Distal-less homeobox 5 is a master regulator of the osteogenesis of human mesenchymal stem cells

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Abstract. Mesenchymal stem cells (MSCs) differentiate into multiple lineages and are a promising source of cells for clinical use. Previously, we found that the gene distal-less homeobox 5 (*DLX5*) is specifically expressed in MSCs with osteogenic potential. Understanding the mechanism of osteogenesis is necessary for successful bone regeneration using MSCs. The aim of this study was to examine the function of the *DLX5* gene in MSCs during osteogenesis (bone development). We analyzed the possible association between *DLX5* expression and osteogenesis-, chondrogenesis- and adipogenesis-related gene expression in different cells isolated from bone marrow and cord blood. Differentiation capacity was assessed by observing morphological changes, monitoring gene expression patterns, and staining with Von Kossa, safranin O, and Oil Red O. Suppression of *DLX5* expression by means of a small interfering RNA (siRNA) downregulated osteogenic markers and reduced the signs of calcium mineralization. Tanshinone IIA is a known small molecule activator of bone morphogenetic protein (*BMP*) signaling. Here, we report that induction of *DLX5* by tanshinone IIA in MSCs enhanced osteogenic differentiation. In addition, we showed that tanshinone IIA (as a mediator of *BMP2* signaling) activates runt-related transcription factor 2 (*RUNX2)* in MSCs and initiates calcium mineralization during osteogenesis. Taken together, these findings indicate that, in MSCs, *DLX5* is a master regulator of osteogenesis. Furthermore, tanshinone IIA may be valuable for stem cell-based therapies of certain bone diseases.

Introduction

Mesenchymal stem cells (MSCs) derived from various sources are valuable in regenerative medicine, including bone repair, because they can differentiate into multiple cell lineages, including osteoblasts, chondrocytes and adipocytes (1,2). MSCs derived from different tissues have similar characteristics, but differ in their molecular profiles and differentiation potential (3).

Recently, MSCs have been applied to bone tissue engineering with a regenerative medicine approach (4). Osteogenic differentiation of MSCs is intricately regulated by multiple transcription factors and various cytokines and hormones (5-7). Previously, we found that distal-less homeobox 5 (*DLX5*), a homeodomain transcription factor encoded by a mammalian homolog of one of the *Drosophila* distal-less (DLL/DLX) genes that regulates the development of multiple cell types, is only expressed in MSCs with osteogenic potential (3). The discovery led us to examine whether *DLX5* is critically involved in the differentiation of MSCs into osteoblasts.

Homeobox-containing genes play a key role as regulators of skeletal development (8). *DLX* genes that encode homeobox-containing transcription factors function in several developmental processes, including osteoblast development (9,10). *DLX5*, which is involved in developing bone, cartilage, and teeth, is a member of the distal-less homeobox domain family (11-14). Overexpression of *DLX5* is known to stimulate bone differentiation, and *DLX5*-null mice exhibit abnormal osteogenesis (15-18). Although numerous studies strongly suggest that *DLX5* is involved in osteogenesis, its functional role in this process is still obscure.

Here, we investigated the regulatory role of *DLX5* in osteogenic differentiation of bone marrow- and cord blood-derived MSCs by examining the effects of *DLX5* inhibition and the expression levels of osteogenesis-associated genes, including bone morphogenetic protein 2 (BMP2) and runt-related transcription factor 2 (*RUNX2*). *BMP2* and *RUNX2* play essential roles in bone development and maintenance by collaborating

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with other signaling molecules; however, they are insufficient to induce osteogenic differentiation (19,20).

The aim of this study was to examine the key regulators of osteogenesis in MSCs. *DLX5* is regulated by *BMP2*, an inducer of osteogenesis (21,22). To investigate the effects of DLX5 on osteogenic differentiation of MSCs, we examined osteogenic factors (*DLX5* and *RUNX2*), chondrogenic factors [*BMP7* and sex determining region Y-box 9 (*SOX9*)], and adipogenic factors [peroxisome proliferator-activated receptor γ (*PPARG*) and CCAAT-enhancer binding protein α (*C/EBPA*)]. We demonstrated that the induction of *DLX5* led to osteoblast differentiation with the expression of several osteoblast markers, whereas the knockdown of *DLX5* expression inhibited the osteogenesis of MSCs. Our data indicate that *DLX5* is the master transcription factor stimulating the osteogenic factor *RUNX2* through *BMP2* signaling during osteogenesis.

Furthermore, we aimed to ascertain whether activation of *DLX5* and/or *BMP2* signaling by certain chemicals could induce osteogenic differentiation in MSCs. Tanshinone IIA is a major active phytochemical derived from phenanthrenequinone, which can be isolated from the roots of *Salvia miltiorrhiza*. It was found to enhance *BMP2*-stimulated differentiation of C2C12 cells into osteoblasts via p38 activation (23). For the first time, we evaluated the effect of tanshinone IIA on the differentiation of MSCs into osteoblasts. This study demonstrated that tanshinone IIA affects osteogenesis from MSCs by augmenting *DLX5*.

These findings may be important for regenerative medicine, facilitating an increase in MSCs with osteogenic potential. Further, tanshinone IIA, as a small-molecule activator of *DLX5* and *BMP* signaling, could be one of the key molecules in *DLX5*-induced osteogenesis of MSCs.

