miR-206 and -486 Induce Myoblast Differentiation by Downregulating Pax7

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Skeletal muscle is largely composed of multinucleated muscle fibers. Postnatal growth or the regeneration capacity of adult skeletal muscle is dependent on satellite cells (15, 36). Satellite cells give rise to myoblasts that undergo multiple rounds of division before terminal differentiation. Skeletal muscle development during embryogenesis and during regeneration in adults requires a delicate balance between myogenic differentiation and self renewal (9).

The paired-box family of transcription factors, specifically Pax3 and Pax7, are important for regulation of the development and differentiation of diverse cell lineages, including skeletal muscle during embryogenesis (26). Pax3 is extensively expressed in the somite, whereas Pax7 expression is restricted to the central part of the dermomyotome (23). The Pax7 transcription factor is required for satellite cell biogenesis, survival, and self renewal and has a crucial role in specifying the satellite cell myogenic lineage, functioning upstream of the MyoD family of basic helix-loop-helix (bHLH) transcription factors (37, 50). Most of the activated satellite cells proliferate, downregulate Pax7, and maintain MyoD to go into differentiation. However, another group of proliferating cells maintain Pax7 expression but downregulate MyoD to remain in the undifferentiated self-renewing state (19, 49). Pax7 upregulation inhibits myogenesis by suppressing MyoD expression and myogenin induction (30). The mechanism by which Pax7 is downregulated during muscle differentiation has not been studied in detail.

Most of our current understanding of muscle differentiation is based on transcriptional regulation by, for example, the MyoD and MEF2 families of transcription factors (7, 33, 36). More recently, we have discovered that specific microRNAs play fundamental roles during muscle proliferation and differentiation by modulating a number of transcription factors and signaling molecules (2, 12, 21, 43, 45). MicroRNAs are a novel class of small, noncoding RNAs of 18 to 25 nucleotides that modulate gene expression by translational repression and mRNA cleavage caused by microRNA-guided rapid deadenylation (5, 20, 46).

The role of microRNAs in muscle differentiation has been recently reviewed (11, 43). In mammals, miR-1 and -133 are expressed in both skeletal and cardiac muscles and miR-206 is specifically expressed in skeletal muscles (12, 21). Overexpression and knockdown experiments investigated the function of these microRNAs in muscle differentiation in a C2C12 model system (12, 21). The effects of miR-1 and -206 were partly mediated by repression of histone deacetylase 4 (HDAC4) and DNA polymerase α (Polα1), respectively. Additional direct targets for miR-206 such as connexin 43 (cx43), follistatin-like 1(Fstl1), utrophin (Utrn), estrogen receptor alpha (ERα), butyrate-induced transcript 1 (Bind1), monocyte-to-macrophage differentiation-associated protein (Mmd), and cMET were identified (1, 2, 21, 35, 40, 44, 48). Targeted deletion of Dicer, an enzyme critical for microRNA biogenesis, in the myogenic compartment caused perinatal lethality with reduced skeletal muscle mass and abnormalities in muscle fiber morphology (31). Intriguingly, deficiency of miR-206 in the amyotrophic lateral sclerosis mouse model accelerated disease progression (44).

For this study, we screened for additional microRNAs and alternative targets involved in skeletal muscle differentiation. Here we report that miR-486 is also upregulated during myoblast differentiation and that miR-206 and -486 accelerate myogenic differentiation by inhibiting Pax7 expression. Pax7 is expressed in proliferating myoblast cells and is rapidly downregulated as these cells differentiate (30, 50). Both the microRNAs are induced by MyoD. The link between MyoD and Pax7 through these microRNAs reveals a bistable switch that distinguishes between two fates: myoblasts and myotubes.
MATERIALS AND METHODS

Cell culture. Mouse skeletal myoblast cell line C2C12 was obtained from the American Type Culture Collection (ATCC) and maintained at subconfluent densities in Dulbecco’s modified Eagle medium (DMEM) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (growth medium [GM]) and 1% penicillin-streptomycin. For myogenic differentiation (from myoblasts into myotubes), DMEM containing 2% heat-inactivated horse serum (differentiation medium [DM]) and 1% penicillin-streptomycin was used. Mouse primary myoblasts (a kind gift from Denis Gattridge, Ohio State University) were cultured in Ham’s F-10 medium supplemented with 20% fetal bovine serum (FBS), HEPES (20 mM), basic fibroblast growth factor (bFGF; 2.5 ng/ml), and 1% penicillin-streptomycin in a collagen type 1-coated plate and differentiated using DM. C3H10T1/2 fibroblast cells were grown in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin.

