TGF-β Regulates miR-206 and miR-29 to Control Myogenic Differentiation through Regulation of HDAC4

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MicroRNAs (miRs) are emerging as prominent players in the regulation of many biological processes, including myogenic commitment and skeletal muscle formation. Members of the TGF-β family can influence the proliferation and myogenic differentiation of cells, although it is presently not clear what role miRNAs play in the TGF-β-mediated control of myogenic differentiation. Here, we demonstrate in the myogenic C2C12 cell line, and in primary muscle cells, that miR-206 and miR-29-two miRs that act on transcriptional events implicated in muscle differentiation are down-regulated by TGF-β. We further demonstrate that TGF-β treatment of myogenic cells is associated with increased expression of histone deacetylase 4 (HDAC4), a key inhibitor of muscle differentiation that has been identified as a target for regulation by miR-206 and miR-29. We confirmed that increased expression of miR-206 and miR-29 resulted in the translational repression of HDAC4 in the presence or absence of TGF-β via interaction with the HDAC4 3′untranslated region. Importantly, we found that miR-206 and miR-29 can attenuate the inhibitory actions of TGF-β on myogenic differentiation. Furthermore, we present evidence that the mechanism by which miR-206 and miR-29 can inhibit the TGF-β-mediated up-regulation of HDAC4 is via the inhibition of Smad3 expression, a transducer of TGF-β signaling. These findings identify a novel mechanism of interaction between TGF-β and miR-206 and -29 in the regulation of myogenic differentiation through HDAC4.

During embryonic development, progenitor cells arising from the somitic mesoderm commit to a program of differentiation that facilitates the formation of skeletal muscle fibers (termed myogenesis). Under the control of a number of extracellular cues, these myogenic precursors adhere to an orchestrated process of mobilization, proliferation, differentiation, and fusion to create the multi-nucleated myotubes that ultimately become mature fibers (1–3). Many of the most important early changes in gene expression that direct the muscle cell lineage are driven by a family of basic helix-loop-helix transcription factors that includes MyoD, myogenin, MRF4, and Myf5, which are therefore commonly referred to as the muscle regulatory factors (MRFs) (4–6).

TGF-β is well characterized as a potent inhibitor of muscle cell differentiation that acts by repressing the transcriptional activity of MRFs (7–9). Interaction between extracellular TGF-β and its membrane-bound receptor complex engages a cascade of intracellular signal transduction that promotes nuclear retention of Smad proteins 2 and 3 in complex with Smad4, which subsequently activates or represses hundreds of TGF-β target genes (10). Of particular relevance to skeletal muscle cell differentiation, the TGF-β signaling protein Smad3 has been shown to physically interact with MRFs in a manner that can inhibit differentiation (9).

microRNAs (or miRs) are single-stranded 21–22-nucleotide noncoding RNAs that are capable of controlling gene expression at a post-transcriptional level by stalling the translation of the cognate mRNA or promoting its degradation in a process referred to as RNA interference (RNAI). Here, individual miRs that have been loaded into a specialized collection of interacting proteins referred to as the RNA-induced silencing complex identify and bind to highly specific sequences featuring within exons or the 3′-untranslated regions of target mRNAs. The degree of pairing complementarity between a microRNA and its target (as well as target location in the transcript) determine whether translation is subsequently repressed or the transcript is degraded (11, 12). In skeletal muscle, specific miRs are increasingly being implicated as key regulators of differentiation, because of their predicted selectivity for genes that are involved in facilitating the myogenic program. Chief among these microRNAs are the so-called “myomiRs,” or muscle-enriched microRNAs (including miR-1/206, -133a, and -133b) that are themselves transcribed as targets of MRF activity. As an example, increased miR-206 levels promote myogenic differentiation in vitro (13, 14) and in vivo (15, 16), whereas inhibiting miR-206 appears capable of delaying or even preventing myogenic differentiation. Ongoing examination is establishing that additional miRs that are expressed in a variety of cell types including but not exclusive to skeletal muscle may also influence the events of differentiation. For instance, the miR-29 family regulates myogenesis by targeting proteins within the NF-κB-YY1 signaling pathway, which is recognized for its role

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2 The abbreviations used are: MRF, muscle regulatory factor; miR, microRNA; HDAC, histone deacetylase; NC, negative control.
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in muscle atrophy and in regulating myogenic differentiation (17, 18). These findings suggest that the full extent of the influence of miRs upon the muscle phenotype has yet to be defined.