Materials and methods

Cells. Bone marrow and umbilical cord blood were collected from healthy donors after obtaining written informed consent. This study was approved by the Institutional Review Boards of Severance Hospital of Yonsei University Health System, Seoul, Korea. As previously described, mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden) and the MSCs were cultured using the plastic adherence method (24). The cells were cultured at 37°C with 5% CO_2, and the medium [DMEM-low glucose supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) (all from Invitrogen, Carlsbad, CA, USA)] was changed every 3 or 4 days. Tanshinone IIA from Sigma-Aldrich (St. Louis, MO, USA) was used for this study. During cultivation, cells were photographed under an inverted phase microscope (Olympus IX-71; Olympus, Tokyo, Japan) to compare morphologies.

Differentiation. To cause MSCs to differentiate into osteoblasts, chondrocytes, and adipocytes, bone marrow- and cord blood-derived MSCs were cultured in osteogenic induction medium, chondrogenic induction medium, and adipogenic induction medium for 3 weeks (Cambrex, Lonza, MD, USA). Osteoinductive medium-treated cells were used as the control. The medium was changed every 3 or 4 days, and the cells intended for chondrogenic differentiation were treated with 10 ng/ml transforming growth factor (TGF)-β3 (Cambrex) whenever the medium was replaced. For analysis, the induced cells were stained by Von Kossa to confirm osteogenesis, safranin O to confirm chondrogenesis, and Oil Red O to confirm adipogenesis. Images of the stained cells were captured using a phase microscope (Olympus IX-71; Olympus).

RT-PCR. Total RNA was extracted using TRIzol reagent, and standard reverse transcription (RT) was carried out using transcriptase II (both from Invitrogen). RT-PCR was performed using PCR primers (Bioneer, Daejeon, Korea) and annealing temperatures listed in Table I. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. The signal intensity of the product was normalized to the respective *GAPDH* signal intensity. Osteoinductive medium-treated cells were used as control.

Small interfering RNA (siRNA) gene silencing. Specific knockdown of gene expression was performed using siRNA (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) specific for DLX5. Briefly, $2x10^5$ MSCs were transfected with 10 μM of negative control or targeted siRNA according to the manufacturer's protocol. Following incubation for 7 h at 37°C and 5% CO_2, normal growth medium was added. After one day, medium was replaced with fresh normal growth medium. The effect of gene knockdown by siRNA was evaluated by RT-PCR assay. MSCs were treated with *DLX5*-siRNA when medium was replaced for the entire induction period.

Cell viability test. The viability of chemically treated cells was analyzed by the trypan blue exclusion method (Invitrogen). Briefly, cells were seeded at a density of $5x10^4$ cells in 12-well plates (Nunc, Roskilde, Denmark). The next day, 3, 6, 12, 24 or 48 μM of tanshinone IIA was added to the cells. After 3 days, the cells were harvested and trypan blue-stained cells were counted.

Analysis of calcium concentration. Following osteogenic induction, the calcium content of cells was determined using a Calcium (CPC) LiquiColor Test (Stanbio Laboratory, Boerne, TX, USA) according to the manufacturer's instructions. Briefly, the cells were washed with phosphate-buffered saline (PBS; Invitrogen) and 0.5 N HCl was added to the cells. The cells were harvested and transferred to a new tube. After shaking for 3 h with an orbital shaker, the supernatant was transferred to a new tube for analysis. Color and base reagents were added to the supernatant, and then absorbances were detected at 550 nm. The cells cultured in DMEM were used as the control.

Statistical analysis. Quantitative data are expressed as the means ± standard deviation (SD). Statistical comparisons were performed by a Student's t-test and one-way analysis of variance (ANOVA) with post-hoc Bonferroni corrections. The differences were considered statistically significant at $P<0.05$.

Results

Characterization of bone marrow- and cord blood-derived MSCs. All MSCs derived from bone marrow and cord blood

Table I. Primer sequences.

Gene name	Primer sequences (5'-3')	Annealing temperature (°C)	Product size (bp)
BMP2	Forward: CGAGGTCCTGAGCGAGTTCGAG Reverse: TGGCAGTAAAAGGCGTGATACC	60	838
RUNX2	Forward: GACCAGTCTTACCCCTCCTACC Reverse: CTGCCTGGCTCTTCTTACTGAG	58	190
DLX5	Forward: ACCATCCGTCTCAGGAATCG Reverse: ACCTTCTCTGTAATGCGGCC	60	384
GAPDH	Forward: GTGGTCTCCTCTGACTTCAACA Reverse: CTCTTCCTCTTGTGCTCTTGCT	62	210
BMP7	Forward: CCAACGTCATCCTGAAGAAATAC Reverse: GCTTGTAGGATCTTGTTCATTGG	60	271
SOX9	Forward: GCCGGGCAAGGCTGACCTGAAG Reverse: TTCTGGTGGTCGGTGTAGTCGT	62	605
PPARG	Forward: TCTCTCCGTAATGGAAGACC Reverse: GCATTATGAGACATCCCCAC	55	474
C/EBPA	Forward: CCAAGAAGTCGGTGGACAAGAA Reverse: TCATTGTCACTGGTCAGCTCCA	62	145
Osterix	Forward: TAATGGGCTCCTTTCACCTG Reverse: CACTGGGCAGACAGTCAGAA	60	161
Osteopontin	Forward: GAGACCCTTCCAAGTAAGTCCA Reverse: GATGTCCTCGTCTGTAGCATCA	62	354
Type I collagen	Forward: CACAGAGGTTTCAGTGGTTTGG Reverse: GCACCAGTAGCACCATCATTTC	62	191
AP2	Forward: AAGAAGTAGGAGTGGGCTTTGC Reverse: CCACCACCAGTTTATCATCCTC	62	381