RNA isolation and RT-PCR. Cells were collected at different days of differentiation, and total RNA was extracted using Trizol reagent (Invitrogen) by following the manufacturer’s instructions. Total RNA from human atrium, breast, brain, colon, heart, kidney, liver, lung, ovary, small intestine, skeletal muscle, and uterus was purchased from Clontech Laboratories, Inc. Ncode microRNA first-strand cDNA synthesis and a quantitative reverse transcription PCR (qRT-PCR) kit (Invitrogen) were used to perform RT-PCR for microRNA detection. For mRNA detection, cDNA synthesis was carried out using the Superscript III first-strand synthesis system for RT-PCR (Invitrogen). Then, quantitative PCR (qPCR) was carried out using Sybr green PCR master mix in an ABIcycler. ABI 7300 software was used for quantification (Applied Biosystems).

Plasmid construction. The Pax7 open reading frame (ORF) (29) and Pax7 ORF with wild-type or mutated untranslated region 2 (UTR2; bp 2521 to 4196) were subcloned to the pMSCV retroviral vector using an EcoRI/Ncol site. Retrovirus was made in HEK-293T cells cotransfected with virus packaging plasmids using a standard protocol. The mouse Pax7 3’ UTR was PCR amplified from C2C12 myoblast genomic DNA and cloned into modified pRL-CMV as described above. The samples were further purified using a Qiagen RNA column, and 5 µg of each sample was sent to Exiqon. Microarray profiling of microRNA was carried out in a locked nucleic acid-based platform by Exiqon.

Immunocytochemistry. Immunostaining was performed as described previously (12, 21). Cells on a sterile glass coverslip were fixed with 2% formaldehyde in phosphate-buffered saline (PBS) for 15 min and were permeabilized with 0.2% Triton X-100 and 1% normal goat serum (NGS) in ice-cold PBS for 5 min. After cells were blocked with 1% NGS in PBS twice for 15 min, incubation with primary antibody (dilutions in 1% NGS: Pax7, 1:10; myogenin, 1:50; MHC1, 400) for 1 h was followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (dilution, 1:500; Dako Cytomation) for 1 h except for Pax7. The Pax7 primary antibody was incubated for 16 h, and secondary antibody was incubated for 2 h. After washes, nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole) (H-1200; Vector Laboratories) for 1 min before mounting. Images were visualized using a microscope (Olympus; Hi-Mag).

RESULTS

miR-206 and -486 are upregulated during myoblast differentiation and are expressed in skeletal muscles. Mouse C2C12 myoblast cells are derived from adult skeletal muscles and mimic skeletal muscle differentiation in cell culture. C2C12 myoblast cells proliferate in the presence of serum and differentiate upon serum withdrawal with upregulation of specific markers such as cell cycle inhibitor p21, myogenin, and myosin heavy chain (MHC) (3, 21). Therefore, C2C12 serves as an excellent model system to study the molecular mechanism underlying skeletal muscle differentiation. We carried out microarray profiling of microRNAs and found that a number of microRNAs were induced more than 2-fold (see Table S1 in the supplemental material). These include miR-206, previously identified as involved in myogenic differentiation, and miR-486. The role of miR-486 in skeletal muscle differentiation was not reported earlier.

qRT-PCR analysis showed that miR-206 and -486 were significantly upregulated during myoblast differentiation (Fig. 1a and b). Consistent with earlier studies (21), a tissue survey showed that miR-206 was expressed only in the skeletal muscle. miR-486 was also expressed abundantly in skeletal muscle as well as in heart and liver (Fig. 1c).

miR-206 and -486 promote myogenesis and cell cycle quiescence. To assess the function of these microRNAs in myogenesis, we transfected synthetic RNA duplexes mimicking the microRNAs into C2C12 cells cultured in growth medium (GM). GL2, a siRNA to luciferase, was used as a negative control. The GM was replaced by differentiation medium (DM), and the cells were collected for immunocytochemistry, RT-PCR, and Western blot analysis at different time intervals.

Transfection of miR-206 increased the number of myogenin- and MHC-positive cells more than 2-fold compared to the GL2 control (Fig. 2a and b; Table 1). Similarly, miR-486 also increased the number of myogenin- and MHC-positive cells compared to GL2 control cells (Fig. 2a and b; Table 1). In both cases the microRNA-transfected cells were elongated and often multinucleated and yielded a brighter immunofluorescence signal than the GL2-transfected cells. Similarly qRT-PCR and Western blot analysis reveal that both myogenin and MHC mRNA and protein levels were upregulated in the miR-206- and -486-transfected cells compared to the GL2 control (Fig. 2c and d). Cell cycle profiling shows that transfection of miR-206 or -486 independently increased the G1 population of cells described above. The samples were further purified using a Qiagen RNA column, and 5 µg of each sample was sent to Exiqon. Microarray profiling of microRNA was carried out in a locked nucleic acid-based platform by Exiqon.
Pax7 is downregulated by miR-206 and -486 during myoblast differentiation. Pax7 is expressed in nearly 100% of C2C12 myoblast cells (Fig. 4a), and by qRT-PCR we have shown that Pax7 mRNA in C2C12 cells is comparable to that in mouse primary myoblasts (Fig. 4b). Similar to what is found for C2C12 cells, when mouse primary myoblasts are induced to differentiate by serum withdrawal, miR-206 and -486 increased and Pax7 mRNA decreased gradually (Fig. 4c and d). Given the similarities between these cell lines, we carried out our experiment using the C2C12 line.

