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MiR-124 Inhibits Myogenic Differentiation of Mesenchymal Stem Cells Via Targeting Dlx5

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ABSTRACT

MicroRNAs (miRNAs), including miR-1, miR-133, and miR-206, play a crucial role in muscle development by regulating muscle cell proliferation and differentiation. The aim of the present study was to define the effect of miR-124 on myogenic differentiation of mesenchymal stem cells (MSCs). The expression level of miR-124 in skeletal muscles was much lower than those in primary cultured bone marrow-derived MSCs and the bone, fat and brain tissues obtained from C57BL/6 mice. Myogenic stimuli significantly decreased the expression levels of miR-124 in mouse bone marrow-derived MSCs and C2C12 cells. Forced expression of miR-124 suppressed the expression of myogenic marker genes such as Myf5, Myod1, myogenin and myosin heavy chain and multinucleated myotube formation. Blockade of endogenous miR-124 with a hairpin inhibitor enhanced myogenic marker gene expression and myotube formation. During myogenic differentiation of MSCs and C2C12 cells, the levels of Dlx5, a known target of miR-124, were inversely regulated with those of miR-124. Furthermore, overexpression of Dlx5 increased myogenic differentiation, whereas knockdown of Dlx5 using siRNA inhibited myogenesis in C2C12 cells. These results suggest that miR-124 is a negative regulator of myogenic differentiation of MSCs and that upregulation of Dlx5 accompanied with downregulation of miR-124 by myogenic stimuli is necessary for the proper progression of myogenic differentiation. J. Cell. Biochem. 115: 1572–1581, 2014. © 2014 Wiley Periodicals, Inc.

KEY WORDS: miR-124; Dlx5; MYOGENESIS; MESENCHYMAL STEM CELLS

The development of skeletal muscle (myogenesis) is a highly regulated, multi-step process in which pluripotent mesodermal cells give rise to the proliferation of myoblast progenitor cells, withdrawal of these cells from the cell cycle, commitment to a myogenic phenotype and subsequent differentiation into the multinucleate syncytia, of which mature muscle cells are comprised [Merlie et al., 1977; Buckingham, 2006]. Myogenesis is mainly orchestrated by the basic helix-loop-helix myogenic regulatory factors, including myogenic factor 5 (Myf5), myogenic differentiation 1 (Myod1) and myogenin (Myog). Myod1 and Myf5 are required for the commitment of progenitor cells to the myogenic lineage, and Myog plays an important role in the maintenance of the muscle phenotype established by Myod1 and Myf5 [Berkes and Tapscott, 2005].

MicroRNAs (miRNAs) are short non-coding RNAs that regulate gene expression at the post-transcriptional level by inducing mRNA degradation and translational inhibition. Aberrant expression patterns of miRNAs have been observed in various muscle diseases, including cardiac hypertrophy, heart failure and skeletal muscle dystrophy, suggesting an important role of miRNAs in the development, maintenance and function of muscles [Eisenberg et al., 2007; Tatsuguchi et al., 2007; Thum et al., 2007]. There are many miRNAs that are involved in the regulation of skeletal myogenesis; miR-1 [Zhao et al., 2005], miR-133 [Chen et al., 2006] and miR-206 [Kim et al., 2006] are specifically expressed in muscles, where they are superimposed on the intricate network of myogenic transcription factors and other regulatory proteins to control proliferation and myogenic differentiation. Another muscle-specific

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miRNA, miR-208, is involved in myosin heavy chain (MHC) production [van Rooij et al., 2007]. MiR-1 and miR-133 also play a role in cardiomyocyte differentiation from embryonic stem cells [Ivey et al., 2008]. Recently, it was reported that miR-124 inhibits cardiomyocyte differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) via targeting Stat3 signaling [Cai et al., 2012].

Distal-less homeobox 5 (Dlx5) plays an important role in the commitment of mesenchymal progenitors into the osteoblast lineage via the upregulation of Runx2 and Osterix expression and inhibition of adipogenic differentiation by inhibiting PPARγ expression [Lee et al., 2013a]. Previously, it was reported that expression *of Dlx5 and Dlx6* is necessary for cephalic neural crest cell-mesoderm interactions that result in myogenic determination, differentiation and patterning [Heude et al., 2010]. However, it remains unclear whether Dlx5 plays a role in the regulation of mesenchymal stem cell differentiation toward the myogenic lineage. We previously demonstrated that Dlx5 is a direct target of miR-124 [Qadir et al., 2013].

In the present study, we investigated the regulatory role of miR-124 and Dlx5 in myogenic differentiation of mouse BMSCs and C2C12 myoblast cell line. In this study, we found that Dlx5 and miR-124 showed a reverse expression pattern during myogenesis such that the expression of Dlx5 increased whereas miR-124 decreased during early myogenic differentiation. Overexpression of miR-124 suppressed myogenic differentiation, whereas knockdown of miR-124 enhanced myogenesis. In contrast, Dlx5 overexpression increased myogenesis, whereas knockdown of Dlx5 decreased myogenesis. These results suggest that miR-124 is a negative regulator of myogenesis and that miR-124 exerts its effect partly via targeting Dlx5.

MATERIALS AND METHODS

MATERIALS

The tissue culture medium was purchased from Sigma (St. Louis, MO). Tissue culture sera were purchased from HyClone (Logan, UT). Anti-Dlx5 antibody was purchased from Millipore (Billerica, MA). Anti-Lamin, anti-Myod1, anti-Myog, anti-Myf5, and anti-MHC antibodies and HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The NE-PER Nuclear and Cytoplasmic Extraction Reagent was obtained from PIERCE Biotechnology (Rockford, IL).