Recently, studies have begun to demonstrate an ability of miRNAs to cross-talk with TGF-β signaling, being both regulated by and regulators of the TGF-β pathway components. These findings likely have implications for all manner of cell types in which signaling driven by TGF-β and related ligands serves a purpose, including skeletal muscle. An example of one such influence is that of miR-24, which appears to exert positive effects on myogenic differentiation but is itself suppressed by TGF-β-driven signaling (19). The findings demonstrate that a better knowledge of the interactions between specific miRs and signal transduction initiated by TGF-β family members is important for our understanding of the mechanisms underlying myogenic differentiation and skeletal muscle remodeling (such as that seen following injury and in degenerative neuromuscular disorders).

One mode of action by which specific miRs are thought to influence skeletal muscle development and plasticity concerns the targeting of histone deacetylases (HDACs) (20, 21). In muscle, specific HDACs appear to inhibit differentiation by forming a co-repressor complex that suppresses the MRFs through direct inhibition of MEF2 (22, 23). TGF-β and HDAC4 may act together to co-regulate the differentiation of bone precursors (24, 25); however, whether a similar co-operative interaction functions to regulate muscle differentiation has not yet been established. Because the literature supports the hypothesis that HDAC4 and TGF-β may act in concert to regulate myogenic differentiation, we sought to determine whether such an axis is also influenced by miRNAs that are implicated as regulators of skeletal muscle development.

Our studies described herein establish that TGF-β can attenuate the differentiation of myogenic cells by increasing expression of HDAC4, a key inhibitor of myogenic commitment. The underlying mechanism involves down-regulated expression of miR-206 and miR-29, which act as translational repressors of HDAC4. These data provide novel insight into a role for miRNAs as regulators of TGF-β effects upon myogenesis.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Antibodies against HDAC4 and Smad3 were obtained from Cell Signaling. The mouse monoclonal myosin heavy chain antibody was a generous gift of Stephen D. Hausschka (University of Washington, Seattle, WA). GAPDH antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Oligonucleotide-based microRNA mimics and inhibitors and matching negative controls (NC) were obtained from Dharmacon (miR-206 experiments) and Ambion (miR-29 experiments). Recombinant TGF-β1 was purchased from Peprotech. SB431542 (a TGF-β1 receptor inhibitor) was acquired from Sigma. A Myc-Smad7 plasmid was purchased from Genscript. Expression levels of relevant miRNAs were determined using Assay on Demand™ kits (Applied Biosystems Inc.).

Cell Culture—C2C12 cells were cultured in DMEM containing 10% FBS (Hyclone), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all culture reagents from Invitrogen except where mentioned). To promote myogenic differentiation and the formation of myoblast fusion into syncytial structures (a process that models the formation of myotubes in vivo), culture medium was substituted with 2% horse serum (Hyclone) in place of FBS for up to 4 days (with medium exchange performed every second day). miRNAs were transfected in 12-well plates using Metafectene Pro (Biontex) or Lipofectamine 2000 (Invitrogen). After 4–6 h, the medium was changed to avoid toxicity, and cells were tracked for up to 72 h post-transfection, before harvest.

For culture of primary muscle cells, 2-week-old C57BL/6 mice were sacrificed by cervical dislocation, and the hind limb and lower back muscles were removed and digested using methods described previously (26). Experiments that involved the use of mice were conducted in accordance with international guidelines including the National Institutes of Health Principles of Laboratory Animal Care and the National Health and Medical Council of Australia Code of Practice for the Care and Use of Animals for Scientific Purposes. Briefly, the muscles were finely minced and digested in 0.2% collagenase II in Hanks’ balanced salt solution for 60 min at 37 °C with frequent agitation and then washed well with PBS before further digestion with 0.25% trypsin for 15 min. After enzymatic depletion with FCS and two washes with PBS, the final pellet was resuspended in 10 ml of growth medium (Ham’s F-10 with 20% FCS and 2.5 ng/ml basic FGF) and plated onto a 100-mm² Petri dish. The cells were incubated at 37 °C for 60 min, at which time unattached myoblasts were transferred to a new dish. Myoblasts were further purified in this manner until all of the fibroblasts were removed from the culture (approximately four passages). Myoblasts were seeded into 12-well plates at 8 × 10⁴ cells/well and treated or transfected 24 h later, as described for C2C12 cells.