When C2C12 cells are induced to differentiate by serum withdrawal, Pax7 protein decreases start from day 1, with the level going below detection by day 4 (Fig. 5a and c). The decrease in Pax7 protein is accompanied by an increased level of MHC expression, indicating that the cells are differentiating (Fig. 5b). Interestingly, qRT-PCR of mRNA shows that Pax7 mRNA declines in a more gradual fashion, with only a 40% reduction after day 3 of serum withdrawal (Fig. 5d). The faster kinetics of repression of Pax7 protein relative to that of the mRNA suggests that posttranscriptional mechanisms may take part in repressing Pax7 during muscle differentiation.

The microRNA target prediction algorithm miRanda suggests that Pax7 is a potential target of miR-206 and -486 (Fig. 6a). We have found two predicted target sites for each of these microRNAs on the basis of seed match (first 8 nucleotides) and one predicted target site for each of these microRNAs on the basis of nonseed match (first 8 nucleotides) and the second part of the 3' UTR (Fig. 6). The two parts of the 3' UTR are from nucleotides 2734 to 2785 (line 1a), 3139 to 3182 (line 1b), and 2671 to 2696 (line 1c). The microRNA binding through nonseed match has been described (24). miR-206 target sites in the Pax7 3' UTR span nucleotides 2641 to 2656 (line 2a), 3154 to 3169 (line 2b), and 3757 to 3779 (line 2c), and miR-486 target sites in the Pax7 3' UTR span nucleotides 1714 to 1738 (line 3a), 2375 to 2399 (line 3b), and 2403 to 2427 (line 3c).

To ascertain whether the 3' UTR of Pax7 mediates the downregulation of the protein, the two parts of the 3' UTR (UTR1, bp 1640 to 2800; UTR2, bp 2521 to 4196) were separately fused to a luciferase reporter gene driven by the cytomegalovirus (CMV) promoter and transfected into C2C12 cells in GM. The relative luciferase activity was gradually downregulated when cells were moved to DM, indicating posttranscriptional regulation through the 3' UTR (Fig. 6b). The importance of the 3' UTR for this microRNA action was corroborated by the relative persistence of the luciferase signal in cells in DM for 1 day and 3 days (DM1 and DM3 cells, respectively) when miR-206-responsive sites were mutated in UTR2 (Fig. 6b). The remaining decrease of luciferase activity with the mutated UTR2 is likely due to the presence of the miR-486 target site (Fig. 6a, line 1c). A luciferase reporter containing the site complementary to miR-206 was similarly downregulated, showing that the assay was capable of detecting an upregulation of miR-206 during differentiation (Fig. 6b).

Only UTR2 has the target sites for miR-206, and both UTR1 and UTR2 have the target site(s) for miR-486. Cotransfection of miR-206 repressed the luciferase activity of the construct containing UTR2 (Fig. 6c). Mutation of each of three miR-206 target sites in UTR2 partially relieved the repression, and three mutations together relieved the repression almost entirely, suggesting that the miR-206 target sites at 2a, 2b, and 2c
are each responsible for the direct repression of Pax7 (Fig. 6c).
In a similar experiment, UTR1 or UTR2 conferred responsiveness to miR-486 (Fig. 6d). Mutation at the UTR1b site alone (MutUTR1b) relieved the repression by miR-486 (Fig. 6d), and there was no additive effect from adding a mutation at UTR1a (data not shown). Like MutUTR1b, MutUTR2-1c (1c site mutated in the UTR2 fragment) also relieved the repression by miR-486 (Fig. 6d). Thus, the miR-486 target sites at 1a and 1c mediate repression by this microRNA. Collectively these results show that Pax7 is a bona fide direct target of miR-206 and -486.

Consistent with this, when C2C12 cells in GM were transfected by miR-206 and -486, endogenous Pax7 protein and mRNA were downregulated by these microRNAs independently (Fig. 6c and f). MicroRNAs are known to decrease the levels of target mRNAs (16, 38). The downregulation of protein level was much greater than the mRNA repression, suggesting that these microRNAs also repress translation of Pax7.
Finally, inhibition of these microRNAs using 2′-O-methyl antisense inhibitors of miR-206 and -486 caused longer persistence of endogenous Pax7 protein during differentiation (Fig. 6g and h). Therefore, the two microRNAs are indeed responsible for the optimal repression of Pax7 protein during differentiation.