CELL CULTURE AND MYOGENIC DIFFERENTIATION

Mouse BMSCs were isolated from the tibias and femurs of C57BL/6 mice as previously described [Lim et al., 2013]. BMSCs were maintained in Dulbecco's modified Eagle's medium (DMEM)-low glucose supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. To induce myogenic differentiation, BMSCs were primed for 24 h in low-glucose DMEM supplemented with 10% FBS, 10 μM 5-azacytidine and 10 ng/ml basic fibroblast growth factor (bFGF), followed by incubation for the indicated periods in myogenic medium (low-glucose DMEM with 2% FBS and 10 ng/ml bFGF).

C2C12 cells were maintained in growth medium consisting of DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. To induce myogenic differentiation, confluent C2C12 cells were incubated in growth medium for 2 days and were further incubated in DMEM supplemented with 2% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (myogenic medium) for 6 days.

Myogenic differentiation of C2C12 cells was confirmed morphologically by an analysis of multinucleated myotube formation under a microscope (200×). C2C12 cells were fixed with 10% formaldehyde in phosphate-buffered saline and stained with 10% hematoxylin. The number of multinucleated myotubes (containing more than two nuclei per myotube) present in a field and the number of nuclei per myotube were quantified in three randomly selected fields.

QUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Isolation of total RNA and quantitative RT-PCR were performed as previously described [Qadir et al., 2013]. Expression levels of examined mRNAs were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Fold differences were then calculated for each treatment group using normalized C_T values for the control. The forward and reverse primers for mouse genes were as follows (5′ → 3′): Dlx5, TCTCTAGGACTGACGCAAACA and GTTACACGCCATAGGGTCGC; Myod1, ACATAGACTTGACAGGCCCCGA and AGACCTTCGATGTAGCGGATGG; Myog, TACGTCCATCGTGGACAGCAT and TCAGCTAAATTCCCTCGCTGG; Myf5, AGGAAAAGAAGCCCTGAAGC and GCAAAAAGAACAGGCAGAGG; myomesin 2 (Myom2), TGCTGCTTTTGCAGAGAAGA and TGAGCTCAATGTCCTTGTCG; myosin light polypeptide 4 (Myl4), AAGATCACCTACGGGCAGTG and CCCTCCACGAAGTCCTCATA; myosin heavy polypeptide 3 (Myh3), GCATAGCTGCACCTTTCCTC and GTCCTCTGGCTTAACCACCA; myosin binding protein H (Myph), TGCCCACTACAGAGCCTTCT and ACTGGGGACATCTTCACTCG; and GAPDH, TCAATGACAACTTTGTCAAGC and CCAGGGTTTCTTACTCCTTGG. For miRNA analysis, the expression levels of miR-124 were normalized using U6 snRNA as a control. For the amplification of miR-124, a universal reverse primer provided from the kit and the specifically synthesized forward primer (5′-TAAGGCACGCGGTGAATGCC-3′) were used.

WESTERN BLOTTING ANALYSIS

Whole cell extracts were prepared by lysing the cells in buffer consisting of 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 0.2 mM Na_3VO_4, 1 mM PMSF, and protease inhibitor cocktail. Nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions. Each sample containing equal amounts of protein was subjected to SDS–PAGE. Proteins were electro-blotted onto PVDF membranes. Immunoblotting and detection of the immune complexes were performed as previously described [Qadir et al., 2013].

TRANSIENT TRANSFECTION

The construction of the HA-tagged Dlx5 and the nucleotide sequence for the Dlx5 small interfering RNA (siRNA) mixture were previously

described [Lee et al., 2013a]. The regulatory role of miR-124 in myogenesis was examined by overexpression of a miR-124 mimic or its hairpin inhibitor. The miR-124 mimic and inhibitor were purchased from Dharmacon (Chicago, IL). Transient transfections of the miRNA mimic and inhibitor and siRNAs were performed using Dharmafect (Dharmacon) according to the manufacturer's instructions. After an overnight incubation, the culture medium was replaced with fresh myogenic medium and the cells were further incubated for the indicated periods. The effect on the myogenic differentiation was then determined by examining the expression levels of myogenic marker genes and multinucleated myotube formation.

STATISTICAL ANALYSIS

Data obtained from quantitative RT-PCR and quantification of myotube numbers are presented as the mean $\pm$ SD. The statistical significance was analyzed using Student's *t*-test. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

EXPRESSION LEVELS OF miR-124 AND Dlx5 ARE INVERSELY REGULATED DURING MYOGENESIS

Tissue specificity of miRNAs contributes to the tissue-specific expression of their target genes [Sempere et al., 2004]. Thus, we investigated the tissue distribution of miR-124 in 4-week-old male C57BL/6 mice. The animals were sacrificed, and the long bones (femur, tibia) and their bone marrow, abdominal fat, leg skeletal muscles, heart, kidney, liver, and brain were harvested. Quantitative RT-PCR results demonstrated that miR-124 was highly expressed in fat, brain and bone marrow, compared to BMSCs (Fig. 1A). In contrast, the expression levels of miR-124 were lower in skeletal muscles, kidney and liver tissues, compared to BMSCs. Interestingly, the levels of miR-124 in skeletal muscles were much lower than those found in the heart. Taken together, these results and a previous report demonstrating that miR-124 is a negative regulator of cardiomyocyte differentiation [Cai et al., 2012] suggested that miR-124 may also exert a negative regulatory effect on skeletal muscle differentiation.

We next examined whether myogenic stimuli regulate the expression levels of miR-124. When C2C12 cells were incubated in myogenic medium, the expression levels of myogenic transcription factors such as Myf5, Myod1, and Myog increased (Fig. 1B,C). However, myogenic stimuli suppressed miR-124 expression, and these suppressive effects were highest on day 1 (Fig. 1B). Expression levels of Dlx5, a known target gene of miR-124, were inversely correlated with those of miR-124; Dlx5 mRNA and protein levels were highest on day 1 (Fig. 1B,C). To investigate the effect of myogenic stimuli on miR-124 and Dlx5 expression in primary cultured cells, mouse BMSCs were incubated for 14 days in the presence or absence of myogenic stimuli. RT-PCR of myogenic transcription factors showed that the expression levels of Myf5, Myod1 and Myog were significantly induced by myogenic medium (Fig. 1D). Similar to the results from C2C12 cells and the mouse miR-124 tissue distribution, myogenic stimuli decreased miR-124 expression levels, which were accompanied by increased levels of Dlx5. These results indicate that during the progression of myogenic differentiation, miR-124 expression decreases whereas Dlx5 expression increases.