BMP2, bone morphogenetic protein; RUNX2, runt-related transcription factor 2; DLX5, distal-less homeobox 5; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SOX9, sex determining region Y-box 9; PPARG, peroxisome proliferator-activated receptor γ; C/EBPA, CCAAT-enhancer binding protein α.

showed a similar spindle-shaped morphology (Fig. 1A). Surface markers of the MSCs were analyzed, and the results showed that all cells exhibited similar immunophenotypic patterns. The cells were positive for CD29, CD44, CD73, CD90 and CD105, all known markers of MSCs, whereas the MSCs were negative for markers of endothelial and hematopoietic cells such as CD14, CD31, CD34, CD45 and CD106 (data not shown). These results confirmed that the cultured cells expressed typical MSC surface markers. To determine their differentiation capacity, the cells were induced to display osteogenic, chondrogenic, or adipogenic phenotypes. Of the MSCs derived from bone marrow and cord blood, one sample of cord blood MSCs (CB-MSC1) did not differentiate into osteoblasts despite a sufficient induction period, whereas the other MSCs exhibited tri-lineage differentiation potential, developing into osteoblasts, chondrocytes and adipocytes (Fig. 1B). Together, these data indicate that not all MSCs with fibroblast-like morphologies and MSC surface proteins have tri-lineage differentiation capacities.

Osteogenesis and DLX5 expression of MSCs. To investigate osteogenic molecular profiles associated with morphological changes, we performed an RT-PCR analysis of specific osteogenesis markers, namely *BMP2*, *RUNX2* and *DLX5*, during the induction of bone marrow- and cord blood-derived MSCs from different donors. *BMP2*, *RUNX2* and *DLX5* were expressed in all MSCs that differentiated into osteoblasts, regardless of the induction period (Fig. 2). However, CB-MSC1, which did not differentiate into an osteogenic phenotype, did not express *BMP2* and *DLX5* at any time in the induction environment. Interestingly, *RUNX2* was independently expressed in all MSCs, regardless of their osteogenic potential (Fig. 2). These results coincide with previous data, confirming *DLX5* as a marker for the osteogenic potential of MSCs (3). Based on these results, we noted that *DLX5* with *BMP2* signaling may be the only critical factors for osteogenesis of MSCs.

Effect of DLX5 knockdown on the differentiation potential of MSCs. To examine the role of *DLX5* in the tri-lineage
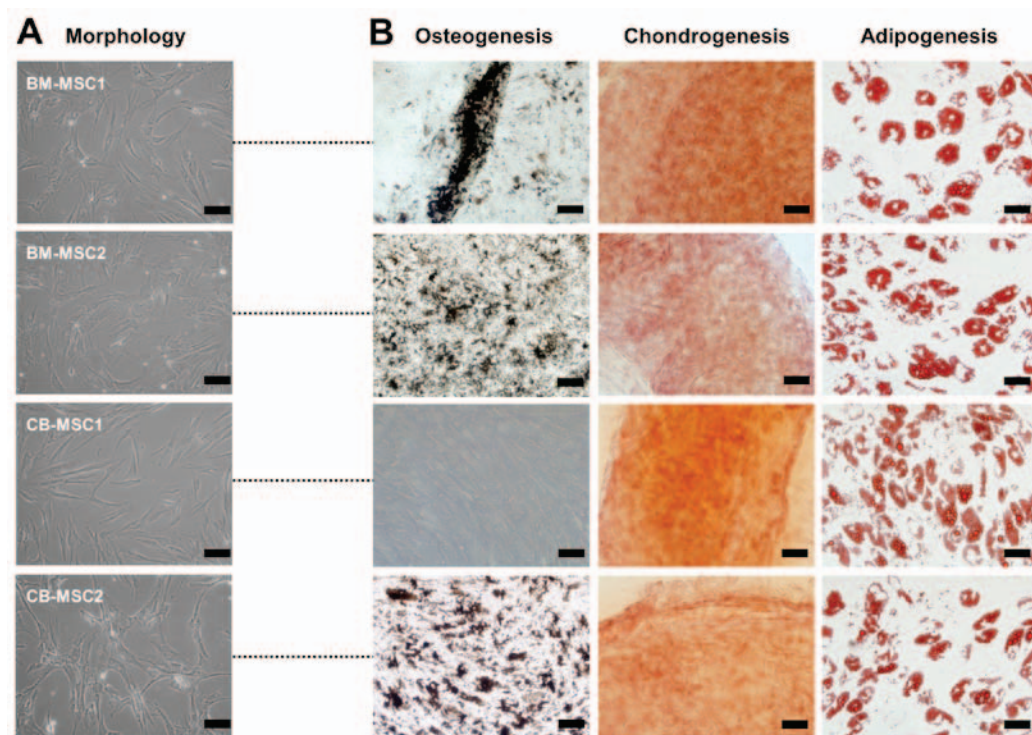
Figure 1. Morphology and differentiation potential of mesenchymal stem cells (MSCs). (A) Phase contrast images of MSCs derived from bone marrow and cord blood (scale bar, 100 μm; magnification, x200). (B) Differentiation capacity was examined after induction. Osteogenesis was verified by Von Kossa staining (scale bar, 100 μm; magnification, x200). Chondrogenesis was investigated by safranin O staining (scale bar, 100 μm; magnification, x200). Adipogenesis was examined by Oil Red O staining (scale bar, 50 μm; magnification, x400).
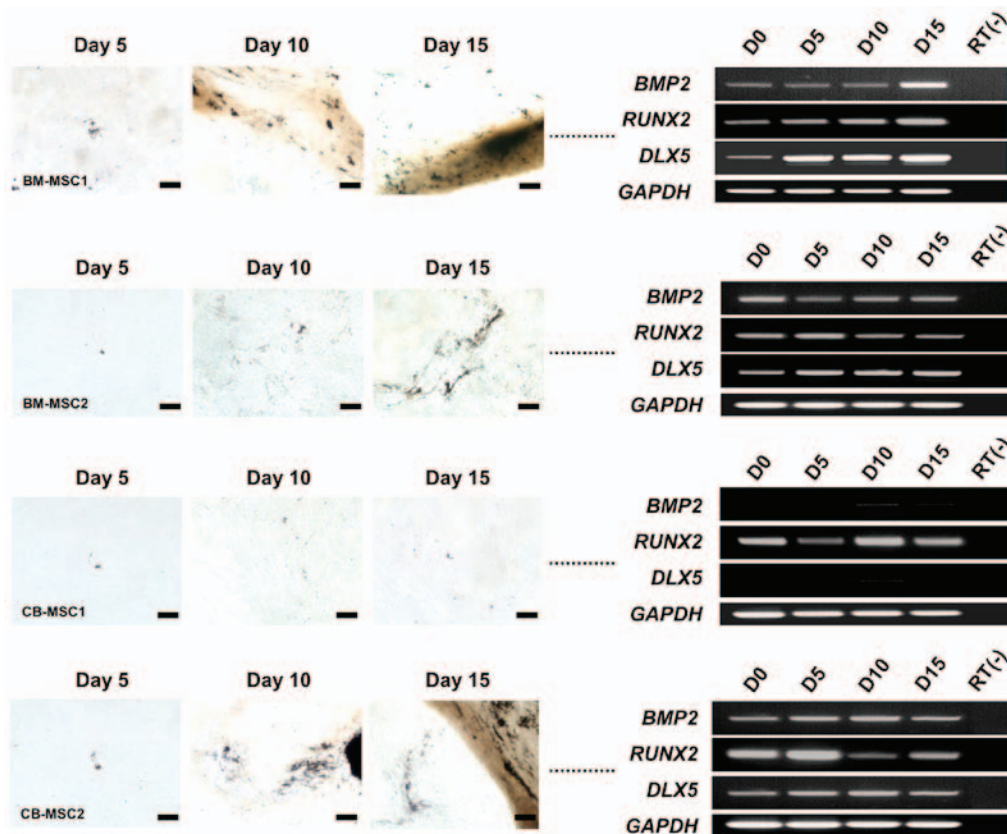