Western Blotting—The cells were lysed using Triton-based lysis buffer (30 mM Hepes, 150 mM NaCl, 1% Triton X-100, 2 mM MgCl₂), with Complete™ EDTA-free protease and phosphatase inhibitor mixture (Roche Applied Science). Lysis was followed by centrifugation at 13,000 × g for 5 min at 4 °C and denaturing of samples for 5 min at 95 °C. Protein concentration was determined using a Pierce micro protein assay kit (Thermo Scientific). Whole proteome fractions from samples were subsequently separated by SDS-PAGE using precast 4–12% Tris gels (Invitrogen), blotted onto nitrocellulose membranes (Bio-Rad), and incubated with the appropriate antibody overnight. After incubation with relevant secondary antibodies, labeled proteins were visualized using an ECL chemiluminescence detection kit (GE Healthcare). Quantification of labeled Western blots was performed using ImageJ pixel analysis (National Institutes of Health Image software), and the data are normalized to the loading control of 1. The data from Western blots are presented as band density normalized to the loading control and are representative of three independent experiments.

Cloning of the HDAC4 3′-UTR—To examine the effect of the miR-29 family, the first 500 bp of the HDAC4 3′-UTR were cloned into a luciferase-based readout vector (Promega). Briefly, this was achieved by cloning nucleotides 4297–4759 of the HDAC4 3′-UTR miRNA sequence from human genomic DNA into the XbaI-NotI site downstream of Renilla luciferase in pCI-neo-hRL35 (27). To examine the effect of miR-206 upon
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HDAC4 translation in separate experiments, the entire 4-kb sequence of the HDAC4 3'-UTR (referred to as full length) was cloned into the pmirGLO™ miRNA target expression vector (Promega). Briefly, the entire HDAC4 3'-UTR sequence (synthesized by GenScript, based on the sequence data deposited in PubMed) was cloned into the pmirGLO vector (Promega) downstream of the luc2 reporter gene, using flanking-introduced XbaI and Sall restriction sites, and the resulting product was then verified by resequencing.

Transfections and Luciferase Assays—C2C12 cells were transfected in 12-well plates either with 50 nM of each of the miR-29 family or 200 nM of miR-206 using Lipofectamine 2000 (Invitrogen) or Metafectene Pro (Biontex). 300 ng/well of HDAC4 3'-UTR was transfected with 200 ng/well of β-galactosidase. The medium was changed 5 h later and supplemented with either vehicle or 5 ng/ml TGF-β for 48 h. The cells were subsequently lysed with commercial cell lysis buffer (Promega), and luciferase activity was measured using a Berthold luminometer according to kit protocols. Luciferase activity was normalized to β-galactosidase activity, which was detected using a Promega β-galactosidase detection assay. Briefly, lysate was frozen and thawed at −80 °C twice, followed by centrifugation at 13,000 rpm and addition of 2× β-galactosidase buffer for 1 h at 37 °C. β-Galactosidase expression was detected at a wavelength of 420 nm. Luciferase activity is presented as the ratio of HDAC 3'-UTR luciferase activity-to-β-galactosidase reporter activity, and the data are representative of three independent experiments. All of the data are expressed relative to the control, which is set at 1.