OVEREXPRESSION OF miR-124 INHIBITS MYOGENIC DIFFERENTIATION, WHEREAS ANTAGONISM OF miR-124 FUNCTION VIA A HAIRPIN INHIBITOR ENHANCES MYOGENIC DIFFERENTIATION

To determine the role of miR-124 in myogenic differentiation, mouse BMSCs and C2C12 cells were transfected with miR-124 mimic and incubated in myogenic media for 7 and 2 days, respectively. Overexpression of miR-124 was confirmed using real-time PCR (Fig. 2A,C). Quantitative RT-PCR results showed that the expression levels of myogenic marker genes were significantly decreased by forced expression of miR-124, compared to the expression in control mimic-transfected cells (Fig. 2A,C). A decrease in the protein levels of myogenic markers such as Myf5, Myod1, Myog, and MHC by the miR-124 mimic was also detected using Western blotting analysis (Fig. 2B,D). Furthermore, the miR-124 mimic significantly decreased C2C12 myocyte differentiation, as indicated by reduced myotube formation and a decreased number of nuclei per myotube (Fig. 2E,F). These results suggest that miR-124 exerts an inhibitory effect on myogenic differentiation in mouse BMSCs and C2C12 cells.

To further determine the effect of endogenous miR-124, BMSCs and C2C12 cells were transfected with a hairpin inhibitor for miR-124 and the expression changes in myogenic marker genes were examined after 7 and 2 days, respectively. Overexpression of the miR-124 inhibitor significantly suppressed miR-124 levels in BMSCs and C2C12 cells (Fig. 3A,C). In addition, the mRNA and protein levels of myogenic marker genes were significantly increased by a miR-124 inhibitor (Fig. 3A–D). Consistent with the marker gene expression levels, the miR-124 inhibitor increased C2C12 myocyte differentiation, as indicated by an increase in the number of myotubes and nuclei per myotube (Fig. 3E,F). These results further confirmed that endogenous miR-124 is a negative regulator of myogenic differentiation in mouse BMSCs and C2C12 cells.

OVEREXPRESSION OF Dlx5 ENHANCES MYOGENIC DIFFERENTIATION, WHEREAS Dlx5 KNOCKDOWN INHIBITS MYOGENIC DIFFERENTIATION

Our results demonstrated that the expression levels of miR-124 and Dlx5 showed an inverse relationship in myogenic conditions. Thus, we next examined whether miR-124 also targets Dlx5 in myogenic conditions. Overexpression with the miR-124 mimic significantly downregulated the levels of Dlx5 mRNA and protein in mouse BMSCs and C2C12 cells (Supplementary Fig. 1A,C). In addition, the miR-124 inhibitor significantly increased the levels of Dlx5 mRNA and protein (Supplementary Fig. 1B,D). Luciferase reporter assays using the wild-type Dlx5 3′UTR reporter demonstrated that the miR-124 mimic suppressed reporter activity, whereas the miR-124 inhibitor significantly increased luciferase activity in myogenic conditions (Supplementary Fig. 1E,F). However, these regulatory effects were not observed in cells transfected with the mutant Dlx5 3′UTR luciferase reporter, which harbors mutations in the miR-124 seed sequence match region (Supplementary Fig. 1E,F). These results indicate that the inverse regulation of expression levels of miR-124