Figure 2. Time course images of osteogenic differentiation and expression of osteogenesis-related markers. Top panels: images of BM-MSC1 induced by osteogenic medium, and RT-PCR analysis of osteogenesis-associated markers in BM-MSC1. Second row: images of BM-MSC2 differentiated by osteogenic medium, and RT-PCR analysis of osteogenesis-related markers in BM-MSC2. Third row: images of CB-MSC1 induced by osteogenic medium, and RT-PCR analysis of osteogenesis-associated markers in CB-MSC1. Lower panels: images of CB-MSC2 induced by osteogenic medium, and RT-PCR analysis of osteogenesis-related markers in CB-MSC2. Osteogenesis was evaluated by Von Kossa staining (scale bar, 100 μm; magnification, x200).
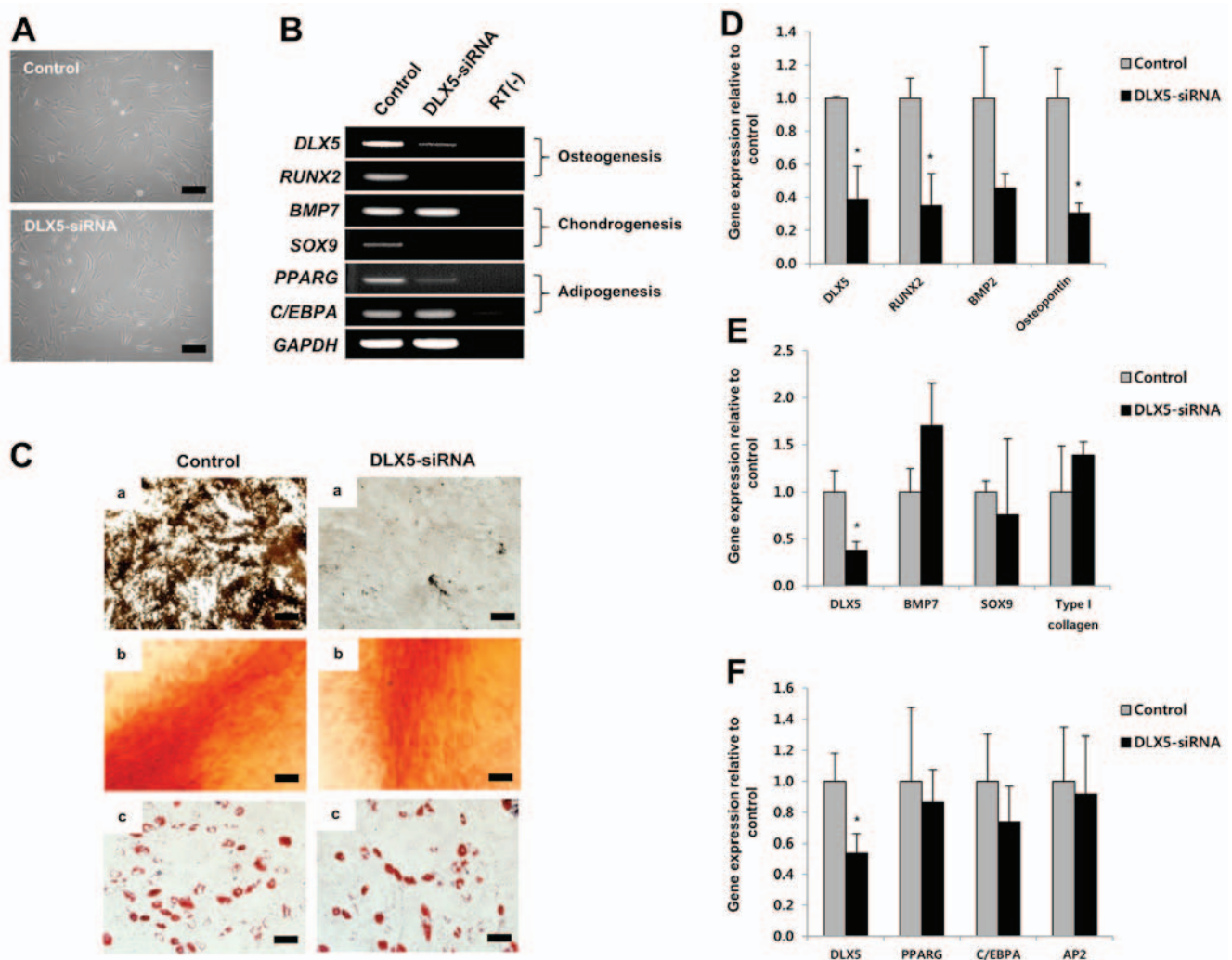
Figure 3. Changes in mesenchymal stem cells (MSCs) following knockdown of distal-less homeobox 5 (DLX5). (A) Phase contrast images before and after silencing of *DLX5* (scale bar, 200 µm; magnification, x100). (B) RT-PCR analysis of osteogenesis-, adipogenesis- and chondrogenesis-associated genes in the control and *DLX5* siRNA-transfected MSCs. Decreased osteogenic differentiation of MSCs following knockdown of *DLX5*. (C) Inhibition of *DLX5* expression prevents osteogenesis of MSCs. The differentiation capacity of MSCs was analyzed after induction. (a) Osteogenesis assessed by Von Kossa staining. (b) Chondrogenesis assessed by safranin O staining. (c) Adipogenesis assessed by Oil Red O staining (scale bar, 100 µm). Relative mRNA expression levels of differentiation-associated markers in the control and *DLX5*-siRNA-transfected MSCs after (D) osteogenic induction, (E) chondrogenic induction, and (F) adipogenic induction. $^{*}P<0.05$.