Quantitative RT-PCR—Total RNA was collected from cells using a TRIzol-based extraction protocol (Invitrogen). 500–1000 ng of RNA was reverse transcribed using the high capacity RNA-to-cDNA kit (Applied Biosystems). cDNA was analyzed for HDAC4 by quantitative RT-PCR using TaqMan assay on Demand™ kits utilizing a specific probe and primer and ABI detection software run on Applied Biosystems hardware (Applied Biosystems Inc.). For analysis of miRNA and pri-miR-206 levels, ABI Assay on Demand™ kits were used according to the manufacturer’s instructions. Pri-miR-29 specific primer mixes were purchased from Qiagen. MyoD transcription was quantitated using primers (forward, AGTGAATGAGGCCTTCGAGA; reverse, GCATCTGAGTCGCCACTGTA) and a SYBR Green-based analysis. Primers for 18 S were used to standardize cDNA concentrations. miRNA expression was normalized to sno135 RNA or 18 S RNA. The data were analyzed using the ΔΔCt method of analysis and are presented as averages of three independent experiments, where the control is standardized to 1.

Statistical Analysis—The Student t test was used to assess the differences in one variable between two groups, and all of the differences reported are p < 0.05 unless otherwise stated. The data are presented as the means ± S.E. unless indicated and are representative of at least three independent experiments.

RESULTS

TGF-β Regulates the Expression of miRNAs Expressed in Skeletal Muscle—In our studies (and in agreement with the work of others), the C2C12 cell line exhibits robustly increased expression of miR-206 and -133 when differentiation is induced by the application of culture medium supplemented with 2% horse serum (Fig. 1a) (14, 28). We also observed elevated levels of other more widely expressed (i.e. not muscle-specific) miRs that have been implicated in myogenic commitment including...
the miR-29 family (17) (Fig. 1a). Because TGF-β is a well-characterized inhibitor of muscle cell differentiation, we investigated whether this growth factor regulates these and other recognized pro-differentiation miRNAs. When C2C12 myoblasts incubated in differentiation media were assessed 48 h after treatment with TGF-β, we found a significant reduction in the endogenous expression of miR-206, -133a, and -133b, as well as the miR-29 family (Fig. 1b). Interestingly, although members of the miR-200 family have been shown to be TGF-β-responsive in epithelial cells (27), TGF-β had no effect on these miRNAs in C2C12 cells. Furthermore, given recent reports that have demonstrated that members of the TGF-β superfamily can regulate miRNAs, including miR-206, at a post-transcriptional level, we assessed whether TGF-β similarly regulated the primary and mature forms of miR-206 (29). We found that TGF-β potently reduced both forms of miR-206, and furthermore, this held true for the effect of TGF-β on pri-miR-29 (Fig. 1c). This indicates that TGF-β, unlike BMP-2 (29), inhibits the processing of the primary form of these miRNAs into their mature forms. Furthermore, we examined whether these findings could be replicated in primary muscle cells subjected to TGF-β treatment for 48 h. These cells exhibited increased proliferation rates (Fig. 1d, right panel, lower image versus upper image). Importantly, we found that in primary muscle cultures, TGF-β also suppressed the mature and primary forms of the miR-29 family and of miR-206, although it had no effect on miR-151, thereby demonstrating that the effects of TGF-β are specific for particular miRs. These data demonstrate that expression of the pro-differentiation miR-206 and miR-29 is altered in myogenic cells by TGF-β treatment, which represents a mechanism by which TGF-β could inhibit myogenesis.

**TGF-β Regulates HDAC4 Expression through Regulation of Its 3’-UTR**—Given the recent reports demonstrating that HDAC4 is a target of miR-206 and the miR-29 family (20, 21) and our data demonstrating that TGF-β can suppress miR-206 and miR-29 expression, we investigated the ability of cellular responses driven by TGF-β exposure to regulate HDAC4 levels. According to existing databases, the human HDAC4 3’-UTR contains three target sites for miR-206 and one for the miR-29 family (Fig. 2a). We cloned short and long versions of the 3’ HDAC4 UTR into a luciferase reporter construct (providing targets for the miR-29 family, as well as miR-206, respectively) and observed that luciferase activity was increased in cells transfected with a Luc-HDAC4 3’-UTR construct, when treated with TGF-β (Fig. 2b). We further tested the effect of TGF-β on the expression of endogenous HDAC4 protein and confirmed that TGF-β exposure induces accumulation of HDAC4 protein in a dose-dependent manner up to doses of 1.25 ng/ml, after which HDAC4 protein induction was maximal (Fig. 2c). We also tested the ability of TGF-β to regulate HDAC4 protein levels in primary muscle cells and found that TGF-β at doses of 0.5 ng/ml could maximally induce HDAC4 protein levels (Fig. 2c). To establish whether TGF-β acts only through post-transcriptional mechanisms to regulate HDAC4 protein expression, the HDAC4 mRNA levels were also examined. Although plasminogen activator inhibitor, a known downstream target of TGF-β (30, 31), was significantly induced by TGF-β, we observed no effect of TGF-β on HDAC4 gene expression. We determined that TGF-β inhibits HDAC4 expression through regulation of its 3’-UTR.
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expression (Fig. 2d). Taken together, these data establish that TGF-β increases expression of HDAC4 protein via a post-transcriptional mechanism that involves the HDAC4 3′-UTR rather than an increase in HDAC4 transcription.

