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miR-124 Negatively Regulates Osteogenic Differentiation and In vivo Bone Formation of Mesenchymal Stem Cells

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ABSTRACT

MicroRNAs are novel key regulators of cellular differentiation. Dlx transcription factors play an important role in osteoblast differentiation, and Dlx5 and Dlx2 are known targets of miR-124. Therefore, in the present study, we investigated the regulatory effects of miR-124 on the osteogenic differentiation and in vivo bone formation of mesenchymal stem cells (MSCs). During osteogenic induction by BMP2, the expression levels of miR-124 were inversely correlated with those of osteogenic differentiation marker genes in human and mouse bone marrow-derived MSCs, MC3T3-E1 cells and C2C12 cells. The overexpression of a miR-124 mimic significantly decreased the expression levels of Dlx5, Dlx3, and Dlx2, whereas the silencing of miR-124 with hairpin inhibitors significantly increased the expression of these Dlx genes. Luciferase reporter assays demonstrated that miR-124 directly targets the 3′UTRs of Dlx3, Dlx5, and Dlx2. The overexpression of a miR-124 mimic suppressed the osteogenic marker gene expression levels, alkaline phosphatase activity and matrix mineralization, which were all significantly increased by the overexpression of a miR-124 inhibitor. When ectopic bone formation was induced by the subcutaneous transplantation of human bone marrow-derived MSCs in nude mice, MSCs overexpressing a miR-124 inhibitor significantly enhanced woven bone formation compared with control MSCs. However, MSCs overexpressing a miR-124 mimic exhibited increased adipocyte differentiation at the expense of ectopic bone formation. These results suggest that miR-124 is a negative regulator of osteogenic differentiation and in vivo bone formation and that the targeting of Dlx5, Dlx3, and Dlx2 genes partly contributes to this inhibitory effect exerted by miR-124. J. Cell. Biochem. 116: 730–742, 2015. © 2014 Wiley Periodicals, Inc.

KEY WORDS: miR-124; Dlx TRANSCRIPTION FACTORS; OSTEOGENIC DIFFERENTIATION; BONE FORMATION

Bone tissue arises from multipotent mesenchymal stem cells (MSCs), which differentiate into the osteoblast lineage via genetic and epigenetic regulatory mechanisms, and osteoblasts play a key role in the production and mineralization of the extracellular matrix [Kirk and Kahn, 1995]. Osteoporosis is becoming a serious public health problem as the aging of the global population accelerates. In addition to the balance between osteoclastic bone resorption and osteoblastic bone formation, the balance between the differentiation of osteoblasts and adipocytes from MSCs in the bone marrow contributes to the maintenance of bone volume. With increasing age, the balance between the differentiation of adipocytes and osteoblasts shifts, resulting in an excessive accumulation of

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bone marrow adipocytes and a decrease in bone volume [Tokuzawa et al., 2010]. Therefore, the balance between bone formation and bone marrow adipogenesis may represent another therapeutic target for the prevention or treatment of osteoporosis [Nuttall and Gimble, 2004].

The distal-less (Dlx) genes encode transcription factors that belong to the homeodomain protein families [Feledy et al., 1999; Panganiban and Rubenstein, 2002]. In vertebrates, there are six Dlx genes arranged in three clusters (Dlx1/Dlx2, Dlx3/Dlx4, Dlx5/Dlx6) [Ghanem et al., 2003]. Dlx genes have been postulated to regulate cellular differentiation toward various lineages, including the osteogenic [Ryoo et al., 1997], chondrogenic [Shea et al., 2003], adipogenic [Lee et al., 2013a], neurogenic [Eisenstat et al., 1999], and myogenic lineages [Qadir et al., 2014]. Among the Dlx genes, Dlx5, Dlx3, and Dlx2 have been implicated in osteoblastic differentiation [Shirakabe et al., 2001; Harris et al., 2003; Lee et al., 2003; Hassan et al., 2004]. Bone morphogenetic protein 2 (BMP2) is one of the powerful growth factors that promotes the differentiation of MSCs into osteoblasts and induces bone formation in vivo [Ruan et al., 2002]. Dlx5 expression is enhanced by osteogenic signals, such as BMP2, and is suppressed by adipogenic stimuli in MSCs [Harris et al., 2003; Lee et al., 2013a]. Dlx5 and Dlx3 have been suggested to play roles as osteoblast lineage determinants in bone marrow-derived MSCs (BMSCs) through the upregulation of Runx2 and the downregulation of PPARγ [Hassan et al., 2006; Lee et al., 2013a]. Dlx2 is also involved in the osteo-inductive cascade of BMP2 [Harris et al., 2003].

Some studies have shown that miRNAs play an important role in osteoblast differentiation and bone formation. It has been reported that 22 miRNAs are downregulated during BMP2-induced osteoblast differentiation, suggesting that these miRNAs may inhibit the function of osteoblast differentiation factors [Cheng et al., 2008]. Several miRNAs, including miR-143, miR-378, miR-27a, miR-26a, miR-29b, miR-125b, miR-133, and miR-135, have been shown to regulate the differentiation of MSCs toward adipocytes or osteoblasts [Esau et al., 2004; Luzi et al., 2008; Mizuno et al., 2008; Balkwill, 2009; Inose et al., 2009; Kim et al., 2010]. miR-124 has been shown to enhance neuronal differentiation by targeting Dlx2 [Cheng et al., 2009]. In addition, we previously demonstrated that miR-124 increases adipogenic differentiation while inhibiting myogenic differentiation by targeting the 3′UTR of Dlx5 [Qadir et al., 2013, 2014]. Because Dlx5 and Dlx2 play important roles in osteogenic differentiation, we investigated the potential involvement of miR-124 during osteoblast differentiation in vitro and in vivo. In this study, we demonstrate that miR-124 acts as an endogenous attenuator of Dlx5, Dlx3, and Dlx2 expression via binding to the 3′UTRs of these genes and that miR-124 inhibits osteoblast differentiation and in vivo ectopic bone formation by BMSCs.

MATERIALS AND METHODS

MATERIALS

Cell culture media, β-glycerophosphate, ascorbic acid, Alizarin red S stain and an ALP staining kit were purchased from Sigma (St. Louis, MO). BMP2 was purchased from R&D Systems (Minneapolis, MN). An anti-Dlx5 antibody was purchased from Millipore (Billerica, MA). Anti-Dlx3, anti-Dlx2, anti-lamin and HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Sensi-view™ Pico ECL Reagent was ordered from Lugen Sci (Bucheon, Korea). The NE-PER Nuclear and Cytoplasmic Extraction Reagent was obtained from Pierce Biotechnology (Rockford, IL). The easy-BLUE™ and *Star*Taq™ reagents were ordered from iNtRON Biotechnology (Sungnam, Korea), and the AccuPower RT-PreMix was purchased from Bioneer (Daejeon, Korea). The microRNA isolation kit was purchased from Ambion (Austin, TX). The Mir-X™ miRNA First-Strand Synthesis Kit and SYBR Advantage miRNA qRT-PCR Kit were obtained from Clontech (Mountain View, CA). The PCR primers were synthesized by TaKaRa Korea (Seoul, Korea). The SYBR Premix Ex Taq™ was purchased from TaKaRa (Otsu, Japan). FBS was purchased from HyClone (Logan, UT) and the Dual-Glo luciferase assay kit was obtained from Promega (Madison, WI).

CELL CULTURE

Human maxillary BMSCs were obtained from patients who underwent third molar removal surgery at Seoul National University Dental Hospital (approved by the Institutional Review Board at Seoul National University School of Dentistry (IRB no. S-D20080009)). The isolation and characterization of human BMSCs were performed as previously described [Seo et al., 2004; Akintoye et al., 2006; Um et al., 2011]. Human BMSCs were cultured in α-MEM supplemented with 15% FBS, 100 μM L-ascorbic acid, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Mouse BMSCs were isolated from the tibiae and femurs of six-week-old C57BL/6 mice and cultured in low-glucose DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Osteogenic differentiation was induced by culturing human and mouse BMSCs in osteogenic medium (growth medium supplemented with 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid).