MicroRNA-resistant form of Pax7 slows myoblast differentiation. Having demonstrated that Pax7 is a cognate target of miR-206 and -486, we next tested whether transfection of the Pax7 ORF, which is resistant to miR-206 and -486 due to the absence of its 3′ UTR, suppresses microRNA-mediated myogenesis. The Pax7 ORF increased the level of Pax7 protein in C2C12 cells (Fig. 7a). We first ensured that the exogenous Pax7 persisted in DM and was resistant to miR-206 and -486 in GM. In DM, Pax7 protein in the parental vector control cells started decreasing on day 1 and no Pax7 protein was detectable from day 3, whereas no significant changes of Pax7 protein were seen in the Pax7 ORF-expressing cells up to day 5 (Fig. 7b). In addition, transfection of miR-206 and -486 in C2C12 (parental vector- and Pax7 ORF-expressing) cells held in GM showed that endogenous Pax7 containing the 3′ UTR disap-

**TABLE 1. Effect of overexpression and inhibition of miR-206 and -486 on myoblast differentiation**

| Transfectant | Myogenin-positive cells | MHC-positive cells |
|--------------|-------------------------|--------------------|
|              | Total count (10 fields) | % of control | Total count (10 fields) | % of control |
| GL2          | 151                     | 100        | 12.53                  | 100         | 26.00    |
| miR-206      | 320                     | 211.92     | 21.85                  | 229         | 57.89    |
| miR-486      | 245                     | 162.25     | 18.75                  | 179         | 43.22    |
| Anti-GL2     | 412                     | 100        | 13.08                  | 165         | 22.35    |
| Antimix      | 211                     | 51.21      | 9.73                   | 89          | 14.12    |

*C2C12 cells were transfected three times at 24-h intervals separately with synthetic RNA duplexes mimicking miR-206, miR-486, or GL2 as a negative control in one set. After an additional 24 h in GM, the cells were transferred to DM and were stained for myogenin at 24 h or MHC at 36 h. In the second set, a 2′-O-methyl antisense oligonucleotide against miR-206 and -486 (antimix) or antisense oligonucleotides against GL2 (anti-GL2) were transfected in GM. After 24 h in GM, the cells were transferred to DM and stained for myogenin or MHC at 48 h. Fractions of myogenin- and MHC-positive cells were determined randomly from 10 fields (magnification, ×200), and data are presented relative to the GL2 control (100%).

**FIG. 3.** Inhibitors of miR-206 and -486 inhibit the myogenesis. (a and b) C2C12 myoblast cells were transfected three times at 24-h intervals with 2′-O-methyl antisense oligonucleotide against GL2 (Anti-GL2) or antisense oligonucleotides against miR-206 and -486 (Anti-Mix). Cells were continuously grown in GM for 24 h and then transferred to DM before staining for MHC (a) or myogenin (b) as in Fig. 2 except that immunofluorescence was done after 48 h. Histograms show fractions of cells staining for myogenin or MHC relative to the fraction in cells transfected with the anti-GL2 control (100%). Values are means ± standard deviations of 10 measurements. *P ≤ 0.001. (c, d) Western blotting for myogenin (c) and MHC (d) after transfection of a 2′-O-methyl antisense oligonucleotide against GL2 or antisense oligonucleotides against miR-206 and -486. C2C12 cells were held in DM for 2 days for myogenin and 3 days for MHC. GAPDH served as the loading control.
appeared at 72 h after transfection, whereas the Pax7 protein expressed from a gene devoid of its 3’/H11032 UTR was not repressed (Fig. 7c).

Upon transfer of the two types of cells to DM, MHC- or myogenin-positive cells appeared with normal kinetics among the parental vector control cells, whereas no MHC- or myogenin-positive cells were seen among Pax7 ORF-expressing cells as late as day 3 or day 2, respectively (Fig. 7d and e). Similarly, Western blot analysis revealed no detectable myogenin and MHC protein levels in Pax7 ORF-expressing cells up to differentiation day 5 (Fig. 7f). These results are consistent with the previous findings that overexpression of Pax7 devoid of its 3’/H11032 UTR delays C2C12 differentiation (28–29).

To further demonstrate that microRNA target sites were important, we overexpressed Pax7 containing UTR2, either wild type or mutant for miR-206-responsive sites in C2C12 cells (Fig. 8a). When these cells were transfected with miR-206 and kept in DM for 48 h, the wild-type UTR2-expressing cells downregulated Pax7 mRNA (Fig. 8b) and differentiated with normal kinetics (Fig. 8c and d). In contrast, cells expressing Pax7 with mutated UTR2 had less downregulation of Pax7 mRNA (Fig. 8b) and less upregulation of myogenin and MHC (Fig. 8c and d).