differentiation of MSCs, we employed siRNA-mediated knockdown of *DLX5*, using *DLX5*-expressing cells. The morphologies of cultured MSCs before induction were unaffected, compared to those of the control, by short-term treatment with *DLX5*-siRNA (Fig. 3A). RT-PCR results showed that *DLX5*-siRNA substantially decreased expression of the *DLX5* gene and completely silenced the osteogenic marker gene *RUNX2* and chondrogenic marker gene *SOX9*. *C/EBPA* of the adipogenic marker genes was unaffected by *DLX5*-siRNA treatment, while *PPARG* expression was slightly decreased (Fig. 3B). These results indicate that osteogenesis of MSCs can be markedly affected by *DLX5*-siRNA knockdown.

We next performed a differentiation assay in the presence of *DLX5*-siRNA. Surprisingly, the osteogenic capacity of MSCs treated with *DLX5*-siRNA was significantly decreased, whereas chondrogenic and adipogenic capacities were similar, relative to that of the control, although MSCs did not express the *SOX9* gene following *DLX5*-siRNA treatment (Fig. 3C). We then analyzed gene expression levels related to tri-lineage

differentiation by RT-PCR after induction. Expression of the following genes was evaluated: *DLX5*, *RUNX2*, *BMP2* and *osteopontin* for osteogenesis; *DLX5*, *BMP7*, *SOX9* and type I collagen for chondrogenesis; and *DLX5*, *PPARG*, *C/EBPA* and *AP2* for adipogenesis. The levels of *RUNX2* and *osteopontin* gene expression were significantly decreased relative to the control by inhibition of *DLX5* (Fig. 3D), whereas no significant differences were detected in the expression of chondrogenesis- (Fig. 3E) or adipogenesis- (Fig. 3F) related genes. Relative gene expression was normalized to that of *GAPDH*, the internal control. These results strongly suggest that *DLX5* is the most powerful and specific transcription factor for osteogenic differentiation.