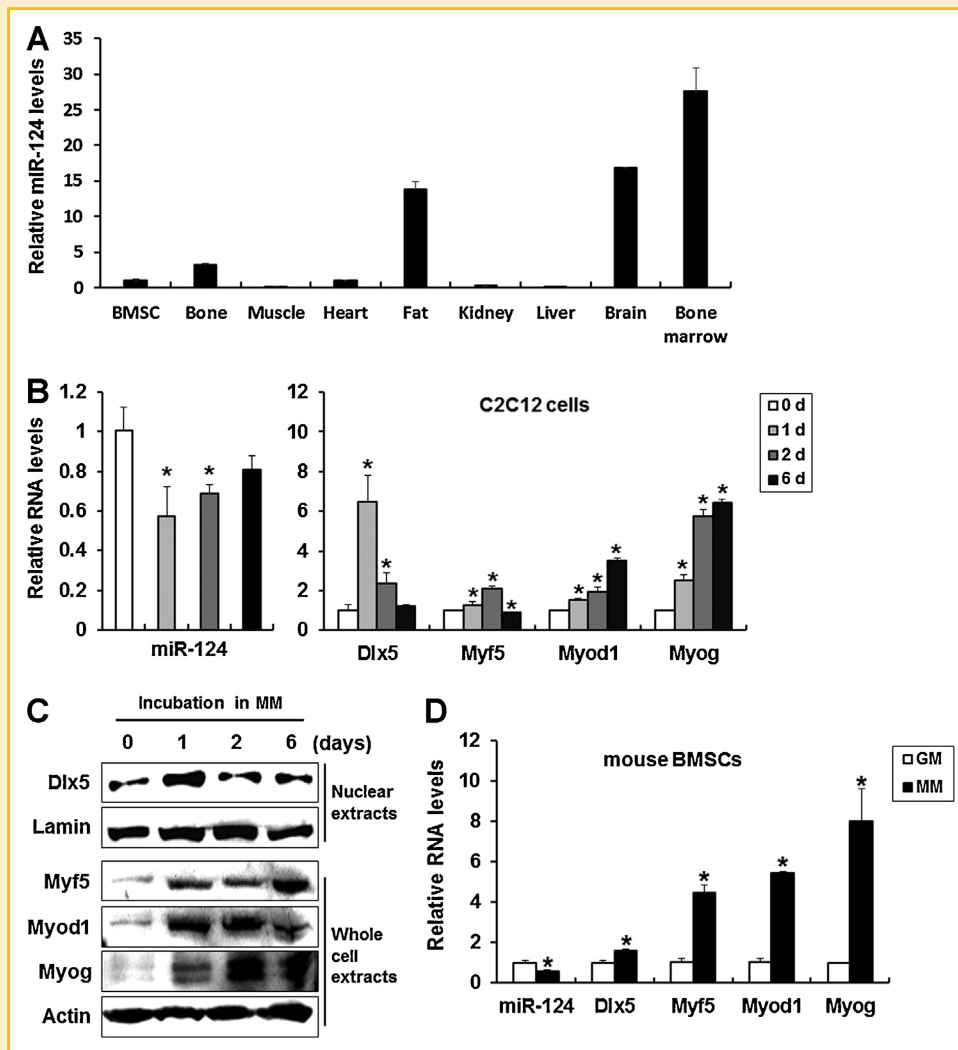

Fig. 1. Myogenic stimuli suppressed miR-124 but increased Dlx5 expression. A: Expression levels of miR-124 were examined in the indicated cells and tissues obtained from 4-week-old male C57BL/6 mice (BMSCs, primary cultured bone marrow-derived mesenchymal stem cells; Muscle, mouse leg skeletal muscles; Fat, abdominal fat). The levels of miR-124 were normalized against U6 snRNA. Data are shown as fold changes of miR-124 compared to BMSCs. Data represent the mean ± SD. B,C: C2C12 cells were incubated in myogenic medium for the indicated periods. At the end of the culture period, quantitative RT-PCR (B) and Western blotting analyses (C) were performed. The transcription factor mRNA levels were normalized against GAPDH, and the data are presented as fold changes compared to those in 0 day samples (*$P < 0.05$, compared to 0 day). D: Mouse BMSCs were cultured in growth medium (GM) or myogenic medium (MM) for 14 days, and quantitative RT-PCR was performed. The levels of miR-124 and transcription factor mRNAs are presented as fold changes compared to those in GM samples (*$P < 0.05$, compared to GM).

and Dlx5 during myogenic differentiation may at least partially result from direct targeting of miR-124 on Dlx5.

We next investigated whether Dlx5 demonstrated any regulatory effects on myogenic differentiation. C2C12 cells were transiently transfected with HA-tagged Dlx5 expression plasmid and incubated in myogenic medium for 2 days to examine myogenic marker gene expression levels and for 6 days to observe myotube formation. Overexpression of Dlx5 was confirmed using RT-PCR (Fig. 4A). Similar to the results observed in miR-124 inhibitor-transfected cells, overexpression of Dlx5 increased the expression levels of myogenic marker genes, compared to those in the pcDNA-transfected control cells (Fig. 4A, B). The stimulatory effect of Dlx5 was further confirmed by observing myotube formation. Dlx5 significantly increased the number of total myotubes and the number of nuclei per myotube (Fig. 4C, D).

Although this gain-of-function study indicated that Dlx5 enhances myogenic differentiation, these results do not necessarily implicate the role of endogenous Dlx5 in this process. To address this issue, we asked whether knockdown of endogenous Dlx5 expression affects myogenic differentiation of C2C12 cells. C2C12 cells were transiently transfected with non-targeting control siRNA or Dlx5 siRNA and myogenic differentiation was induced by incubation in myogenic medium for 2 or 6 days. Knockdown of Dlx5 was confirmed at both the mRNA and protein levels (Fig. 5A, B). Similar to the results of miR-124 overexpression, knockdown of Dlx5 significantly decreased the expression levels of myogenic marker