C2C12 cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. To induce the osteogenic differentiation of C2C12 cells, the culture medium was changed to DMEM supplemented with 5% FBS, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid. MC3T3-E1 cells were maintained in α-MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Osteogenic differentiation of MC3T3-E1 cells was induced by culturing confluent cells for an additional two days and subsequent incubation for the indicated time period in osteogenic medium (α-MEM supplemented with 10% FBS, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid). Where indicated, BMP2 was added to the osteogenic medium. For human and mouse BMSCs, BMP2 was used at a concentration of 100 ng/ml, whereas C2C12 and MC3T3-E1 cells were treated with 50 ng/ml BMP2.

BIOINFORMATICS METHODS

Dlx5 and Dlx2 are known targets of miR-124 [Cheng et al., 2009; Okamoto et al., 2012; Qadir et al., 2013, 2014]. The potential targeting of osteogenic transcription factors and other Dlx family

genes by miR-124 was screened with computer-aided algorithms from TargetScan (http://www.targetscan.org/vert_42/) and miRanda (http://www.microrna.org/microrna/).

QUANTITATIVE RT–PCR

The total RNA was isolated using easy-BLUE™ RNA extraction reagents. Complementary DNA was synthesized from 2 μg of total RNA using Accu Power RT-PreMix. Real-time PCR was performed using SYBR Premix Ex Taq™ and an AB 7500 Fast Real-Time system. Each sample was analyzed in triplicate, and the expression levels of the target genes were normalized to a reference housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The fold differences for each treatment group were then calculated using the normalized C_T values for the control. The forward and reverse primers for the amplification of mouse genes were as follows (5′→3′): Dlx5, TCTCTAGGACTGACGCAAACA and GTTACACGCCATAGGGTCGC; Dlx3, ATGAGTGGCTCCTTCGATCGCAAGCT and TCAGTACACAGC-CCCAGGGTTGGGC; Dlx2, GGCTCCTACCAGTACCAC and GTAGCC-CAGGTCGTAGCT; Runx2, TTCTCCAACCCACGAATGCAC and CAG-GTACGTGTGGTAGTGAGT; osterix, CCCACCCTTCCCTCACTCAT and CCTTGTACCACGAGCCATAGG; osteocalcin (OCN), CTGACAAAGCC-TTCATGTCCAA and GCGCCGGAGTCTGTTCACTA; alkaline phosphatase (ALP), CCAACTCTTTTGTGCCAGAGA and GGCTACATTGGT-GTTGAGCTTTT; and GAPDH, TCAATGACAACTTTGTCAAGC and CCAGGGTTTCTTACTCCTTGG. The forward and reverse primers for the amplification of human genes were as follows (5′→3′): DLX5, CAACTTTGCCCGAGTCTTC and GTTGAGAGCTTTGCCA-TAGG; DLX3, TACCCTGCCCGAGTCTTCTG and TGGTGGTAGGTG-TAGGGGTTC; DLX2, GCCTCAACAACGTCCCTTACT and GGGAGCG-TAGGAGGTGTAGG; and GAPDH, CCATCTTCCAGGAGCGAGATC and GCCTTCTCCATGGTGGTGAA.

For miRNA analysis, microRNA was extracted using the mirVana microRNA isolation kit according to the manufacturer's instructions. Complementary DNA was synthesized using the Mir-X™ miRNA First-Strand Synthesis Kit, and quantitative PCR was performed using the SYBR Advantage miRNA qRT-PCR Kit. The relative expression levels were normalized using U6 snRNA as a control. For the amplification of miR-124, a forward primer was designed to specifically bind to miR-124, and a universal reverse primer was provided in the kit. The nucleotide sequence of the forward primer for mouse and human miR-124 is as follows (5′→3′): TAAGGCACGCGGTGAATGCC.

WESTERN BLOT ANALYSIS

Nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions. The protein concentration was determined using the BioRad protein assay reagent. Each sample, which contained equal amounts of protein, was subjected to SDS–PAGE. The proteins were electro blotted onto a PVDF membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 and incubated with the relevant primary antibody and then with an HRP-conjugated secondary antibody. Immune complexes were visualized using the Sensi-view™ Pico ECL Reagent, and the luminescence was detected with a MicroChemi imaging system (DNR; Jerusalem, Israel). The quantification of the labeled bands was performed using Image J pixel analysis (NIH Image software), and the data are presented as relative band densities normalized to lamin.

DETERMINATION OF ALP ACTIVITY AND MATRIX MINERALIZATION

ALP staining has been widely accepted as a simple and easy approach for determining osteogenic differentiation. Cultured cells were stained with ALP staining reagent (Sigma) according to the manufacturer's instructions. For the quantification of ALP activity, the cells were inoculated into 24-well plates and cultured in osteogenic medium in the presence or absence of BMP2 for 48 h (MC3T3-E1 and C2C12 cells) or seven days (mouse BMSCs). At the end of the culture period, cell lysates were prepared, and ALP activity was determined using the TRACP & ALP kit (TaKaRa). The absorbance at 405 nm was measured, and the data are presented as relative ALP activities normalized to that of the control cells.

To observe matrix mineralization, Alizarin red S staining was performed. Mouse BMSCs and MC3T3-E1 cells were cultured in osteogenic medium for 21 and 10 days, respectively. At the end of the culture period, the cells were washed twice with PBS and fixed with ice-cold 70% ethanol. The fixed cells were stained with Alizarin red S solution, washed with water and observed under a microscope. For quantitation, the Alizarin red stain was eluted with 0.5 N HCl/5% SDS, and the absorbance at 415 nm was measured. The data are presented as relative optical densities (O.D.) normalized to that of the control cells.

OVEREXPRESSION OF A MIRNA MIMIC AND A HAIRPIN INHIBITOR

To examine the regulatory role of miR-124, the cells were transiently transfected with a miR-124 mimic oligonucleotide (mimic-124) or a hairpin inhibitor of miR-124 (anti-124). Control oligonucleotides (mimic-con or anti-con) were used as negative controls. The small RNA mimic and hairpin inhibitor were obtained from Dharmacon (Chicago, IL). Transfection into mouse BMSCs, MC3T3-E1 and C2C12 cells was performed using Dharmafect (Dharmacon) according to the manufacturer's instructions. After transient transfection, the culture medium was replaced with fresh osteogenic medium, and the cells were incubated in the presence or absence of BMP2 for the indicated periods of time.

LUCIFERASE REPORTER ASSAY

A luciferase reporter construct containing the wild-type mouse Dlx5 3′UTR sequence downstream of a luciferase sequence (Dlx5 3′UTR WT) was described previously [Qadir et al., 2013]. To prepare a luciferase reporter construct that contained the mouse Dlx3 3′UTR sequence, the 3′UTR region of mouse Dlx3 (NM_010055.3) was amplified by PCR using the following primers: forward 5′-CCAAATGAGGGAACCTTTGA-3′ and reverse 5′-TGGTCCAGTAA-GAAAGCAGAAA-3′. The purified PCR product was then amplified with the following primers containing an *Xba*I restriction site: forward 5′-GCCGTGTAATTCTAGAGGGCTTCCAAGGGAAG AC-3′ and reverse 5′-CCGCCCCGACTCTAGACAGAAATGAGAA-GGCTGGGGAG-3′. The Dlx3 3′UTR WT luciferase reporter construct was produced by subcloning the Dlx3 3′UTR PCR fragments downstream of the luciferase gene in the pGL3 vector (Promega) after digestion with the *Xba*I enzyme. The 3′UTR of mouse Dlx2

(NM_010054.2) was also amplified by PCR using the following primers: forward 5′-CACGCGTCCTGTAGCTAGAG-3′ and reverse 5′-AGTATGTTGCTTTCATAATA-3′. The purified PCR product was then amplified with the following primers containing *Mlu*I and *Hind*III restriction sites: forward 5′-TACACGCGTCCTGTAGCTAGAG-3′ and reverse 5′-TAAAGCTTGTATGTTGCTTTCATAATA-3′. The Dlx2 3′UTR WT luciferase reporter construct was produced by subcloning the Dlx2 3′UTR PCR fragments downstream of the luciferase gene in the pGL3 vector after digestion with the *Mlu*I and *Hind*III enzymes.