Tanshinone IIA induces DLX5 through BMP2 signaling in MSCs. Tanshinone IIA, a major active phytochemical, is involved in bone metabolism. It has a wide range of biological activities, including anti-inflammation and anti-oxidation (25-27). Moreover, tanshinone IIA is known to
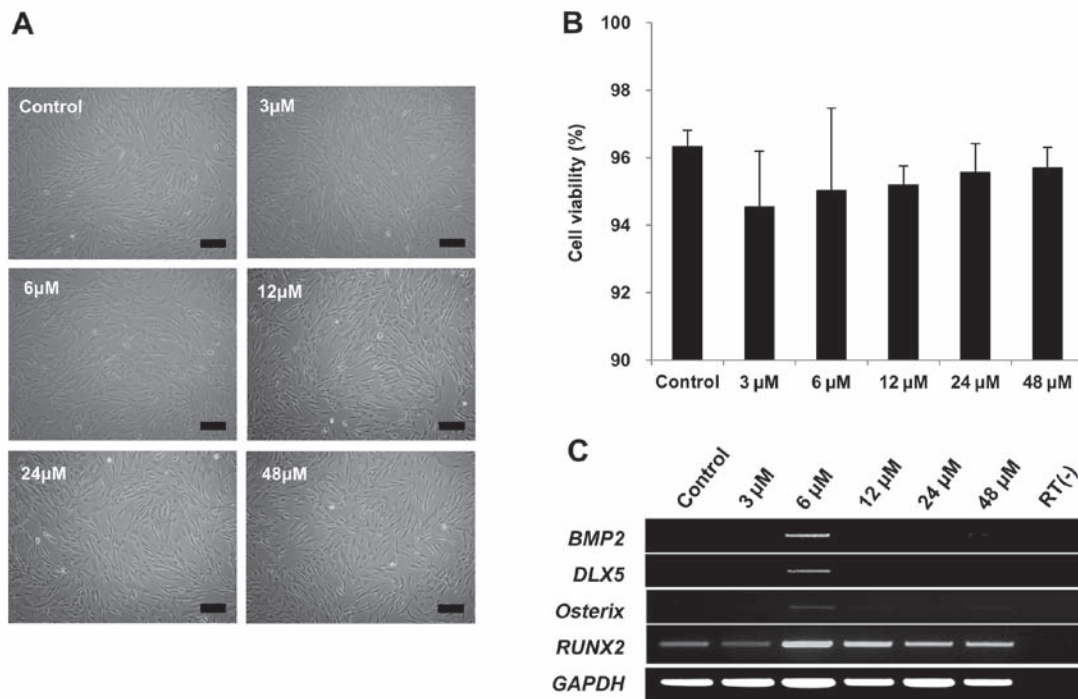
Figure 4. Effect of tanshinone IIA on osteogenesis. (A) Mesenchymal stem cells (MSCs) treated with tanshinone IIA were photographed after 3 days (scale bar, 200 μm; magnification, x100). (B) Cell viability was determined by trypan blue staining. The cell viability of the tanshinone IIA-treated MSCs was >90%. (C) Induction of osteogenesis-associated genes by tanshinone IIA treatment. Treatment only with 6 μM tanshinone IIA induced *BMP2*, distal-less homeobox 5 (DLX5) and *osterix* genes.

enhance BMP-2 stimulation of cells to differentiate into osteoblasts (23). Ultimately, stimulation by tanshinone IIA induces osteogenesis via regulation of osteogenic factors, including *BMP2* and *DLX5*.

Therefore, we tested whether tanshinone IIA could induce *DLX5* in *DLX5* not-expressing MSCs. Cell morphologies were not affected by 3, 6, 12, 24 or 48 μM of tanshinone IIA treatment (Fig. 4A). In addition, cell viability of >90% was maintained with tanshinone IIA treatment (Fig. 4B). Remarkably, *BMP2*, *DLX5* and *osterix* genes were only induced in response to 6 μM of tanshinone IIA treatment, as early as after 3 days of cultivation, indicating the activation of *DLX5* by the *BMP2* pathway. However, *RUNX2* was similarly expressed (Fig. 4C). Taken together, these results show that tanshinone IIA can induce DLX5, as well as the most prominent factors of osteogenesis.

Tanshinone IIA enhances osteogenesis of MSCs by inducing DLX5 with BMP2. We next treated *DLX5* not-expressing MSCs with 6 μM tanshinone IIA to induce osteogenesis. After tanshinone IIA treatment, we did not find any differences in our morphological investigation. Subsequently, the effect of tanshinone IIA on the osteogenic potential of MSCs was evaluated by analyzing the expression levels of genes associated with osteogenic differentiation and by visualizing the staining of induced cells. Tanshinone IIA significantly induced *BMP2* and *DLX5*, as well as upregulated *RUNX2* genes involved in osteogenesis. The expression of *SOX9*, which is involved in chondrogenesis, was similarly upregulated, compared to that of the control, despite tanshinone IIA treatment, whereas *BMP7* expression was decreased (Fig. 5A). Interestingly, expression of *C/EBPA*, a transcription factor for adipogenesis,

was completely inhibited by tanshinone IIA (Fig. 5A). The differentiation assay revealed that tanshinone IIA specifically enhanced osteogenesis of MSCs (Fig. 5B). This finding was confirmed by calcium deposition assay, as shown in Fig. 5C. Furthermore, tanshinone IIA-mediated enhancement of *DLX5* through the induction of *BMP2* upregulated mRNA expression of *RUNX2* and *osteopontin* during osteogenic differentiation of MSCs (Fig. 5D). As shown in Fig. 5A, we again confirmed that the expression of *BMP7* was decreased significantly during chondrogenesis (Fig. 5E), and that *C/EBPA* expression was suppressed by tanshinone IIA during adipogenic differentiation (Fig. 5F). Relative gene expression in the differentiated MSCs was normalized to *GAPDH*, the internal control. Surprisingly, tanshinone IIA-treated MSCs differentiated into chondrocytes and adipocytes despite the suppression of *BMP7* and *C/EBPA* genes (Fig. 5B). Taken together, these results indicate that tanshinone IIA induces osteogenesis in *DLX5* not-expressing MSCs by activating *DLX5* through *BMP2* expression.