**Regulation of HDAC4 Is via the Canonical TGF-β Pathway**—Having observed an effect upon HDAC4 levels associated with TGF-β treatment, we sought to determine whether the regulation of HDAC4 by TGF-β was dependent on canonical TGF-β signaling, by investigating the capacity of other TGF-β superfamily members to regulate HDAC4 3′-UTR luciferase activity. Over the growth factor concentrations used, we found that TGF-β1 and TGF-β2 treatment significantly increased Luc-HDAC4 3′-UTR activity, whereas BMP-7 and activin-A had no significant effect (Fig. 3a). These findings suggest that the effect of TGF-β upon HDAC4 is contingent on signaling situated primarily downstream of the TGF-β receptor complex and is unlikely to involve Smad proteins-1 and -5 that are more closely associated with BMP-dependent signaling operating through distinct receptor complexes. To further characterize the effect of TGF-β on the HDAC4 3′-UTR, we assessed the ability of TGF-β to induce luciferase activity in the presence of selected TGF-β pathway inhibition. We found that increased expression of the inhibitory Smad7 abrogated the ability of TGF-β to increase luciferase activity (Fig. 3b), lending further support to the induction of HDAC4 expression being dependent on canonical (i.e. Smad2/3-based) TGF-β signaling. In a complementary set of experiments, we observed that chemical inhibition of the type I receptor with SB431542 at 1 or 10 μM also abrogated TGF-β-induced 3′-UTR HDAC4 luciferase activity (Fig. 3c).

miR-206 and miR-29 Inhibit TGF-β-mediated Induction of HDAC4—Given that TGF-β can influence HDAC4 expression and HDAC4 3′-UTR luciferase activity (Fig. 2) and that miR-206 and miR-29 levels change with TGF-β treatment, we investigated the relationship between TGF-β, the HDAC4 3′-UTR, and the aforementioned miRs. Consistent with recent reports that miR-206 and miR-29abc target the 3′-UTR of HDAC4, we observed that cells transfected with synthetic miR-29abc and -206 mimics demonstrated reduced Luc-HDAC4 3′-UTR activity (Fig. 4, a and b) and conversely that cells transfected with miR-29abc- and miR-206-specific anti-miRs or hair-pin inhibitors displayed increased Luc-HDAC4 3′-UTR activity (Fig. 4, c and d). Importantly, we noted that increases in luciferase activity following TGF-β treatment were potently inhibited when cells were co-transfected with either miR-29 or miR-206 mimics (Fig. 4, a and b). Furthermore, we demonstrated that inhibition of miR-29abc or miR-206 with specific inhibitors potentiated the luciferase activity in cells treated with TGF-β (Fig. 4, c and d). These data confirm that the HDAC4 3′-UTR is a target of both miR-29 and miR-206 and, more importantly, demonstrate the ability of these miRNAs to potently modulate TGF-β-induced changes in HDAC4 expression.