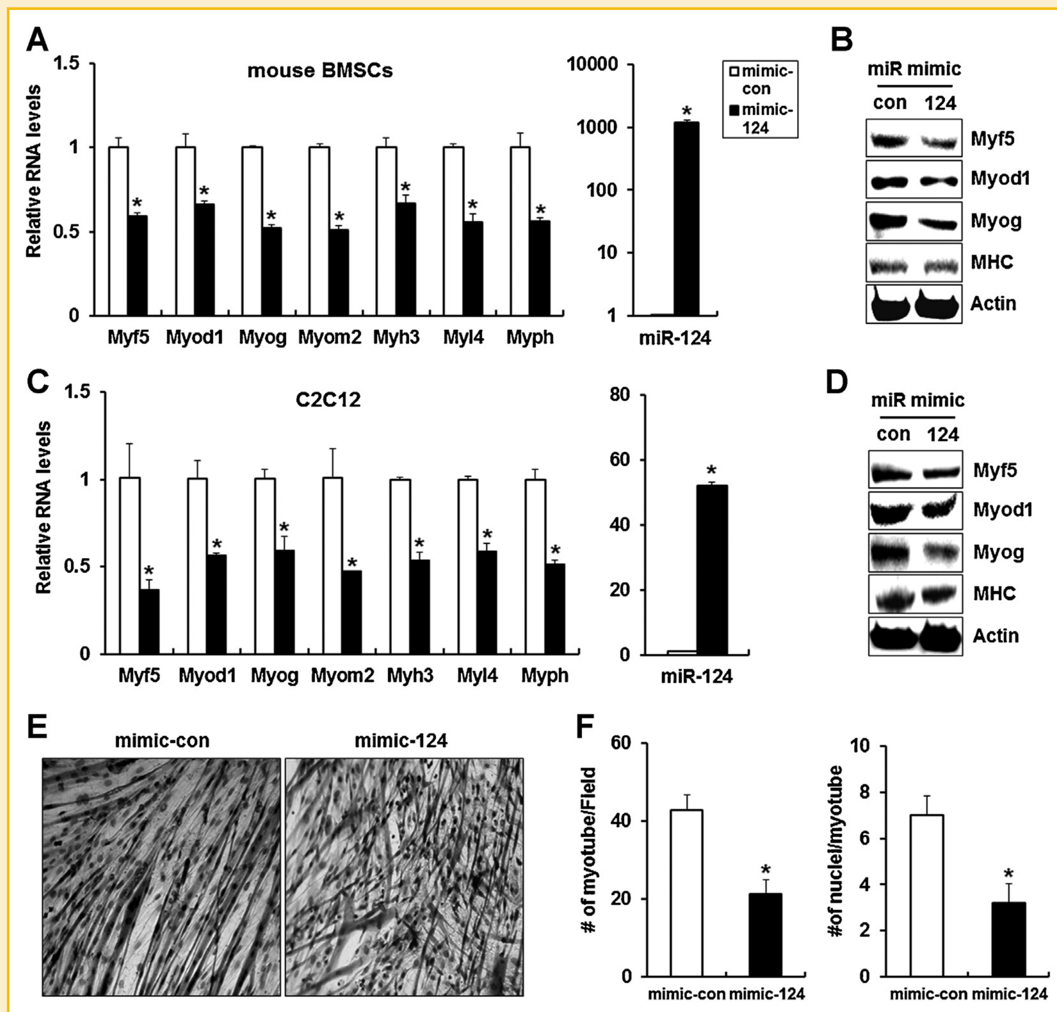

Fig. 2. Overexpression of miR-124 mimic inhibited myogenic differentiation. Mouse BMSCs (A, B) and C2C12 cells (C–F) were transiently transfected with 100 nM of control miRNA mimic (mimic-con) or miR-124 mimic (mimic-124) and incubated in myogenic medium for 7 days for mouse BMSCs (A, B) and 2 days for C2C12 cells (C, D). The relative RNA levels (A, C) were measured using quantitative RT-PCR, and protein levels were examined using Western blotting analysis (B, D). Multinucleated myotube formation in C2C12 cells was examined after myogenic induction for 6 days (E, F). The cells were hematoxylin-stained and observed under a phase-contrast microscope (200×, E). The number of multinucleated myotubes (more than two nuclei per myotube) present in a field and the number of nuclei in each myotube were quantified from three randomly selected fields (F). $^{*}P < 0.05$, compared to mimic-con.

genes, compared to those in control siRNA-transfected cells (Fig. 5A, B). Furthermore, Dlx5 knockdown significantly reduced the number of myotubes and number of nuclei per myotube (Fig. 5C, D). Taken together, these results further suggest that endogenous Dlx5 is a positive regulator of myogenic differentiation of C2C12 cells and that Dlx5 may be a target that mediates the regulatory effect of miR-124 on myogenesis.

DISCUSSION

In the present study, we identified miR-124 as a novel negative regulator of skeletal muscle differentiation. Several lines of evidence support this hypothesis: (i) myogenic stimuli downregulated miR-124 expression; (ii) forced expression of miR-124 suppressed myogenic differentiation of mouse BMSCs and C2C12 cells, while inhibition of endogenous miR-124 enhanced myogenic differentiation; and (iii) miR-124 expression in skeletal muscles was much lower than the expression in BMSCs. In addition, we also provided evidence demonstrating that Dlx5 is involved in myogenic differentiation of mesenchymal stem cells and that miR-124 regulates, at least partially, myogenic differentiation via down-regulation of Dlx5 expression.

It was previously reported that miR-124 expression was down-regulated during cardiomyocyte differentiation of BMSCs and that forced expression of miR-124 suppressed cardiomyocyte differentiation [Cai et al., 2012]. Thus, we confirmed the tissue distribution pattern of miR-124 in C57BL/6 mice to associate the expression level of miR-124 with its inhibitory role in myogenesis. In a previous report, Northern blot analysis results showed that miR-124 was strongly expressed in brain tissues but was not detected in the liver, heart, skeletal muscle, lung, and kidney [Sempere et al., 2004].