Discussion

MSCs derived from various tissues have become a preferred cell type in the field of regenerative medicine due to their plastic and immunosuppressive properties (28). Although stem cells hold great promise for future therapeutic applications, clinical applications using these cells have been stymied by an insufficient understanding of stem cell biology, including the complex genetic processes in these cells. Therefore, further characterization of stem cells via diverse approaches such as genomics and proteomics will be critical for a better understanding and utilization of stem cells.
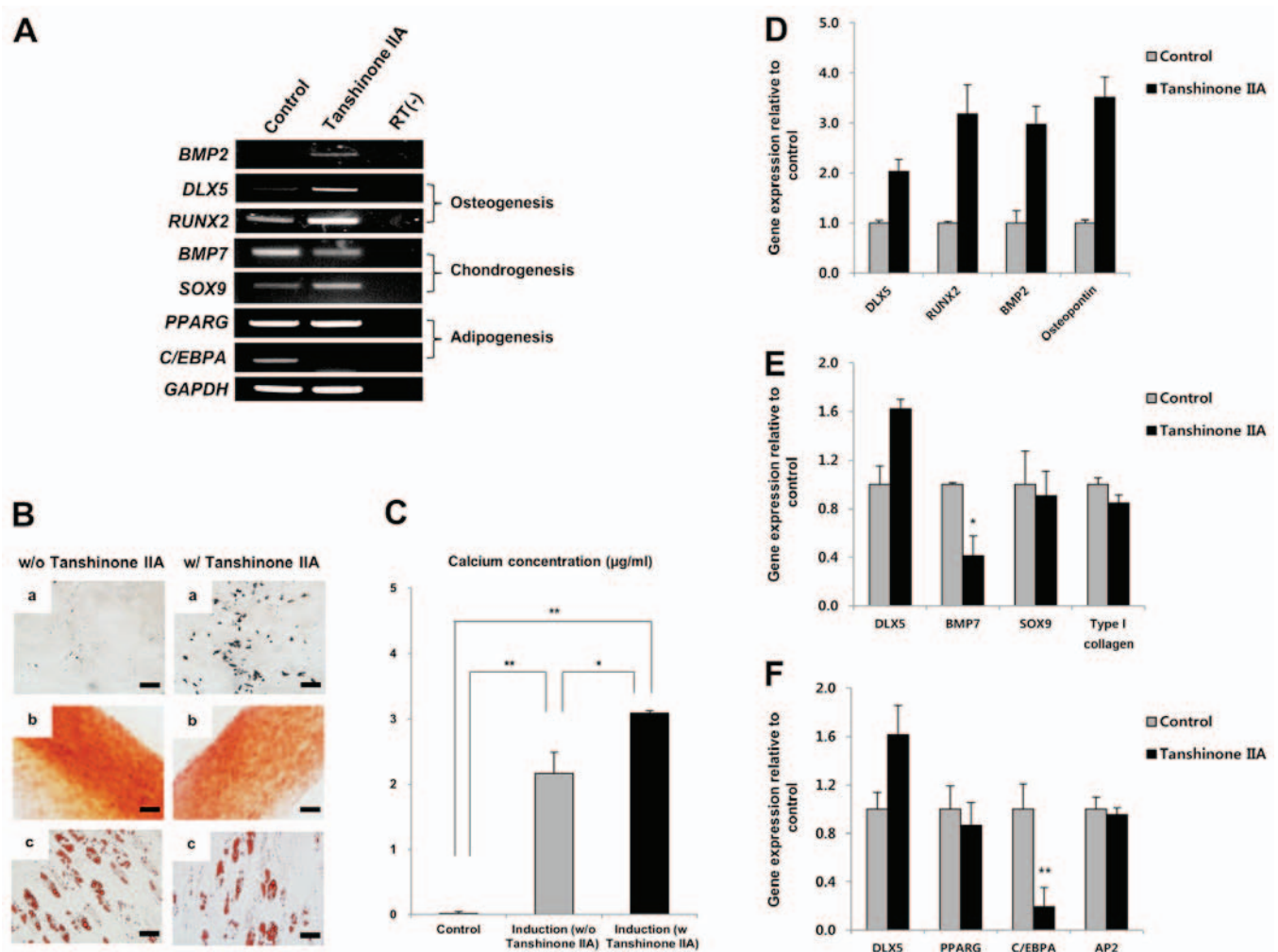
Figure 5. Changes in mesenchymal stem cells (MSCs) following tanshinone IIA treatment. (A) RT-PCR analysis of osteogenesis-, chondrogenesis- and adipogenesis-associated genes in the control and tanshinone IIA-induced MSCs. Increased osteogenic differentiation of MSCs by tanshinone IIA. (B) Induction of distal-less homeobox 5 (DLX5) expression enhanced osteogenesis of DLX5 not-expressing MSCs. The differentiation capacity of the cells was analyzed after induction. (a) Osteogenesis assessed by Von Kossa staining (scale bar, 50 µm). (b) Chondrogenesis assessed by safranin O staining (scale bar, 100 µm). (c) Adipogenesis assessed by Oil Red O staining (scale bar, 50 µm). (C) Calcium concentrations were measured in triplicate using a Calcium LiquiColor test. Relative mRNA expression levels of differentiation-associated markers in the control and DLX5-induced MSCs after (D) osteogenic induction, (E) chondrogenic induction, and (F) adipogenic induction. *P<0.05 and **P<0.01.