Previous studies have demonstrated that TGF-β exerts effects that can inhibit muscle differentiation through actions upon Smad3 (9). We therefore considered whether the inhibition of myogenic differentiation could be mediated by the effect of miR-29 and miR-206 on Smad3, hypothesizing that this may act as a mechanism by which these miRs suppress the effect of TGF-β on the 3′-UTR of HDAC4. We found that ectopic expression of miR-29 (but not miR-206) attenuated basal levels of Smad3 protein (Fig. 4e). We then tested the effect of these miRNAs on the induction of Smad3 by TGF-β and found that both miR-206 and miR-29 prevented the induction of Smad3 following the exposure of cells to TGF-β (Fig. 4f). The results suggest that differential modes of Smad3 regulation are exerted by miR-29 and miR-206 and that the miR-29 family serves a particularly significant role in regulating basal Smad3 levels in muscle. Moreover, the data indicate that although TGF-β acts to elevate HDAC4 expression, miR-29 and miR-206, as two modulators of the program contributing to myogenic differentiation, have the ability to suppress cellular effects following TGF-β treatment via mechanisms that can interfere with the induction of Smad3. The mechanisms of this precise effect remain incompletely defined, however, because neither miR-206 nor miR-29abc is presently predicted to strongly target the murine Smad3 3′-UTR or open reading frame (although a predicted miR-206 target exists in the human and primate Smad3 3′-UTR).
Regulation of HDAC4 Protein Expression by TGF-β and miR-206 and -29 Is Independent of Transcription—To determine whether miR-206 and miR-29 regulate HDAC4 expression at a post-transcriptional level, we assessed HDAC4 gene expression in response to ectopic expression of these miRNAs in the absence or presence of TGF-β. As shown in Fig. 5 (a and b), both TGF-β and the miRs-29 and -206 had little effect on HDAC4 mRNA levels, whereas HDAC4 protein levels were clearly induced in the presence of TGF-β, and this response was attenuated by ectopic expression of miR-29 or miR-206 (Fig. 5, c and d). These findings are consistent with the inability of TGF-β to induce HDAC-3 3’-UTR-driven luciferase activity in the presence of these miRNAs, as in Fig. 4.

miR-206 and miR-29 Can Partially Rescue the Inhibitory Effect of TGF-β on Skeletal Muscle Cell Differentiation—Our data indicate that TGF-β prevents the differentiation of myogenic cells by processes that include increasing expression of HDAC4, an established inhibitor of muscle differentiation. This mechanism involves the down-regulation of miR-206 and miR-29, which can each act as translational repressors of HDAC4 by targeting the 3’-UTR of this gene. The stimulatory effects of TGF-β upon HDAC4 protein expression can be attenuated by elevated expression of miR-206 and miR-29, which, apart from preventing TGF-β-mediated induction of HDAC4 expression, can also interfere with Smad signaling that occurs downstream of the TGF-β receptor. To confirm the effects of TGF-β on myogenic differentiation in the presence of these miRNAs, we examined the levels of MyoD and Mef-2 because these regulatory factors are integral players in control of myogenic programming and are under direct repression by HDAC4 and also examined the expression of myosin heavy chain protein, as a marker of muscle differentiation.

Although TGF-β acts as a potent inhibitor of muscle cell differentiation and fusion (Fig. 6, a and b, panel 1 versus panel
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These data further demonstrate that increasing miR-206 and miR-29 can override (at least to a degree) the inhibitory effect of TGF-β upon induction of the myogenic program.

DISCUSSION

This study has established that TGF-β inhibits the differentiation of myogenic cells (i.e. both the C2C12 cell line and murine primary myoblasts) in part by decreasing the expression of the pro-differentiation microRNAs miR-29 and miR-206, which act as translational repressors of HDAC4 by targeting the 3′-UTR of this gene. This mechanism allows TGF-β to increase expression of HDAC4 (to inhibit gene expression required for myogenic differentiation), and this response can be prevented by interventions that reduce TGF-β signaling, such as drug-based inhibition of receptor activation or overexpression of Smad7. Moreover, the effects of TGF-β were attenuated by increasing concentrations of miR-29 and miR-206, which, apart from repressing the effect of TGF-β on HDAC4 translation, can also interfere with Smad3 signaling downstream of the TGF-β receptor. The forced expression of miR-29 and miR-206 in myogenic cells affects the ability of TGF-β to inhibit differentiation, highlighting a novel pathway through which TGF-β cross-talks with microRNAs to regulate commitment of cells to myogenic differentiation and cell fusion.