These data suggest that the 3’/H11032 UTR of Pax7 is critical for the proper differentiation of myoblasts. Since miR-206 and -486 are induced during differentiation and target the Pax7 3’ UTR, we suggest that the microRNAs contribute to myoblast differentiation by specifically repressing Pax7 protein and mRNA.

miR-206 and -486 downregulate Pax7 target genes. If miR-206 and -486 repress Pax7, one expects them to repress known targets of Pax7 (22, 29, 42). The mRNA levels of four such
miR-206 and -486 downregulate Pax7 by directly targeting its 3’ UTR. (a) miR-206 and -486 target sequence alignment in the Pax7 3’ UTR and mutations created. Three predicted target sites for miR-206 (2a, 2b, and 2c) and -486 (1a, 1b, and 1c) in the 3’ UTR of mouse Pax7 are shown. Mutations in the target sites are indicated. (b) Activity of a Renilla luciferase reporter fused to Pax7 3’ UTR fragments transfected into C2C12 cells that were kept in GM or moved to DM. UTR1, bp 1640 to 2800; UTR2, bp 2521 to 4196; 206cs, complementary sequence to miR-206 in the 3’ UTR of the luciferase gene; UTR2 Mut, bp 2521 to 4196, where miR-206-responsive sites were mutated. A firefly luciferase plasmid was cotransfected with a Renilla luciferase construct as a transfection control, and the results are expressed as Renilla luciferase activity (rr) relative to firefly luciferase activity (pp). The ratio of rr/pp for a given plasmid is expressed after normalizing to the ratio in GM. Values are means ± standard deviations of three measurements. (c and d) Luciferase assays were performed to measure the effect of miR-206 (c) and -486 (d) on a Renilla luciferase reporter fused to the indicated segments of the Pax7 3’ UTR as described in Materials and Methods. The results are expressed relative to the ratio of rr/pp for the control Renilla luciferase plasmid without a Pax7 3’ UTR segment. Values are means ± standard deviations of three measurements. (e) Transfection of miR-206 and -486 downregulates Pax7 protein as detected by immunoblotting. GAPDH served as a loading control. (f) Transfection of miR-206 and -486 downregulated Pax7 mRNA, as detected by qRT-PCR. Results are expressed relative to the GAPDH mRNA level and normalized to the relative Pax7 level seen in C2C12 cells in GM or GL2 transfection. GL2, miR-206-, and miR-486-transfected cells were held in GM. Values are means ± standard deviations of three measurements. (g) Western blot for Pax7 and GAPDH. 2’O-Methyl antisense oligonucleotides against miR-206, and -486 (Anti-mix) cause longer persistence of Pax7 protein level in C2C12 cells held in DM for 1 or 3 days compared to cells transfected with the anti-GL2 control oligonucleotide. (h) Quantification of Pax7 protein normalized to GAPDH from panel g.
targets, PlagL1, Id1, Id2, and Id3, were measured after transfection of miR-206 or miR-486 into myoblasts in GM (Fig. 9a to d). Consistent with the downregulation of Pax7 during differentiation, moving myoblasts from GM to DM decreased the levels of all four mRNAs. Interestingly, all four genes were downregulated by the transfection of miR-206 or -486 into myoblasts growing in GM, consistent with the hypothesis that the microRNAs downregulate the Pax7 transcription factor.

To test whether the downregulation of these four genes was mediated by the repression of Pax7 by these microRNAs, we transfected Pax7 ORF-expressing cells with miR-206 or -486 or with GL2 as a negative control and held the cells in GM for 72 h (Fig. 9e). PlagL1, Id1, Id2, and Id3 were no longer repressed (Fig. 9e). These findings demonstrate that downregulation of PlagL1 and Id1 to -3 in the control cells by miR-206 and -486 is through Pax7 downregulation. Earlier we had shown that miR-206 downregulates Id1 to -3 in C2C12 cells but that the Id genes were not direct targets of this microRNA (21). Here, we demonstrate that the downregulation of Id1 to -3 by miR-206 (and miR-486) is mediated indirectly through Pax7 downregulation.

A bistable switch exists between Pax7 and MyoD via miR-206 and -486. The next question we addressed was how these microRNAs were regulated during myogenesis. miR-206 has already been shown to be responsive to MyoD (34, 35, 39, 44), and we wondered if that was true for miR-486 as well. Ank1.5 is a variant transcript that is found exclusively in muscle (17), and miR-486 is found in an intron of Ank1.5. The mRNA level of Ank1.5 increases during C2C12 differentiation as we found for miR-486 (Fig. 10a). We therefore thought that miR-486 may be controlled at the transcriptional level along with of Ank1.5.

By analyzing previously published MyoD chromatin immunoprecipitation-DNA sequencing (ChIP-seq) data (10),
we found a MyoD binding peak in the promoter of Ank1.5 (Fig. 10b). Within that binding peak there were two well-conserved E boxes (Fig. 10c). The enhancer activity of this region was tested by cloning the fragment upstream from a simian virus 40 (SV40) promoter-driven luciferase. Transcription was greatly activated by transfecting MyoD and E12 into C3H10T1/2 fibroblasts (Fig. 10d). This activation was greatly attenuated by mutating either of the E boxes. This result is consistent with previous findings that MyoD requires multiple E boxes to function as a transcriptional activator (41). We conclude that miR-486 levels are regulated directly by MyoD activity. In cells overexpressing Pax7, the Ank1.5 level accumulated much more slowly during differentiation (Fig. 10e).