C2C12 cells were plated at an initial density of 1×10^4 cells per well into 96-well plates. The cells were transfected with 0.2 μg of the reporter construct using lipofectamine 2000 (Invitrogen). Where indicated, the cells were transfected with the miRNA-mimic or hairpin inhibitor 24 h after transfection with the reporter construct and incubated for an additional 24 h. To normalize the transfection efficiency, a *Renilla* luciferase-expressing plasmid was used as an internal control. The cells were then treated with or without BMP2 for 24 h and the luciferase activity was then measured using a Dual-Glo luciferase assay kit. The data are presented as relative luciferase activities normalized to that of the control cells.

IN VIVO ECTOPIC BONE FORMATION ASSAY

To examine whether changes in the miR-124 level in BMSCs regulate in vivo bone formation, human BMSCs were transiently transfected with 100 nM mimic-con, mimic-124 or anti-124 by electroporation two days before transplantation. The in vivo transplantation of human BMSCs was performed as previously described [Um et al., 2011]. In brief, human BMSCs (5×10^6 cells per transplantation) were collected, re-suspended in 500 μl of medium and incubated for 1 h with 40 mg of hydroxyapatite/tricalcium phosphate particles (HA/TCP) (Zimmer, Warsaw, IN) at 37°C in a 5% CO_2 incubator. The cells with HA/TCP were then transplanted into the dorsal surface of six-week-old immunocompromised beige nude mice (three transplantations per group; NIH-bg-nu-xid, Harlan Sprague Dawley, Indianapolis, IN). The transplants were removed after eight weeks, fixed in 4% paraformaldehyde, decalcified in formic acid (pH 8.0), and then embedded in paraffin. The sections were de-paraffinized and stained with hematoxylin and eosin. The percentage of woven bone area per total tissue area was quantified using three serial sections obtained from the center of each specimen as previously described [Bork et al., 2011]. All of the procedures in this animal experiment were approved by the Institute of Laboratory Animal Resources at Seoul National University (SNU-121116–4).

STATISTICAL ANALYSIS

The data are presented as the mean ± SD. The statistical significance was analyzed by Student's *t*-test. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

BMP2 SUPPRESSES THE EXPRESSION LEVELS OF MIR-124 BUT INCREASES THE EXPRESSION LEVELS OF DLX5, DLX3 AND DLX2

To confirm the stimulatory effects of BMP2 on osteoblast differentiation under the culture conditions used in this study, post-confluent human BMSCs, mouse BMSCs, MC3T3-E1, and C2C12 cells were incubated in the presence or absence of BMP2 (100 or 50 ng/ml). Because Runx2, osterix, ALP, and OCN represent well-established and reliable markers for examining the progress of osteoblast differentiation, the levels of these genes were examined in the following experiments. As expected, BMP2 significantly increased the mRNA levels of these genes in all of the cells examined (Suppl. Fig. 1A–C). The Western blot analysis results confirmed that BMP2 increased the protein levels of Runx2 and osterix in MC3T3-E1 and C2C12 cells (Suppl. Fig. 1D). These data indicate that BMP2 exerts stimulatory effects on osteoblastic differentiation in all cells used in this study.

We then examined the effects of BMP2 on the expression levels of miR-124, Dlx5, Dlx3, and Dlx2. BMP2 caused a considerable decrease in the expression levels of miR-124 while inducing Dlx5, Dlx3, and Dlx2 mRNA expression (Fig. 1). BMP2 also increased the protein levels of Dlx5, Dlx3, and Dlx2 in MC3T3-E1 and C2C12 cells (Figs. 1B and C). The regulatory effects of BMP2 on these Dlx genes and miR-124 were significant in MC3T3-E1 and C2C12 cells even after incubation for 12 h. These results indicate that BMP2 downregulates miR-124 expression and that there is an inverse correlation between the expression levels of miR-124 and those of Dlx5, Dlx3, Dlx2 and osteogenic marker genes.

It has been previously reported that BMP2 downregulates the expression of miR-141 and miR-200a, which are other miRNAs targeting the Dlx5 3′UTR [Itoh et al., 2009]. Therefore, the levels of miR-141 and miR-200a were also examined in MC3T3-E1 and C2C12 cells. Consistent with a previous report, BMP2 decreased the expression of miR-141 and miR-200a in both MC3T3-E1 and C2C12 cells (Suppl. Fig. 2).

OVEREXPRESSION OF A MIR-124 MIMIC DOWNREGULATES THE EXPRESSION LEVELS OF DLX5, DLX3, AND DLX2

It was previously reported that Dlx5 and Dlx2 are the direct targets of miR-124 [Cheng et al., 2009; Qadir et al., 2013]. Further analysis using target prediction algorithms demonstrated that there is a single binding site for the miR-124 seed sequence in the 3′UTR of Dlx3 and that this binding site is also highly conserved in many vertebrates (Suppl. Fig. 3A). Therefore, we examined whether miR-124 is able to downregulate Dlx5, Dlx3, and Dlx2 expression under osteogenic conditions. Mouse BMSCs, MC3T3-E1 and C2C12 cells were transiently transfected with mimic-con or mimic-124 and further incubated for seven days (mouse BMSCs) or two days (MC3T3-E1 and C2C12 cells). The quantitative RT-PCR results confirmed that miR-124 was highly expressed in mimic-124-transfected cells (Fig. 2A, C, and E, right panels). The overexpression of mimic-124 significantly suppressed the BMP2-induced expression of Dlx5, Dlx3, and Dlx2 mRNA in all of the cells examined (Fig. 2A, C, and E, left panels). The western blot data also showed that the mimic-124/BMP2 group expressed lower levels of Dlx5, Dlx3, and Dlx2 proteins compared with the mimic-con/BMP2 group (Fig. 2B, D, and F).

To verify whether miR-124 is able to recognize the 3′UTR of Dlx5, Dlx3, and Dlx2 under osteogenic conditions, we performed luciferase reporter assays using reporter constructs containing the 3′UTR sequences of mouse Dlx5, Dlx3 or Dlx2. Consistent with our results showing that BMP2 downregulates miR-124 expression, BMP2 significantly increased the luciferase activity of all three