Members of the TGF-β superfamily are widely involved in the regulation of organ development, cellular adaptation, and the origin of disease. In the context of skeletal muscle, altered TGF-β levels (or TGF-β dependent signaling) can exert potent effects on myogenic differentiation (9,32). With interest rapidly growing in the biological roles of microRNAs, studies have begun to establish mechanisms of cross-talk and feedback between TGF-β signaling and microRNA networks, which appear to provide an added level of regulatory control over cellular processes. In various models, signaling driven by TGF-β superfamily members has been shown to influence the transcription and processing of microRNAs (33–35). Furthermore individual microRNAs have been observed to manipulate the TGF-β signaling transduction network, and its profile of transcriptional targets (27,36,37). Because the effects of TGF-β can differ between cell/tissue types, it is important to define what interplay exists between TGF-β signaling and microRNAs specifically in myogenic cells, to understand the mechanisms that govern the formation and maintenance of muscle. Our data have provided a new insight into the manner in which TGF-β and muscle-enriched microRNAs interact to control induction of the myogenic program.

Precedence exists for interaction between the TGF-β pathway and microRNAs in the regulation of the myogenic program. For instance, it has been shown that a Smad3 signaling process driven by TGF-β can inhibit miR-24 transcription, which is otherwise increased during myoblast differentiation (19). Also BMP2, another TGF-β superfamily member, has been shown to negatively regulate miR-206 (a key muscle-enriched microRNA) by blocking its maturation (29). These processes have significant implications for induction of the myogenic program, because it has been established that the expression of individual microRNAs can influence and is simultaneously influenced by MRFs (13,38,39). We further support

FIGURE 5. The regulation of HDAC4 by TGF-β and microRNAs -206 and -29 is through post-transcriptional mechanisms. Cells treated with TGF-β alone or in combination with either miR-206 (a) or miR-29 (b) do not exhibit significant changes in HDAC4 transcription (HDAC4 mRNA levels are expressed relative to the negative control), even though the cells treated with TGF-β alone exhibit increased quantities of HDAC4 protein. Overexpression of either miR-206 (c) or miR-29 (d) degrades protein levels of HDAC4 and suppresses the induction of HDAC4 in the presence of TGF-β (*, p < 0.01 versus NC; +, p < 0.05 versus NC).

2), the inhibition of differentiation by TGF-β is reduced in the presence of miR-206 or the miR-29 family (Fig. 6, a and b, panel 2 versus panel 4). These morphological effects were also confirmed by determining the expression levels of MyoD transcripts. Although MyoD gene expression was reduced in cells after administration of TGF-β, the effect of TGF-β upon cells simultaneously administered miR-29 (and to a lesser degree miR-206) was reduced (Fig. 6, c and d). Similar trends were observed of Mef-2 and myosin heavy chain protein levels in cells treated with TGF-β in the presence and absence of increased concentrations of miR-29 and 206 (Fig. 6, e and f).
the ability of TGF-β to regulate miRNA expression in skeletal muscle by presenting data that demonstrate that TGF-β can also regulate miR-29 and miR-206 during skeletal muscle differentiation.

Although our work was conducted using isolated primary myoblasts and the established C2C12 myogenic cell line in vitro, the findings have implications for the development and remodeling of muscle in vivo. An important role has already been established for miR-29 and miR-206 in cell commitment, such that development of rhabdomyosarcoma can be inhibited with forced expression of these miRs (15, 17). Interestingly, rhabdomyosarcoma tumors express high levels of TGF-β and Smad4, as well as concomitant reductions in miR-29 and miR-206 (15, 17, 40). Those findings agree with our observa-
tions that increased TGF-β signaling can contribute to suppression of miR-29 and miR-206, with consequences for cellular differentiation.