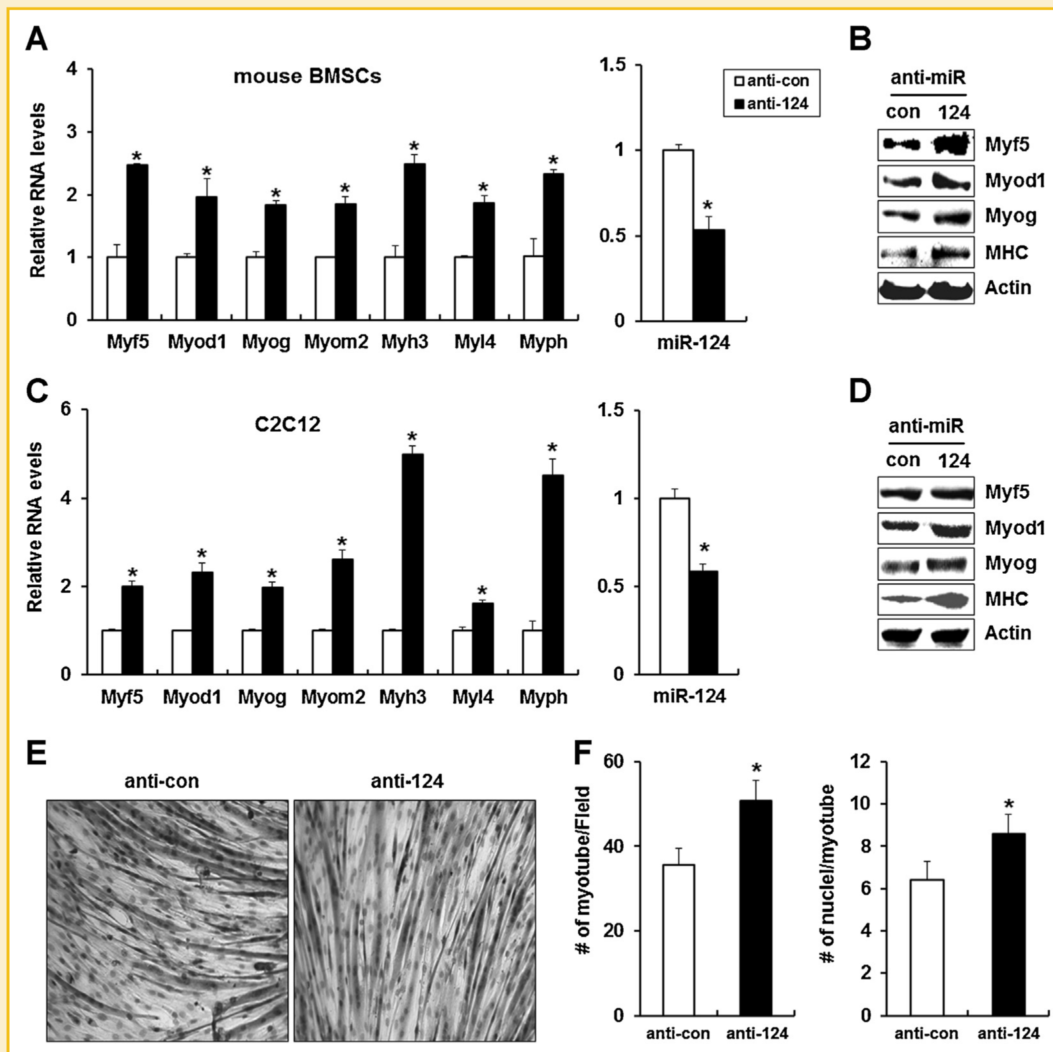

Fig. 3. Knockdown of miR-124 enhanced myogenic differentiation. Mouse BMSCs (A, B) and C2C12 cells (C–F) were transiently transfected with 100 nM of non-targeting control hairpin inhibitor (anti-con) or miR-124 inhibitor (anti-124) and incubated in myogenic medium for 7 days for mouse BMSCs (A, B) and 2 days for C2C12 cells (C, D). The relative RNA levels (A, C) were measured using quantitative RT-PCR, and protein levels were examined using Western blotting analysis (B, D). Multinucleated myotube formation in C2C12 cells was examined after myogenic induction for 6 days (E, F). $^{*}P < 0.05$, compared to anti-con.

Consistent with this report, the expression levels of miR-124 were high in brain but low in the skeletal muscles, heart, liver and kidney. In addition, consistent with our previous report demonstrating that adipogenic differentiation increased miR-124 expression, miR-124 was highly expressed in abdominal fat tissues [Qadir et al., 2013]. Interestingly, our results showed that expression levels of miR-124 in heart were similar to those in BMSCs, but miR-124 levels in skeletal muscles were much lower than those in the heart and BMSCs. These results suggest a potential role of miR-124 in the negative regulation of skeletal myogenesis.

Skeletal muscle development in vertebrates is an evolutionarily conserved process that involves myoblast fusion into multinucleated myotubes. It has been reported that mesenchymal stem cells derived from multiple regions can differentiate into muscle lineages, including skeletal muscle cells [Wakitani et al., 1995; Ferrari et al., 1998], smooth muscle cells [Lee et al., 2006; Rodriguez et al., 2006] and cardiac muscle cells [Planat-Benard et al., 2004]. The chemical 5-azacytidine is a strong DNA demethylating agent that is utilized extensively to induce stem cells to differentiate into muscle cells. Previous studies have shown that 5-azacytidine inhibits the methylation of CpG islands, resulting in the reactivation of muscle-specific genes that had been silenced, such as Myod1 [Balana et al., 2006]. In the present study, 5-azacytidine was used to prime mouse BMSCs to differentiate toward a myogenic lineage. After priming for 24 h with 5-azacytidine, further incubation of BMSCs in the presence of bFGF for 14 days significantly increased the expression levels of myogenic marker genes, including Myf5, Myod1, and Myog. In contrast to the induction of myogenic marker gene expression, a significant reduction in miR-124 expression was observed in the myogenic condition. A similar phenomenon was also observed in C2C12 cells. The availability of myoblast C2C12 cells, which can recapitulate the skeletal myogenic process in vitro, has