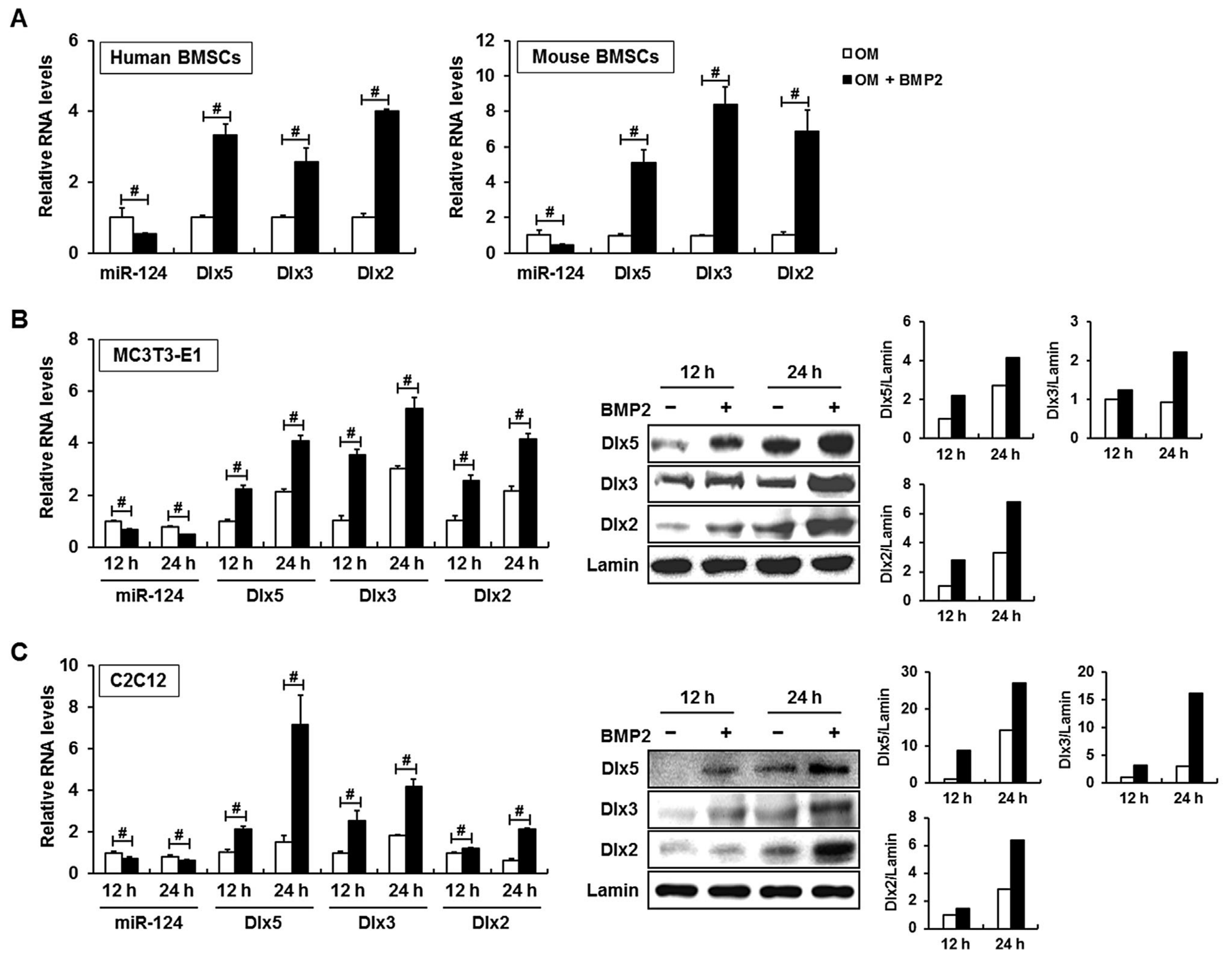

Fig. 1. BMP2 downregulates miR-124 expression while inducing the expression of Dlx5, Dlx3, and Dlx2. (A) Human BMSCs and mouse BMSCs were cultured for 21 and 14 days, respectively, in osteogenic medium (OM) in the presence or absence of BMP2 (100 ng/ml). The expression levels of miR-124 and Dlx5, Dlx3, and Dlx2 mRNA were examined by quantitative RT-PCR. The data represent the mean ± SD from triplicate samples (# $P < 0.05$). (B, C) MC3T3-E1 and C2C12 cells were cultured for the indicated periods of time in OM in the presence or absence of BMP2 (50 ng/ml). The RNA expression levels were examined by quantitative RT-PCR (left panels). The protein levels of Dlx5, Dlx3, and Dlx2 were examined via western blot analysis (right panels). The protein band densities were quantified, and the data are presented as relative band densities normalized to that of lamin.

reporters in the mimic-con-transfected C2C12 cells compared with the vehicle-treated mimic-con cells (Fig. 2G). Furthermore, the overexpression of mimic-124 significantly decreased both basal and BMP2-induced luciferase activity (Fig. 2G). These results suggest that miR-124 directly recognizes and binds to the 3′UTR of Dlx5, Dlx3, and Dlx2 and thereby decreases the mRNA and protein levels of Dlx5, Dlx3, and Dlx2.

To confirm the specificity of the interaction between miR-124 and the Dlx3 3′UTR target site, a luciferase reporter containing mutations in this seed match region was prepared (Dlx3 3′UTR MT reporter, Suppl. Fig. 3A). Unlike the effect exerted on the wild-type luciferase reporter, the overexpression of mimic-124 did not suppress the luciferase activity of the mutant reporter compared with the mimic-con-transfected cells (Suppl. Fig. 3B). However, the BMP2-induced increase in luciferase activity was maintained with the mutant reporter, suggesting that BMP2 regulates multiple miRNAs targeting the Dlx3 3′UTR. These results indicate that miR-124 directly recognizes and binds to the 3′UTR of Dlx3 and thereby contributes to the downregulation of Dlx3 mRNA and protein expression.

OVEREXPRESSION OF A MIR-124 MIMIC SUPPRESSES OSTEOGENIC DIFFERENTIATION

To determine the regulatory role of miR-124 on osteogenic differentiation, mouse BMSCs, MC3T3-E1, and C2C12 cells were transfected with mimic-con or mimic-124 and incubated in osteogenic medium in the presence or absence of BMP2. For RNA isolation, ALP staining and ALP activity assay, the cells were cultured for two days (MC3T3-E1 and C2C12 cells) or seven days (mouse BMSCs). For Alizarin red staining, the cells were cultured for 10 days (MC3T3-E1) or 14 days (mouse BMSCs). The overexpression of mimic-124 significantly suppressed both the basal and the