HDACs exert their effect by promoting histone deacetylation and chromatin remodeling, which in turn inhibits the access of transcriptional regulators to promoter regions within the genome (41). HDAC4 is highly expressed in the heart and skeletal muscles and is well characterized as an inhibitor of muscle differentiation, by way of negatively regulating the expression of genes associated with the myogenic program (22, 23, 42, 43). For the first time, we show that HDAC4 is regulated post-transcriptionally by TGF-β in myogenic cells, as part of the regulatory program that controls myogenic differentiation. The literature supports our hypothesis of cross-talk between HDAC4 and TGF-β in myogenic differentiation, because it has been demonstrated that similar effects of TGF-β upon HDACs can inhibit osteoblastic differentiation (24). Also, administration of HDAC inhibitors can attenuate the inhibition of differentiation by TGF-β in lung-derived fibroblasts (44). Given that TGF-β and certain HDACs can both regulate key muscle regulatory factors (9, 23, 45, 46), it would appear that the TGF-β-HDAC axis may serve as a fundamental regulator of cellular commitment and differentiation, the ultimate effects of which are tuned (e.g. muscle versus bone) according to the specific cell lineage (committed myogenic or osteoblastic progenitor). These studies and our findings substantiate a role for cooperation between TGF-β and HDAC4, specifically as inhibitors of skeletal muscle differentiation.

Interestingly, we found that miR-29 and miR-206 could suppress HDAC4 protein expression via direct targeting of the 3′-UTR of this gene, even in the presence of TGF-β treatment. This observation demonstrates that these miRNAs can dominantly suppress various aspects of TGF-β signaling, including the ability to induce HDAC4. We therefore assessed Smad3, which is essential for TGF-β to mediate its inhibitory effects on differentiation (9). We found that the increased presence of miR-29 inhibited Smad3 expression and also inhibited the induction of Smad3 in response to TGF-β. It has been shown that miR-29 can inhibit the expression of TGF-β3 (21), which may establish one example of a mechanism by which miR-29 can regulate the TGF-β axis and suppress basal Smad3 levels. By comparison, miR-206 did not affect basal expression of Smad3 in our studies but did blunt the ability of TGF-β to induce Smad3. Our findings suggest that miR-29 and miR-206 may regulate TGF-β signaling through different mechanisms.

These studies have confirmed that HDAC4 is a bona fide target of miR-29 and miR-206 in cells of a committed myogenic lineage. Previously, it was shown that HDAC4 is a target of miR-206 in a model of ALS, where the key role identified related to the facilitation of neuronal regeneration (20). Elsewhere, up-regulated expression of miR-29 was shown to target HDAC4 as a means of osteoblast differentiation (21). Consistent with these findings, we found that miR-29 and miR-206 inhibited HDAC4 protein expression in the myogenic C2C12 line, through direct targeting of the HDAC4 3′-UTR. Although miR-206 can target transcripts from several genes that are associated with the myogenic program (13) in a manner that would influence the efficiency of protein translation, it is clear that a direct effect upon HDACs would provide an effective means of influencing the transcriptional activity of particular genes. Thus, a regulatory loop can be established that conceivably controls the levels of particular proteins in muscle cells. Although less is known about how miR-29 controls muscle cell differentiation, one study has shown that this miRNA regulates the NF-κB-Y1 axis in muscle (17). Importantly, we have identified a level of cross-talk between these miRNAs and TGF-β that shows that they can at least partially prevent the ability of TGF-β to inhibit muscle differentiation. The over-riding of effects typically exerted by TGF-β that we observed is consistent with work that found that miR-24 could promote myogenic differentiation and expression of the myogenic program that was otherwise inhibited in the presence of TGF-β (19). The literature and our findings are therefore consistent in supporting the role of miRNAs in antagonizing the effect of TGF-β on muscle differentiation (Fig. 7).

In summary, this study reveals a mechanism whereby TGF-β inhibits myogenic differentiation by suppressing levels of miR-206 and miR-29. This mechanism allows TGF-β to augment HDAC4 expression, with negative consequences for expression of muscle-specific genes. From a different perspective, in the course of myogenic differentiation (as might be observed in muscle regeneration for instance), the increased expression of miR-206 and miR-29 ensures that HDAC4 levels are kept low to encourage induction of muscle-specific genes despite potentially elevated levels of TGF-β. This study therefore reveals a
new role of miR-206 and miR-29 in the control events driven by TGF-β that can affect the differentiation of cells within the skeletal muscle lineage.

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