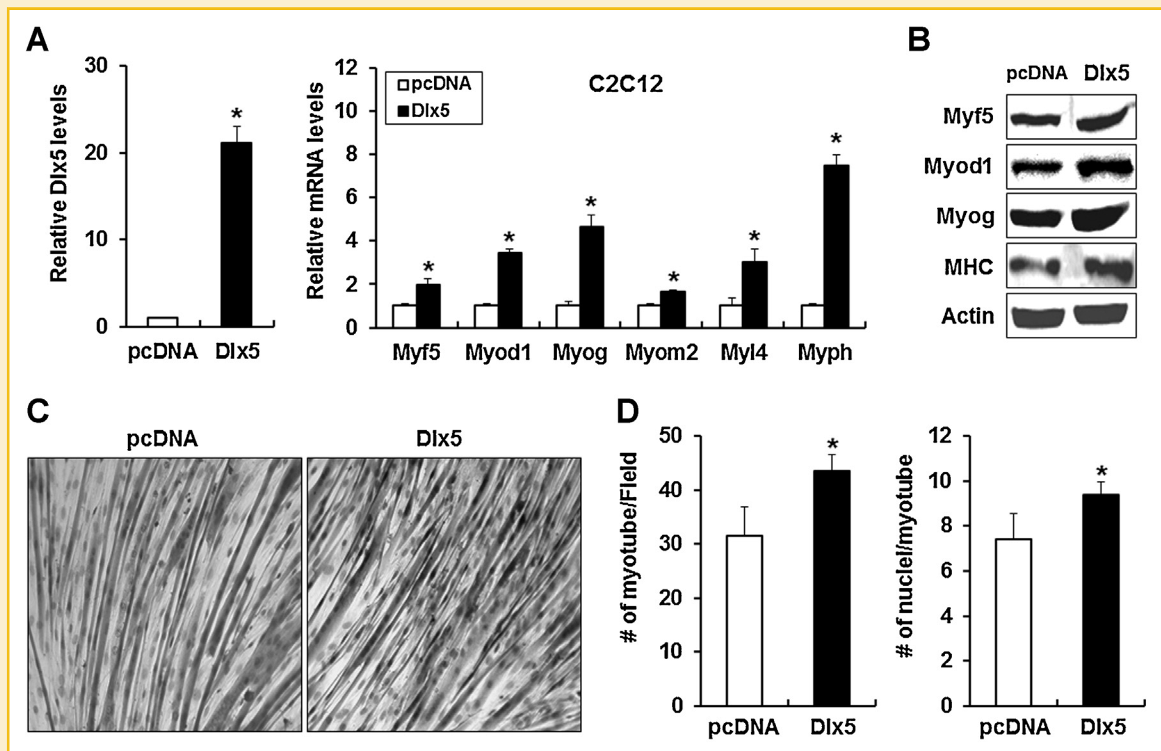

Fig. 4. Overexpression of Dlx5 enhanced myogenic differentiation of C2C12 cells. C2C12 cells were transiently transfected with HA-tagged Dlx5 or pcDNA plasmids and incubated in myogenic medium for 2 days (A, B) or 6 days (C, D). Expression levels of myogenic marker genes were examined using quantitative RT-PCR (A) and Western blotting analyses (B). Overexpression of Dlx5 was confirmed using RT-PCR (A). Cells were hematoxylin-stained and observed under the microscope (200×, C), and the number of myotubes per field and number of nuclei per myotube were quantified from three randomly selected fields (D). *$P < 0.05$, compared to pcDNA-transfected cells.

provided a convenient way to study associated developmental processes. In C2C12 cells, myogenic stimuli decreased the expression levels of miR-124, which were recovered to control levels after incubation for 6 days in myogenic medium. It is difficult to compare the results from BMSCs and C2C12 cells side-by-side. However, quite many myotubes were observed in C2C12 cells at day 6, whereas there were no clear myotubes in BMSCs at day 14 (data not shown). Thus, it is likely that reduced levels of miR-124 expression were maintained during the early phase of myogenic differentiation but they were recovered to the control levels after myotubes were formed.

To address the role of miR-124 in myogenesis, we induced forced expression of miR-124 or silencing of endogenous miR-124 and observed the effect on myogenic differentiation. These results support the negative regulatory role of miR-124 in myogenic differentiation by demonstrating that miR-124 overexpression suppressed myogenic marker gene expression and myotube formation, whereas the miR-124 hairpin inhibitor increased myogenic marker gene expression and myotube formation. Thus, it is suggested that downregulation of miR-124 expression by myogenic stimuli is necessary for the proper progression of myogenic differentiation of precursor cells, and that elucidation of the regulatory mechanism for miR-124 expression may provide a novel way to control skeletal muscle regeneration.

Because overexpression of miR-124 decreased the expression levels of myogenic transcription factors such as Myf5, Myod1, and Myog, we examined whether miR-124 directly targets any of these myogenic transcription factors using several miRNA target prediction programs. However, we could not obtain any evidence demonstrating that these transcription factors are a direct target of miR-124. Previous studies have demonstrated that miR-124 targets Dlx5 in mouse induced pluripotent stem cells, MC3T3-E1 preosteoblasts and 3T3-L1 preadipocytes [Okamoto et al., 2012; Qadir et al., 2013]. In this study, we confirmed that miR-124 downregulates the expression levels of Dlx5 mRNA and protein in both BMSCs and C2C12 cells that were incubated in myogenic medium. Thus, we examined the potential role of Dlx5 in myogenic differentiation. The requirement for Dlx5 in osteoblast differentiation and bone formation is well established [Robinson and Mahon, 1994; Ryoo et al., 1997; Holleville et al., 2007], but we could not find any previous report demonstrating that Dlx5 directly regulates myogenic differentiation of BMSCs. In the present study, the expression pattern of Dlx5 was inversely regulated with that of miR-124 during myogenic differentiation: myogenic stimuli upregulated Dlx5 expression and downregulated miR-124 in both BMSCs and C2C12 cells. In addition, overexpression of Dlx5 enhanced myogenic differentiation, whereas Dlx5 knockdown suppressed myogenic differentiation. Our data showed that myogenic stimuli increased Dlx5 expression, which was consistent with that of a previous report demonstrating that 5-azacytidine induced Dlx5 expression in C2C12 cells in the absence of any osteogenic stimuli [Hupkes et al., 2011]. Taken together, this report and our data suggest that Dlx5 plays a positive modulatory role in myogenic differentiation