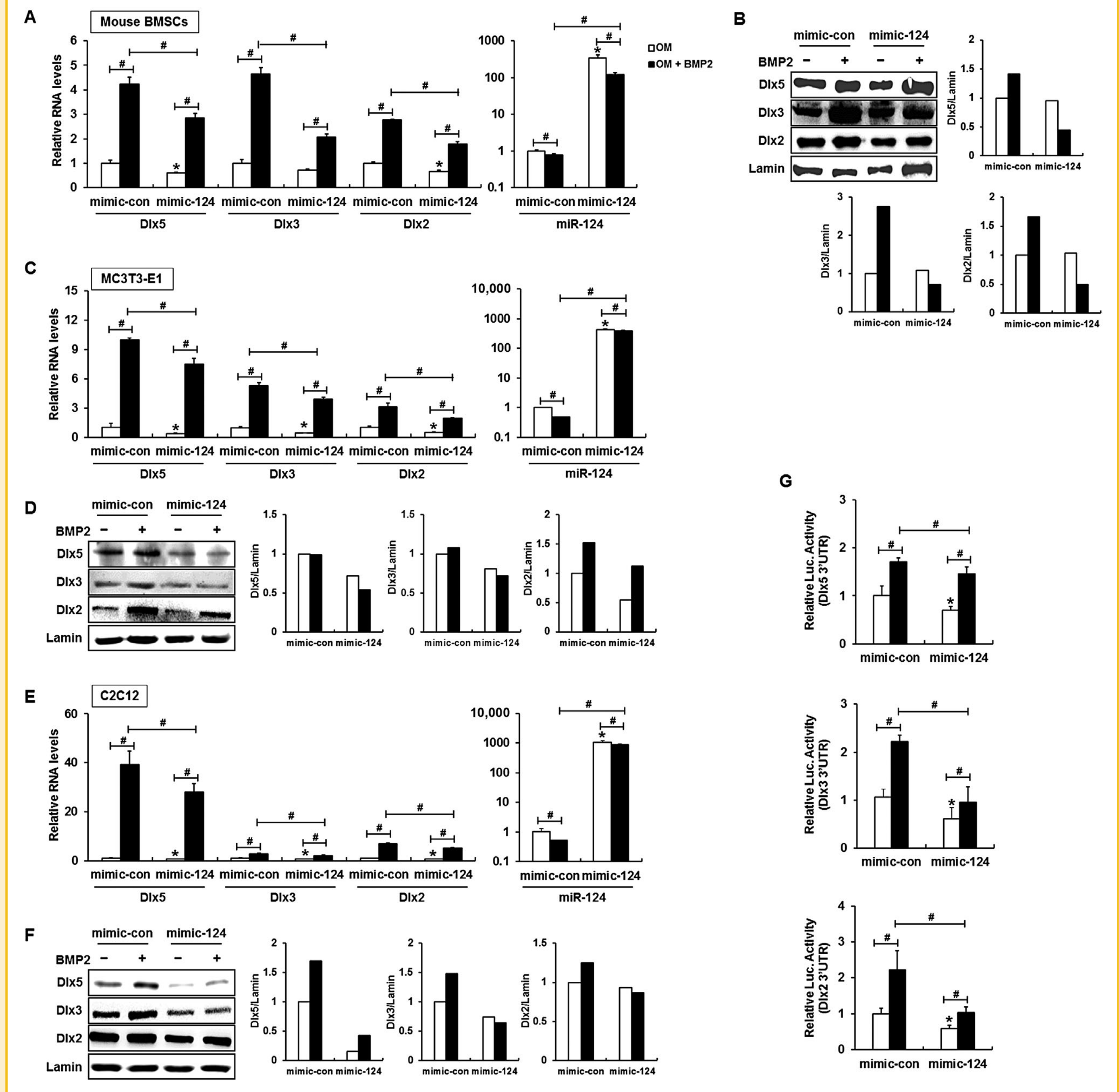

Fig. 2. Overexpression of a miR-124 mimic inhibits the expression of Dlx5, Dlx3, and Dlx2. (A–F) Cells were transiently transfected with 100 nM control mimic (mimic-con) or miR-124 mimic (mimic-124) and incubated for two days (MC3T3-E1 and C2C12 cells) or seven days (mouse BMSCs) in OM in the presence or absence of BMP2. The RNA expression levels of Dlx5, Dlx3, Dlx2, and miR-124 in mouse BMSCs (A), MC3T3-E1 (C) and C2C12 cells (E) were examined via quantitative RT-PCR. The data represent the mean ± SD from triplicate samples (*$P < 0.05$ compared with the mimic-con-transfected cells without BMP2 treatment; #$P < 0.05$ compared with the indicated sample). The protein levels of Dlx5, Dlx3, and Dlx2 in mouse BMSCs (B), MC3T3-E1 (D) and C2C12 cells (F) were examined via western blot analysis. (G) Mimic-con or mimic-124 was transfected into C2C12 cells that expressed luciferase reporter genes containing one of the Dlx5 3′UTR, Dlx3 3′UTR or Dlx2 3′UTR sequences. The cells were then incubated for 24 h in OM or OM + BMP2. The transfection efficiency was normalized by the *Renilla* luciferase activity. The data represent the mean ± SD from triplicate samples (*$P < 0.05$ compared with the mimic-con-transfected cells without BMP2 treatment; #$P < 0.05$ compared with the indicated sample).

BMP2-induced expression of osteoblast marker genes in all of the cells examined (Fig. 3A, C, and E). The ALP staining and ALP activity assay results also demonstrated that the inhibitory effects of mimic-124 on osteoblast differentiation were significant in both the presence and absence of BMP2 (Fig. 3B, D, and F). Furthermore, the Alizarin red staining results clearly showed that the overexpression of mimic-124 suppressed the basal and BMP2-induced matrix mineralization in mouse BMSCs and MC3T3-E1 cells (Fig. 3G). These results indicate that miR-124 is a negative regulator of osteogenic differentiation.

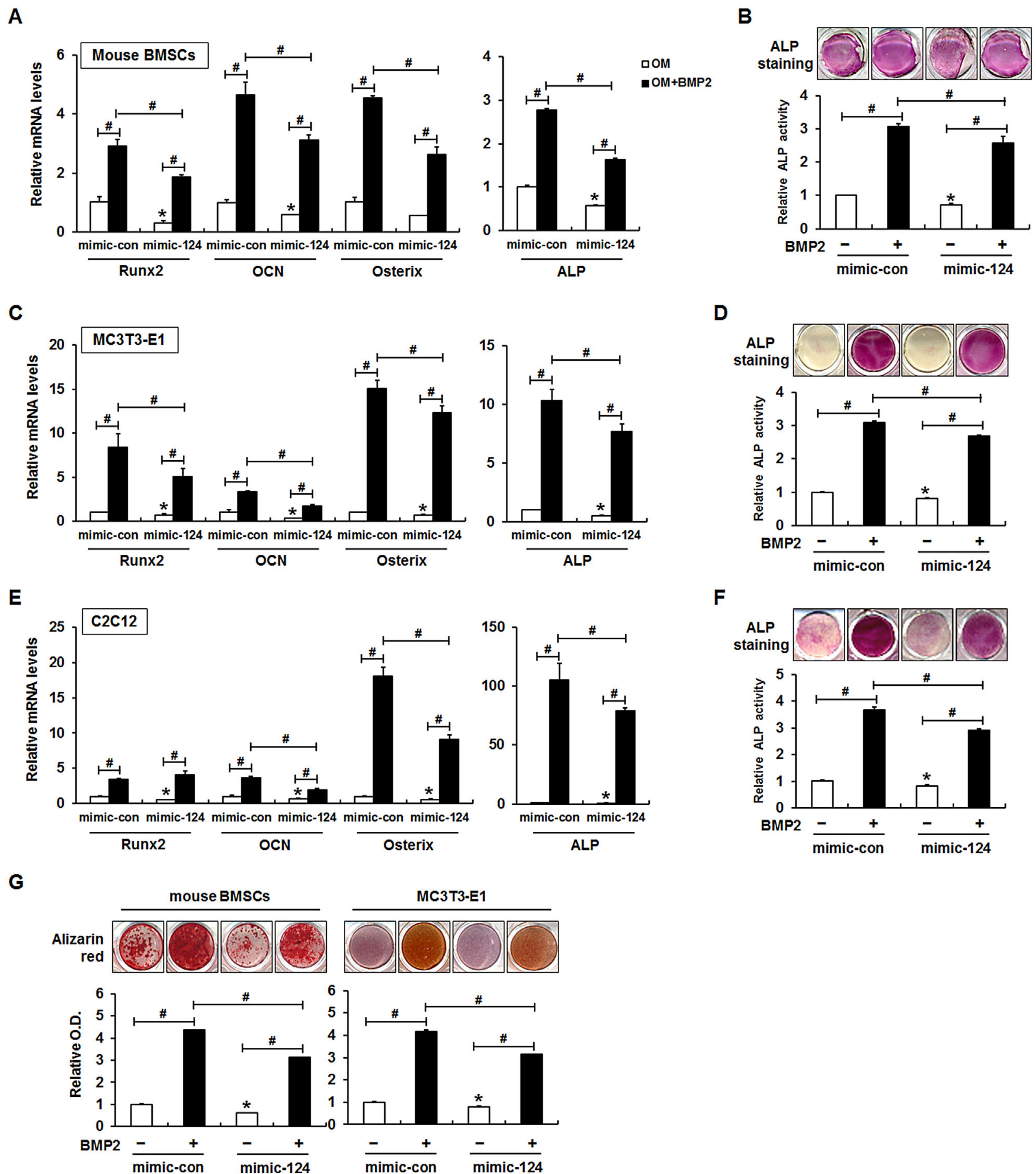

Fig. 3. Overexpression of a miR-124 mimic inhibits osteogenic differentiation. (A–F) Mouse BMSCs, MC3T3-E1 and C2C12 cells were transiently transfected with 100 nM mimic-con or mimic-124 and incubated for seven days (BMSCs) or 48 h (MC3T3-E1 and C2C12 cells) in OM or OM + BMP2. The expression levels of Runx2, osteocalcin (OCN), osterix and ALP in BMSCs (A), MC3T3-E1 (C) and C2C12 (E) were then examined by quantitative RT-PCR. The ALP activity in BMSCs (B), MC3T3-E1 (D) and C2C12 cells (F) was determined via ALP staining or quantitative ALP assay. (G) Matrix mineralization was examined by Alizarin red S staining of cells cultured for 14 days (BMSCs) or 10 days (MC3T3-E1). The Alizarin red staining was quantified by measuring the optical density of the eluent. $^{*}P < 0.05$ compared with the mimic-con-transfected cells without BMP2 treatment; $\#P < 0.05$ compared with the indicated sample.