Our next question was whether Pax7 prevented its own repression via inhibition of miR-206 and -486. Pax7 directly upregulates a number of repressors of myogenesis, including Id2 and Id3 (22) (Fig. 9). Id2 binds to E12/47 and sequesters it away from MyoD binding sites, thereby repressing MyoD activity (8). Therefore, we expected and observed that overexpression of Pax7 prevented the increase of MyoD targets miR-206 and -486 (Fig. 11a and b). Knockdown of Id2 in Flag-Pax7 ORF-expressing C2C12 cells relieved the repression of miR-206 and -486 (Fig. 11c and d) and traditional markers of terminal myogenesis like myogenin and MHC (Fig. 11e and f). These results are consistent with the hypothesis that Pax7 inhibits miR-206 and -486 through the inhibition of MyoD by the activation of Id2.

**DISCUSSION**

Most of the current understanding of skeletal muscle differentiation is based on transcriptional regulation by the MyoD family of myogenic transcription factors and the MEF2 family of MADS box transcription factors (7, 33, 36). More recently, studies have shown that specific microRNAs play fundamental roles during muscle proliferation and differentiation by modulating a number of transcription factors and signaling molecules (2, 12, 21, 45). Here we report that miR-206 and -486 are...
induced during myoblast differentiation and promote muscle differentiation by directly targeting and downregulating Pax7 protein and mRNA. Transfecting miR-206 or -486 independently increased the G1 phase population and decreased the S phase population of myoblast cells, indicating that these microRNAs also promote cell cycle quiescence (Fig. 2). Expression of either of these microRNAs in myoblasts accelerates differentiation, whereas inhibition of these microRNAs causes persistence of Pax7 protein and inhibits differentiation (Fig. 2 to 6). Furthermore, we have shown that a microRNA-resistant form of Pax7 that lacks its 3′ UTR or is mutated in all miR-206 sites delays the differentiation (Fig. 7 and 8). Thus, microRNAs contribute to myoblast differentiation by specifically repressing Pax7 protein and mRNA.

Pax7 is an important regulator of skeletal muscle development required for maintenance of the satellite cells that are responsible for postnatal muscle growth and regeneration (32, 37). Pax7 is upregulated in progenitor cells that have migrated to the limbs to activate the myogenic program. Pax7-positive myoblast cells proliferate rapidly, but they downregulate Pax7 at the onset of differentiation. However, the mechanism of Pax7 downregulation during myoblast differentiation was not clearly understood. Our study strongly suggests that Pax7 downregulation is microRNA mediated. While our paper was under review another paper reported that miR-1 and -206 repressed Pax7 during muscle differentiation (13).

The potential role of microRNAs in regulating Pax7 adds a new dimension to how microRNAs sculpt the myogenic gene expression program. Very recently miR-27 was reported to repress Pax3, suggesting that the direct repression of antidifferentiation transcription factors could be quite widespread (14). Due to the repression of Pax7, we find that several inhibitors of differentiation, Id1, -2, and -3, are also repressed by the muscle differentiation-induced microRNAs (Fig. 9). These data suggest that extensive cooperation between several microRNAs and several transcription factors is necessary to execute the complete differentiation program.

We have seen in our previous study that miR-206 promotes myogenesis by inhibiting DNA polymerase α (21). B-ind1, c-MET, Cx43, HDAC4, Fstl1, and Mmd were the other identified targets for miR-206, although repression of some of these did not stimulate muscle differentiation. For example, a block in DNA synthesis through the direct downregulation of DNA polymerase α affected DNA replication but was not sufficient.
to promote differentiation (our unpublished data). This result suggested that there were undiscovered targets for miR-206. The results in this paper suggest that Pax7 is one such target.

We have also found an intricate regulatory network between Pax7 and miR-206 and -486 that is at least partially mediated via the Pax7 target gene Id2 and muscle regulatory factor MyoD (Fig. 11). This finding suggests a tight control in the muscle differentiation pathways for normal development and function. Clearly, the cells can be in a Pax7-positive myoblast state when Id2 is activated and MyoD and the muscle differentiation-induced microRNAs are repressed. Conversely, once MyoD has gained the upper hand, the induction of miR-206 and -486 and repression of Pax7 shift the equilibrium toward the MyoD-active myotube state.

Chromosomal translocation of Pax7 and aberrant expression of the fusion of Pax7 with FKHR cause rhabdomyosarcoma, again indicating that fine tuning of Pax7 expression and its target genes may be important for normal skeletal muscle differentiation (4, 6, 18). The translocation not only deletes the C-terminal transactivation domain of Pax7 to replace it with a more active FKHR transactivation domain but also deletes the 3′ UTR of Pax7. Interestingly, the miRanda target prediction algorithm can detect putative miR-206 binding sites in the human Pax7 3′ UTR (see Fig. S1 in the supplemental material). Our studies suggest that the deletion of the Pax7 3′ UTR and escape of the fusion transcript from repression by muscle differentiation-induced microRNAs could be important players in the deregulation of Pax7. This is very similar to the way that oncogene HMGAl2 escapes from repression by let-7 (27) and may be a common mode by which potential oncogenes escape repression from microRNAs.

