection reagent and incubated for 30 min. Finally, 100 μ L of microRNA complex was injected into the TA muscle of one leg, and the control microRNA complex was injected into the contralateral leg 3 d after the CTX injection. Mice were anesthetized and sacrificed by cervical dislocation to harvest muscle samples.

Immunocytochemistry and immunohistochemistry

Immunocytochemistry was carried out as described previously (Kim et al. 2006). Cells were grown on sterile glass coverslips and fixed with 2% formaldehyde in PBS for 15 min. Next, the cells were permeabilized 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. Cells were incubated with primary antibody (myogenin [1:50; Santa Cruz Biotechnology] and MHC [1:400; Sigma], in 1% NGS) for 1 h. After washing twice with 1× PBS, FITC-conjugated anti-mouse IgG (dilution 1:500; Dako Cytomation) was incubated for another 1 h. Again, after two washes, nuclei were counterstained with DAPI, and the coverslips were mounted on a glass slide (H-1200, Vector Laboratories). Images were captured with an Olympus Hi-Mag microscope. Immunostaining of mouse tissue sections was carried out with slight modifications of the protocols as described by others: anti-laminin and anti-desmin (Liu et al. 2012). The primary antibodies used were rat anti-laminin (1:100; Millipore) and mouse anti-desmin (1:200; Dako). The secondary antibodies used were Alexa Fluor 594 goat anti-rat IgG1 (1:400; Invitrogen) and Alexa Fluor 488 goat anti-mouse IgG (1:400; Invitrogen). Images were taken using a Zeiss LSM-700 confocal microscope. H&E staining was carried out using a standard protocol of the University of Virginia histology core facility. Bright-field images were captured using an Olympus microscope.

Statistical analyses

Data are presented as the mean \pm standard error of mean (SEM) of three or more biological replicates. Two-tailed Student's *t*-test was employed to determine *P*-values.

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