OVEREXPRESSION OF A MIR-124 INHIBITOR INCREASES THE EXPRESSION LEVELS OF THE DLX5, DLX3 AND DLX2 GENES

To further confirm the effects of miR-124 on Dlx5, Dlx3, and Dlx2 expression under osteogenic conditions, mouse BMSCs, MC3T3E1 and C2C12 cells were transfected with anti-124 or anti-con and incubated for two days (MC3T3E1 and C2C12 cells) or seven days (mouse BMSCs) in osteogenic media in the presence or absence of BMP2. Compared with the anti-con transfected cells, the overexpression of anti-124 decreased the expression levels of miR-124 in both the presence and absence of BMP2 (Fig. 4A, C, and E, right panels). Compared with the anti-con transfected cells, the overexpression of anti-124 significantly increased the expression levels of Dlx5, Dlx3, and Dlx2 mRNA in both the presence and absence of BMP2 (Fig. 4A, C, and E, left panels). Anti-124 also increased the protein levels of Dlx5, Dlx3 and Dlx2 in both the basal and BMP2-treated conditions (Fig. 4B, D, and F). Furthermore, the overexpression of anti-124 significantly increased the basal and BMP2-induced luciferase activity of Dlx5, Dlx3 and Dlx2 3′UTR WT reporters (Fig. 4G). The overexpression of anti-124 did not exert any effects on the luciferase activity of Dlx3 3′UTR mutant reporter in both the presence and absence of BMP2 (Suppl. Fig. 3C). These results further support the notion that miR-124 inhibits the expression of Dlx5, Dlx3 and Dlx2 by targeting their 3′UTR sequences.

KNOCKDOWN OF MIR-124 INCREASES OSTEOGENIC DIFFERENTIATION

To further examine the effects of endogenous miR-124 on osteogenic differentiation, anti-con or anti-124 was transfected into mouse BMSCs, MC3T3-E1 and C2C12 cells, and the expression levels of osteogenic marker genes and matrix mineralization were examined. The quantitative RT-PCR results demonstrated that anti-124 overexpression increased the expression levels of Runx2, osterix, ALP and OCN compared with the anti-con-transfected cells, and these stimulatory effects were observed in both the presence and absence of BMP2 (Fig. 5A, C, and E). The ALP assay and Alizarin red staining results also indicated that anti-124 increased the basal and BMP2-induced osteogenic differentiation of mouse BMSCs, MC3T3-E1, and C2C12 cells (Fig. 5B, D, F, and G). These results further support the notion that miR-124 is a negative regulator of osteoblastic differentiation.

MIR-124 SUPPRESSES IN VIVO ECTOPIC BONE FORMATION BY HUMAN BMSCS

Because the results described above demonstrated that miR-124 exerts a negative regulatory effect on the osteogenic differentiation of BMSCs in vitro, we next explored whether the regulation of the miR-124 expression levels in BMSCs also exerts an effect on in vivo bone formation. To induce ectopic bone formation, human BMSCs were loaded on an HA/TCP scaffold and subcutaneously transplanted in nude mice, as depicted in Figure 6A. Eight weeks after transplantation, woven bone formation was observed in the decalcified tissue specimens. Compared with mimic-con-transfected cells, the anti-124-transfected BMSCs showed significantly enhanced ectopic bone formation, whereas mimic-124 suppressed ectopic bone formation (Fig. 6B and C). Interestingly, the mimic-124 specimens demonstrated a marked increase in adipocytes (Fig. 6B, middle panel). Furthermore, many osteoclast-like multinucleated cells were observed around the scaffolds (Fig. 6B, middle panel; closed arrow-heads). These results suggest that miR-124 inhibits the differentiation of BMSCs toward the osteogenic lineage but enhances adipogenic differentiation, thereby suppressing in vivo bone formation.

DISCUSSION

It has been previously demonstrated that miR-124 is highly expressed in fat and nervous tissues, but very low levels of miR-124 are expressed in skeletal/cardiac muscles and bone; furthermore, miR-124 is a positive regulator of adipogenic and neurogenic differentiation but a negative regulator of myogenic differentiation [Cheng et al., 2009; Qadir et al., 2013, 2014]. In the present study, we demonstrated that miR-124 inhibits the osteogenic differentiation of BMSCs and in vivo bone formation. Several lines of evidence support this notion: (i) BMP2, a strong osteogenic stimulus, downregulated miR-124 expression; (ii) the forced expression of miR-124 suppressed the osteogenic differentiation of BMSCs, MC3T3-E1, and C2C12 cells, whereas the inhibition of endogenous miR-124 further enhanced the osteogenic differentiation of these cells; and (iii) the overexpression of miR-124 inhibited bone formation but enhanced adipocyte differentiation of subcutaneously transplanted human BMSCs in nude mice, whereas the overexpression of anti-124 significantly enhanced ectopic bone formation.

Many miRNAs have been shown to control the proliferation and differentiation of cells in the osteoblast lineage: miR-29a was identified as a negative regulator of Wnt antagonists, Dkk1, Kremen2, and sFRP2 [Kapinas et al., 2010]; miR-2861 promotes osteoblast differentiation by repressing histone deacetylase 5 expression [Balkwill, 2009] and miR-206 inhibits osteoblast differentiation by suppressing the expression of connexin 43 [Inose et al., 2009]. In addition, miR-196a [Inose et al., 2009], miR-208 [Itoh et al., 2010], miR-29b [Balkwill, 2009], miR-204 [Huang et al., 2010], and miR-133/135 [Cheng et al., 2008] also play a role in osteoblast differentiation by targeting the various transcriptional factors involved in osteogenic differentiation. miR-204/211 inhibit osteogenic differentiation but stimulate adipocyte differentiation [Huang et al., 2010]. Conversely, miR-128a inhibits mesoderm-type lineage differentiation (osteoblast, adipocyte and chondrocyte) and maintains MSCs in undifferentiated states [Huang et al., 2010].

To explore the molecular mechanisms through which miR-124 inhibits osteogenic differentiation, we examined Dlx transcription factors as targets of miR-124. Dlx5 is one of the well-known target genes of miR-124 during adipogenesis, myogenesis and osteogenesis [Okamoto et al., 2012; Qadir et al., 2013, 2014]. A regulatory connection between miR-124 and Dlx2 has also been reported in subventricular zone stem cells [Cheng et al., 2009]. However, no previous study has examined the regulation of Dlx2 by miR-124 under osteogenic conditions. We also investigated whether other Dlx family genes or other osteogenic transcription factors are potential targets of miR-124 using the TargetScan and miRanda programs. The target prediction programs showed that Dlx3 can also be a target of miR-124. In the present study, we demonstrated that Dlx3 is a