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REFERENCES

1. Adams, B. D., D. M. Cowee, and B. A. White. 2009. The role of miR-206 in the epidermal growth factor (EGF) induced repression of estrogen receptor- 

alpha (ERalpha) signaling and a luminal phenotype in MCF-7 breast cancer 
cells. Mol. Endocrinol. 23:1215–1230.
2. Anderson, C., H. Catoe, and R. Werner. 2006. MIIR-206 regulates connexin43 expression during skeletal muscle development. Nucleic Acids Res. 34:5863– 

5871.
3. Andres, V., and K. Walsh. 1996. Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. J. Cell Biol. 132:671–666.
4. Barry, F. G., L. E. Nauta, R. J. Davis, B. W. Schafer, L. M. Nyceum, and J. A. Biegel. 1996. In vivo amplification of the PAX3-FKHR and PAX7-FKHR fusion genes in alveolar rhabdomyosarcoma. Hum. Mol. Genet. 5:15–21.
5. Bartel, D. P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and func-
tion. Cell 116:281–297.
6. Bennicelli, J. L., S. Advani, B. W. Schafer, and F. G. Barr. 1999. PAX3 and PAX7 exhibit conserved cis-acting transcription repression domains and utilize a common gain of function mechanism in alveolar rhabdomyosarcoma. Oncogene 18:4348–4356.
7. Berkes, C. A., and S. J. Taspott. 2005. MyoD and the transcriptional control of myogenesis. Semin. Cell Dev. Biol. 16:585–595.
8. Biggs, J., E. V. Murphy, and M. A. Israel. 1992. A human Id-like helix-loop- helix protein expressed during early development. Proc. Natl. Acad. Sci. U. S. A. 89:1512–1516.
9. Buckingham, M. 2006. Myogenic progenitor cells and skeletal myogenesis in vertebrates. Curr. Opin. Genet. Dev. 16:525–532.
10. Cao, Y., Z. Yao, D. Sarkar, M. Lawrence, G. J. Sanchez, M. H. Parker, K. L. MacQuarrie, J. Davison, M. T. Morgan, W. L. Ruzzo, R. C. Gentleman, and S. J. Taspott. 2010. Genome-wide MiR-206 binding in skeletal myoblast cells: a potential for broad cellular reprogramming. Dev. Cell 18:662–674.
11. Chen, J. F., T. E. Callis, and D. Z. Wang. 2009. microRNAs and muscle disorders. J. Cell Sci. 122:13–20.
12. Chen, J. F., E. M. Mandel, J. M. Thomson, Q. Wu, T. E. Callis, S. M. Hammond, F. L. Conlon, and D. Z. Wang. 2006. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat. Genet. 38:228–233.
13. Chen, J. F., Y. Tao, J. Li, Z. Deng, Z. Yan, X. Xiao, and D. Z. Wang. 2010. microRNA-1 and microRNA-206 regulate skeletal muscle satellite cell proliferation and differentiation by repressing Pax7. J. Cell Biol. 190:867–879.
14. Crist, G. D., B. Montarras, G. Pallafacchina, D. Rocancourt, A. Cumano, S. J. Conway, and M. Buckingham. 2009. Muscle stem cell behavior is modified by microRNA-27 regulation of Pax3 expression. Proc. Natl. Acad. Sci. U. S. A. 106:13383–13387.
15. Dhawan, J., and T. A. Rando. 2005. Stem cells in postnatal myogenesis: molecular mechanisms of satellite cell quiescence, activation and replenishment. Trends Cell Biol. 15:666–673.
32. Farh, K. K., A. Grimm, C. Jan, B. P. Lewis, W. K. Johnston, L. P. Lim, C. B. Burge, and D. P. Bartel. 2005. The widespread impact of mammalian microRNAs on mRNA repression and evolution. Science 310:1817–1821.

31. Gallagher, P. G., and B. G. Forget. 1998. An alternate promoter directs expression of a truncated, muscle-specific isoform of the human ankyrin 1 gene. J. Biol. Chem. 273:1339–1348.

30. Gallego Melcon, S., and J. Sanchez de Toledo Codina. 2007. Molecular biology of rhabdomyosarcoma. Clin. Transl. Oncol. 9:415–419.

29. Hafey, O., Y. Pietrusi, M. Z. Allouh, B. W., Rosser, Y. Rinkevitch, R. Reshef, I. Rozenboim, M. Wielinski-Lee, and Z. Yalohonka-Neuveni. 2004. Pattern of Pax7 expression during myogenesis in the posthatch chicken establishes a model for satellite cell differentiation and renewal. Dev. Dyn. 231:489–502.