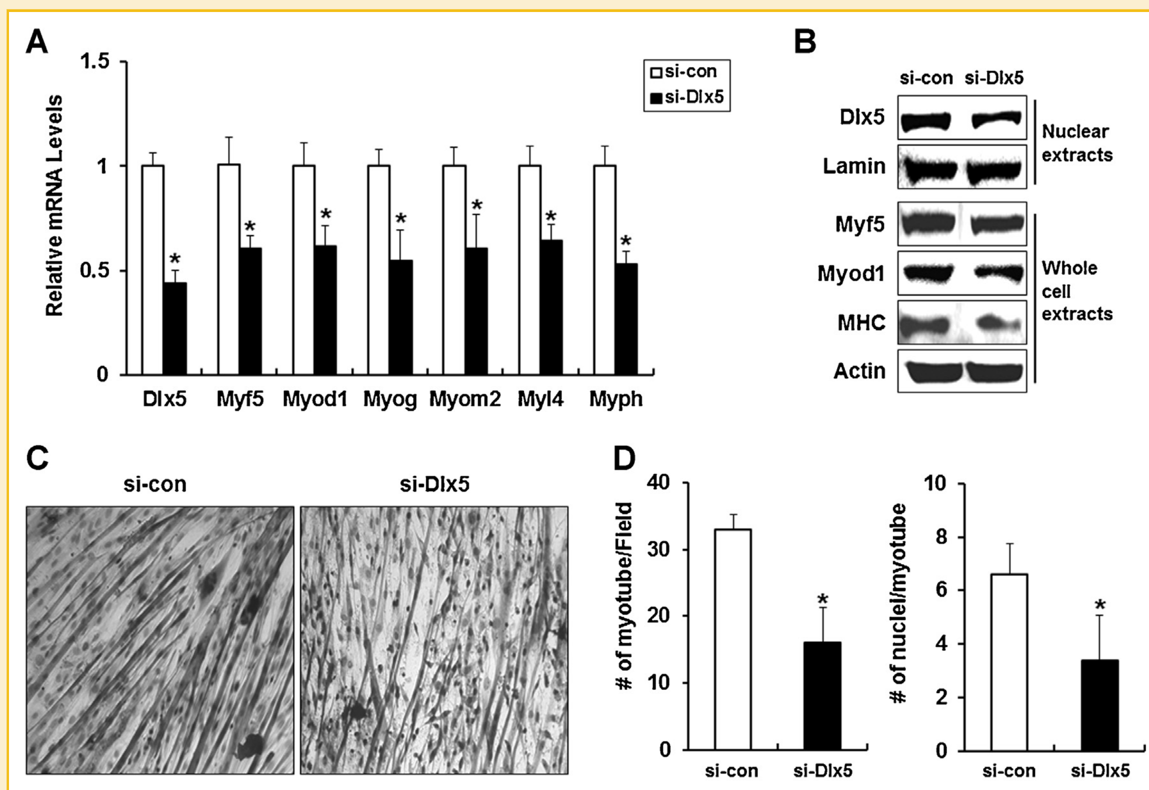

Fig. 5. Knockdown of Dlx5 inhibited myogenic differentiation of C2C12 cells. C2C12 cells were transiently transfected with 100 nM of non-targeting control siRNA (si-con) or Dlx5 siRNA (si-Dlx5) and incubated in myogenic medium for 2 days (A, B) or 6 days (C, D). Expression levels of myogenic marker genes were examined using quantitative RT-PCR (A) and Western blotting analyses (B). Knockdown efficiency of Dlx5 siRNA was examined using RT-PCR (A) and Western blotting analyses (B). Myotube formation was observed under the microscope (200×, C), and the number of myotubes and number of nuclei per myotube were quantified from three randomly selected fields (D). *$P < 0.05$, compared to si-con.

and that miR-124 regulates, at least in part, myogenesis via targeting Dlx5.

Transcription factors such as bone morphogenetic protein (BMP)-activated Smads stimulate the differentiation of mesenchymal stem cells to an osteogenic lineage while blocking differentiation into the myogenic lineage [Amthor et al., 1998, 1999]. However, some transcription factors support differentiation into both osteogenic and myogenic lineages. Canonical Wnt/β-catenin signaling is required for both osteoblast and myoblast differentiation [Ridgeway et al., 2000; Schmidt et al., 2000; Gong et al., 2001]. Overexpression of Myod1, a master transcription factor for myogenesis, in C3H10T1/2 and C2C12 cells stimulated osteogenic differentiation, whereas silencing of Myod1 inhibited BMP2-induced osteoblast differentiation [Hewitt et al., 2008]. Furthermore, muscle satellite cells derived from Myod1-deficient mice showed severely impaired osteogenic induction by BMP7, and overexpression of Myod1 increased the expression of osteoblast marker genes such as osterix and alkaline phosphatase [Komaki et al., 2004]. Interestingly, Pax7, a marker of muscle satellite cells, was co-expressed with alkaline phosphatase in human myogenic progenitor cells in regenerating muscles [Hashimoto et al., 2008]. Data obtained from the present study provide evidence supporting that Dlx5 is another transcription factor that enhances the differentiation of mesenchymal stem cells into both osteogenic and myogenic lineages.

MiR-124 has been shown to inhibit cardiomyogenesis via targeting Stat3 signaling [Cai et al., 2012]. Jak1/Stat1/Stat3 signaling is involved in myoblast proliferation and prevents premature differentiation [Sun et al., 2007]. However, Jak2/Stat2/Stat3 appears to positively regulate differentiation, indicating that Stat3 elicits context-dependent responses during myogenesis [Wang et al., 2008]. NFATc1 and other NFAT signaling regulators, such as CAMTA1 and PTBP1, have been shown to be targeted by miR-124 in osteoclasts and pulmonary artery smooth muscle cells [Kang et al., 2013; Lee et al., 2013b]. Calcineurin/NFAT signaling is required for primary myogenesis by increasing Myog expression in differentiating myoblasts [Armand et al., 2008]. Thus, further study is necessary to elucidate whether Stat3, NFATc1, and other miR-124 target genes are also involved in miR-124 inhibition of myogenic differentiation.

In summary, this study demonstrated a novel role of miR-124 and Dlx5 in regulating the differentiation of BMSCs toward the myogenic lineage, that is, miR-124 inhibits myogenic differentiation partially through targeting Dlx5 expression. Taken together, our data and those of others suggest that miR-124 has functional overlaps in the regulation of neurogenesis, adipogenesis, osteogenesis, osteoclastogenesis, and myogenesis. Thus, further investigations are required to elucidate the physiological roles of miR-124 in vivo and the potential implication of miR-124 targeting therapy in enhancing skeletal muscle regeneration.

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