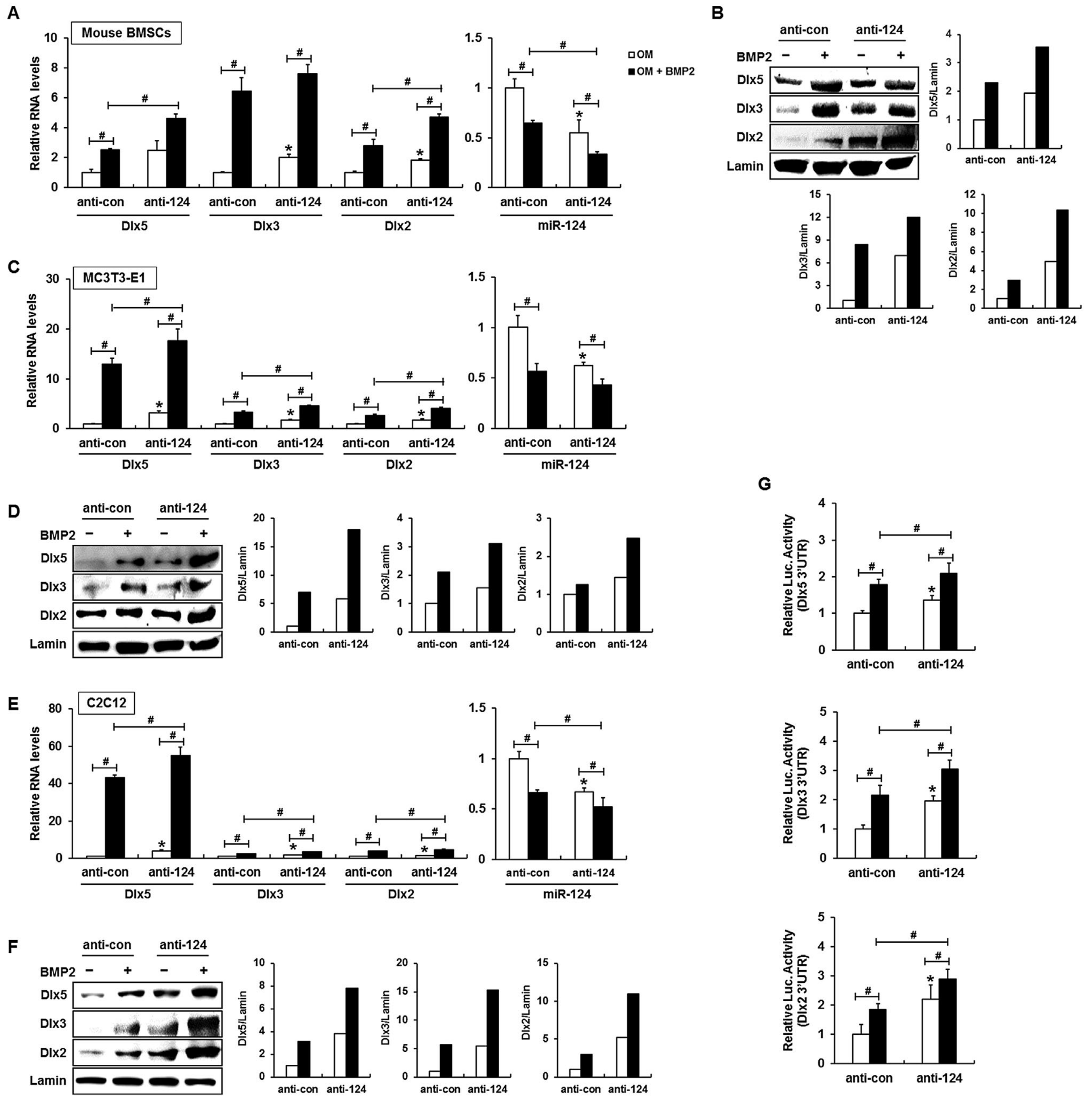

Fig. 4. Functional blocking of miR-124 with a hairpin inhibitor increases the expression levels of Dlx5, Dlx3, and Dlx2. (A–F) Cells were transiently transfected with 100 nM control inhibitor (anti-con) or miR-124 inhibitor (anti-124) and incubated for two days (MC3T3-E1 and C2C12 cells) or seven days (mouse BMSCs) in OM in the presence or absence of BMP2. The RNA expression levels of Dlx5, Dlx3, Dlx2, and miR-124 in mouse BMSCs (A), MC3T3-E1 (C) and C2C12 cells (E) were examined by quantitative RT-PCR. The data represent the mean ± SD from triplicate samples (*p<0.05 compared with the anti-con-transfected cells without BMP2 treatment; #*P* < 0.05 compared with the indicated sample). The protein levels of Dlx5, Dlx3, and Dlx2 in mouse BMSCs (B), MC3T3-E1 (D) and C2C12 cells (F) were examined via western blot analysis. (G) Anti-con or anti-124 was transfected into C2C12 cells expressing luciferase reporter genes containing one of the Dlx5 3′UTR, Dlx3 3′UTR or Dlx2 3′UTR sequences. The cells were then incubated for 24 h in OM or OM + BMP2. The transfection efficiency was normalized by the *Renilla* luciferase activity. The data represent mean ± SD from triplicate samples (**P* < 0.05 compared with the anti-con-transfected cells without BMP2 treatment; #*P* < 0.05 compared with the indicated sample).

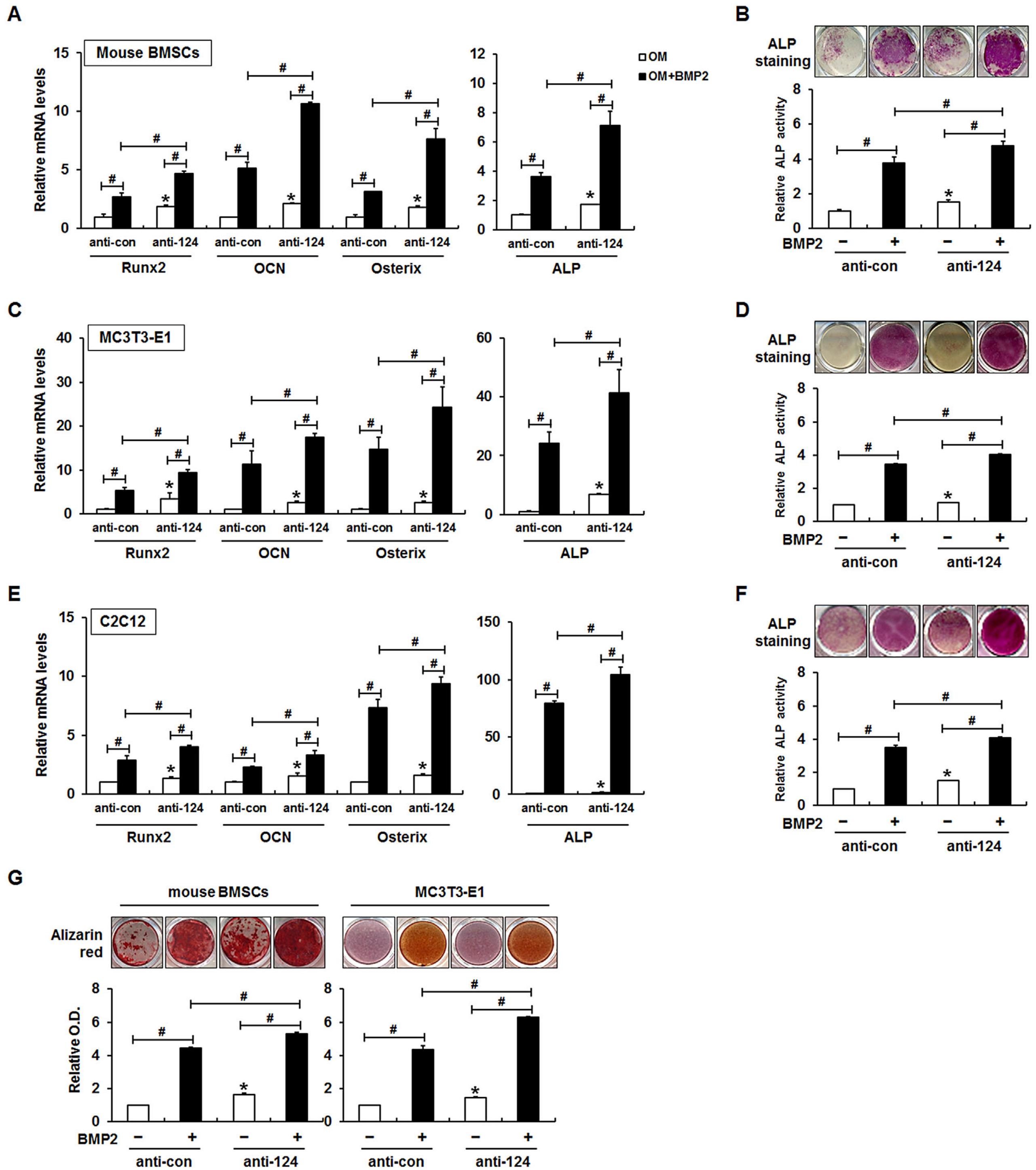

Fig. 5. Knockdown of miR-124 with a hairpin inhibitor enhances osteogenic differentiation. (A–F) Mouse BMSCs, MC3T3-E1, and C2C12 cells were transiently transfected with 100 nM anti-con or anti-124 and incubated for seven days (BMSCs) or 48 h (MC3T3-E1 and C2C12 cells) in OM or OM + BMP2. The expression levels of osteogenic marker genes in BMSCs (A), MC3T3-E1 (C) and C2C12 cells (E) were examined via quantitative RT-PCR. The ALP activity in BMSCs (B), MC3T3-E1 (D) and C2C12 cells (F) was determined by ALP staining or a quantitative ALP assay. (G) Matrix mineralization was examined by Alizarin red S staining of cells cultured for 14 days (BMSCs) or 10 days (MC3T3-E1). *$P < 0.05$ compared with the anti-con-transfected cells without BMP2 treatment; #$P < 0.05$ compared with the indicated sample.