28. He, L., and G. J. Hannon. 2004. MicroRNAs: small RNAs with a big role in gene regulation. Nat. Rev. Genet. 5:522–531.

27. Kim, H. K., Y. S. Lee, U. Sivaprasad, A. Malhotra, and A. Dutta. 2006. Muscle-specific microRNA miR-206 promotes muscle differentiation. J. Cell Biol. 174:677–687.

26. Kumar, D., J. L. Shadradch, A. J. Wagers, and A. B. Lassar. 2009. Id3 is a direct transcriptional target of Pax7 in quiescent satellite cells. Mol. Biol. Cell 20:3170–3177.

25. Lagha, M., T. Sato, L. Bajard, P. Daubas, M. Esner, D. Montarras, F. Yelamos, C. B. Burge, and D. P. Bartel. 2005. The tumor suppressor microRNA let-7 regulates the cell cycle and propagation of myogenic satellite cells but not their specification. EMBO J. 24:3430–3439.

24. Mayr, C., M. T. Hemann, and D. P. Bartel. 2005. Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. Science 310:1817–1821.

23. McKinnell, I. W., J. Ishibashi, F. Le Grand, V. G. Punch, G. C. Addicks, J. F. Greenblatt, F. J. Dilworth, and M. A. Rudnicki. 2004. Pax7 directs postnatal renewal of muscle-specific microRNA miR-206 blocks human rhabdomyosarcoma growth in xenotransplanted mice by promoting myogenic differentiation. J. Clin. Invest. 119:2366–2378.

22. Weintraub, H., R. Davis, D. Lockshon, and A. Lassar. 1990. MyoD binds cooperatively to two sites in a target enhancer sequence: occupancy of two sites is required for activation. Proc. Natl. Acad. Sci. U. S. A. 87:5623–5627.

21. White, R. B., and M. R. Ziman. 2008. Genome-wide discovery of Pax7 target genes during development. Physiol. Genomics 33:41–49.

20. Williams, A. H., G. Valdez, V. Moresi, X. Qi, J. McAnally, J. L. Elliott, R. Tapscott, and S. Tapscott. 2008. MicroRNA-1/206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. Science 320:1549–1554.

19. Wu, L., J. Fun, and J. G. Belasco. 2006. MicroRNAs direct rapid deadenylation of mRNA. Proc. Natl. Acad. Sci. U. S. A. 103:4034–4039.

18. Yaffe, D., and O. Saxel. 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature 276:725–727.

17. Yan, D., X. D. Dong, X. Chen, L. Wang, C. Lu, J. Wang, J. Qu, and L. Tu. 2009. MicroRNA-1/206 targets c-Met and inhibits rhabdomyosarcoma development. J. Biol. Chem. 284:29596–29604.

16. O’Rourke, J. R., S. A. Georges, H. R. Seay, S. J. Tapscoff, M. T. McNamus, D. J. Goldhamer, M. S. Swanson, and B. D. Harfe. 2007. Essential role for Dicer during skeletal muscle development. Dev. Biol. 311:359–368.

15. Oustanina, S., G. Hause, and T. Braun. 2004. Pax7 directs postnatal renewal and propagation of myogenic satellite cells but not their specification. EMBO J. 23:3430–3439.

14. Potthoff, M. J., and E. N. Olson. 2007. MEF2: a central regulator of diverse developmental programs. Development 134:4131–4140.

13. Rao, P. K., R. M. Kumar, M. Farkhondeh, S. Baskerville, and H. F. Lodish. 2006. Myogenic factors that regulate expression of muscle-specific microRNAs. Proc. Natl. Acad. Sci. U. S. A. 103:8721–8726.

12. Rosenbargh, M. L., S. A. Georges, A. Mawasaki, and J. A. Iwano, and S. J. Tapscoff. 2006. MyoD inhibits Fstl1 and Uttrn expression by inducing transcription of microRNA-206. J. Cell Biol. 175:77–85.

11. Rudnicki, M. A., F. Le Grand, I. McKinnell, and S. Klua. 2008. The molecular regulation of muscle stem cell function. Cold Spring Harbor Symp. Quant. Biol. 73:323–331.

10. Seale, P., I. A. Sabourin, A. Girgis-Gabardo, A. Mansouri, P. Gruss, and M. A. Rudnicki. 2000. Pax7 is required for the specification of myogenic satellite cells. Cell 102:777–786.

9. Selbach, M., B. Schwanhaesser, N. Thirheimer, F. Sengstag, R. Kehn, and N. Rajewsky. 2008. Widespread changes in protein synthesis induced by microRNAs. Nature 455:58–63.

8. Sweetman, D., K. Gokbel, T. Rathjen, S. Oustanina, T. Braun, T. Dalmay, and A. Munsterberg. 2008. Specific requirements of MRFs for the expression of muscle specific microRNAs miR-1, miR-206 and miR-133. Dev. Biol. 321:491–499.