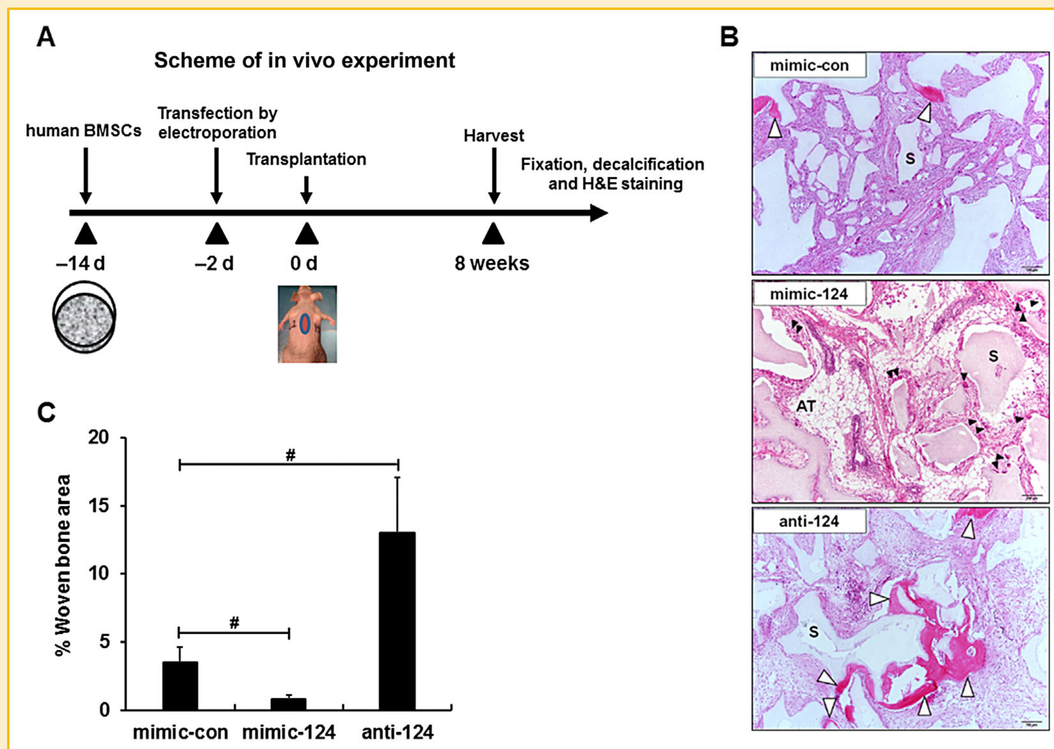

Fig. 6. Overexpression of mimic-124 inhibits but anti-124 enhances ectopic bone formation of human BMSCs. (A) Scheme for assessing human BMSC-based ectopic bone formation. Human BMSCs were amplified, transfected with mimic-con, mimic-124 or anti-124 and incubated for two days. The cells were then harvested, mixed with HA/TCP and transplanted into nude mice. After eight weeks, the transplanted specimens were removed, fixed, decalcified and stained with H&E. (B) Representative image from each sample. S, HA/TCP scaffold; AT, adipocytes; open arrow-head, woven bone; closed arrow head, osteoclast-like multinucleated cells. (C) The percentage of woven bone area per total specimen area was quantified from three sections per specimen (three specimens per each group). The data represent the mean ± SD (# $P < 0.05$ compared with the indicated sample).

novel target of miR-124 by showing that (i) the Dlx3 3′UTR sequence contains a sequence that is complementary to the miR-124 seed sequence; (ii) the overexpression of mimic-124 resulted in the downregulation of Dlx3, whereas the functional inhibition of miR-124 by anti-124 increased the Dlx3 expression levels in mouse BMSCs, MC3T3-E1 and C2C12 cells; and (iii) luciferase reporter assays confirmed that the mimic-124-mediated inhibition of Dlx3 3′UTR reporter activity was abolished by the insertion of mutations into the miR-124 seed binding site.

All Dlx genes are expressed by embryonic stem cells and play roles in the control of craniofacial development and neurogenesis [Merlo et al., 2000; Perera et al., 2004] and in the formation of the distal regions of extending appendages [Merlo et al., 2000]. The regulation of the Dlx5, Dlx3, and Dlx2 genes is well characterized during osteogenic differentiation and bone formation [Merlo et al., 2000; Hassan et al., 2004; Ryoo et al., 2006]. Dlx5 and Dlx3 are the immediate early targets of BMP2 signaling [Hassan et al., 2004]. Dlx5 and Dlx3 activities are required for BMP2-induced osteoblast differentiation and Runx2 expression, indicating a critical role for Dlx genes in the progression of the osteogenic differentiation of MSCs [Hassan et al., 2006; Ryoo et al., 2006]. We also confirmed that BMP2 significantly increased the expression levels of the Dlx5, Dlx3, and Dlx2 genes. The mechanisms through which BMP2 upregulates the expression of these genes have not been clearly elucidated. In the present study, we demonstrated that BMP2 increases the expression levels of Dlx5, Dlx3, and Dlx2 at least partly through the downregulation of miR-124 expression.

A previous study demonstrated that Dlx5 is a direct target of miR-141 and miR-200a, which are downregulated by BMP2 [Itoh et al., 2009]. Our data also showed that BMP2 significantly decreased the expression levels of miR-141, miR-200a, and miR-124. In the present study, although the overexpression of mimic-124 significantly suppressed BMP2-induced Dlx5 expression compared with the mimic-con-transfected cells, the overexpression of miR-124 did not completely overcome BMP2 induction of Dlx5 expression and Dlx5 3′UTR reporter activity. These results indicate that BMP2 simultaneously downregulates multiple miRNAs that target independent sequences in the Dlx5 3′UTR and thereby significantly increases the Dlx5 expression levels. These results further suggest that in addition to transcriptional control mechanisms, epigenetic regulation via miRNA expression is an important mechanism involved in BMP2 induction of Dlx gene expression during the osteogenic differentiation.

Few studies have examined the role of miRNAs during in vivo bone formation or during the development of the osteoblast phenotype. In this study, the overexpression of mimic-124 in BMSCs led to increased adipocyte differentiation at the expense of osteoblastic differentiation and bone formation, whereas the

overexpression of anti-124 significantly enhanced ectopic bone formation. We have previously demonstrated that miR-124 expression is induced by adipogenic stimuli and that the over-expression of mimic-124 enhances the adipogenic differentiation of 3T3-L1 cells [Qadir et al., 2013]. Similarly, the overexpression of mimic-124 in mouse BMSCs significantly increased adipogenic marker gene expression, whereas anti-124 overexpression suppressed adipogenic marker gene expression (Suppl. Fig. 4). These results suggest that the miR-124 expression levels exert important effects on the lineage commitment of MSCs toward the osteogenic versus adipogenic lineages in vivo.

It has been previously reported that miR-124 inhibits osteoclastogenesis by decreasing the expression of NFATc1, a key transcription factor for osteoclastogenesis [Lee et al., 2013b]. Interestingly, our in vivo experiment demonstrated that the overexpression of mimic-124 in human BMSCs significantly increased the number of osteoclast-like multinucleated cells around the HA/TCP scaffold. Because osteoclasts/macrophages originate from hematopoietic cells, it is likely that the overexpression of mimic-124 in BMSCs may increase the expression levels of chemotactic factors or osteoclastogenic factors, thereby increasing the recruitment and/or differentiation of multinucleated osteoclast-like cells. Further studies are necessary to elucidate the molecules involved in this phenomenon.

In conclusion, we demonstrated that BMP2 downregulates the expression of miR-124, which acts as a negative regulator of osteoblast differentiation via the targeting of the Dlx5, Dlx3, and Dlx2 transcription factors. We also showed that the functional inhibition of miR-124 can accelerate osteogenic differentiation and in vivo bone formation. These results suggest the potential application of miR-124 inhibitors to promote the regeneration of localized bone defects. Although we focused on Dlx transcription factors as targets of miR-124, the possibility that miR-124 also targets other genes that are involved in osteogenesis cannot be ruled out because miRNAs usually target multiple genes to regulate specific pathways. The involvement of other potential targets during osteogenesis and bone formation should be elucidated in future studies.

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