Previous research on MSCs from different sources has documented their variable differentiation potential and has shown that this variation in osteogenic potential depends on DLX5 gene expression (3). Consistent with the results of a previous study, DLX5 expression was not detected in MSCs that did not have the capacity to differentiate into osteoblasts. In addition, BMP2 gene expression was not observed in this study when DLX5 not-expressing MSCs were maintained in an osteogenic environment. Differentiation of stem cells is a complex process governed by various genetic networks, and the biological functions of genes associated with MSC differentiation remain unclear. In the present study, we aimed to investigate the precise role of the DLX5 gene during the osteogenesis of MSCs, including whether the DLX5 gene is important for initiating osteogenesis and whether the gene is sufficient to completely drive osteogenesis from MSCs.

DLX5, a member of the DLX family of homeobox genes, is known to be a key regulator of differentiation involved in developing skeletal elements and of osteogenesis and chondrogenesis in the formation of hard tissues (29). Several studies suggest that DLX5 acts as a modulator of osteogenesis in various cell types (18,30). However, the mechanism underlying osteogenic differentiation, including the role of DLX5, is still controversial, especially in MSCs. In this study, we used MSCs derived from bone marrow and cord blood, less than 5 passages, and expressing and/or not expressing the DLX5 gene to identify the effects, including the effects on genes activated and inactivated by DLX5 in the course of differentiation. In order to investigate the role of DLX5, we profiled morphological and gene expression changes associated with osteogenesis of MSCs. As mentioned above, DLX5 not-expressing MSCs without BMP2 expression failed to differentiate into osteoblasts. However, RUNX2 was consistently expressed during osteogenic induction, irrespective of the expression of BMP2 and DLX5. To further examine the effect of DLX5 on osteogenesis, siRNA, which targets the DLX5 gene, was used to inhibit endogenous DLX5 expression in MSCs. Knockdown of DLX5 using siRNA did not alter the morphology and the proliferation rate of the cells. Seventy-two hours after siRNA transfection, RUNX2 and SOX9 genes specific for osteogenesis and chondrogenesis,
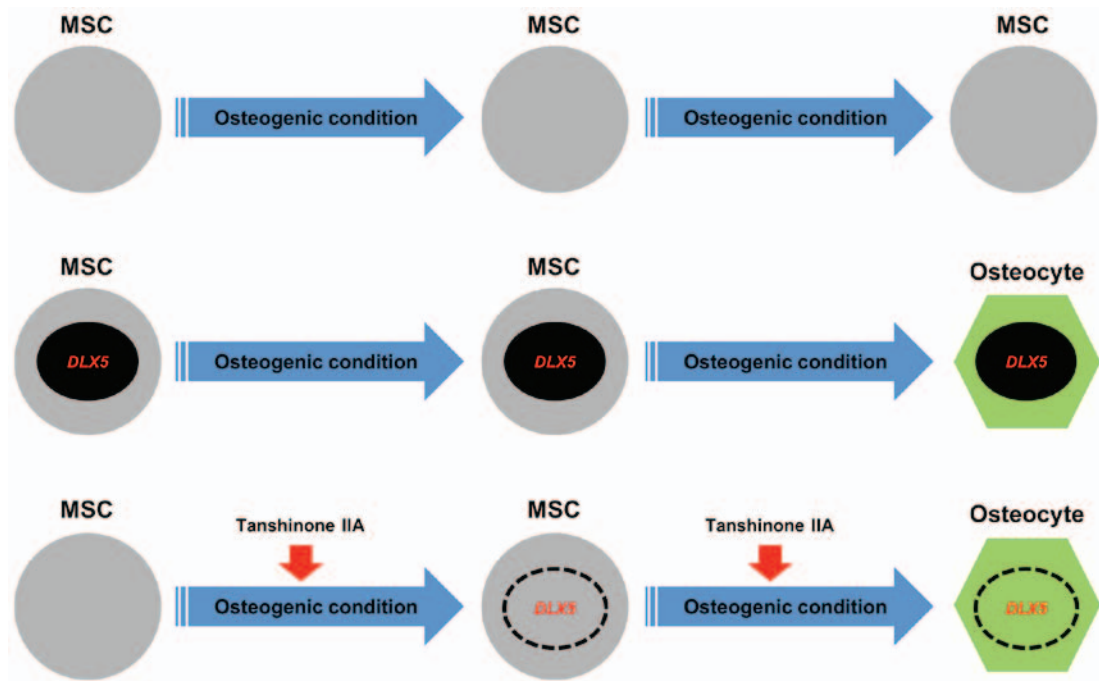
Figure 6. Model summarizing osteogenesis in mesenchymal stem cells (MSCs). Distal-less homeobox 5 (*DLX5*) and *DLX5* inducer, tanshinone IIA, promote the differentiation of MSCs into osteoblasts by upregulating osteogenesis-related genes.

respectively, were inhibited in the cultured cells. In addition, osteogenic differentiation of MSCs was significantly suppressed by *DLX5*-siRNA, with a decrease in *osteopontin* gene expression compared to the control. In contrast, the chondrogenic and adipogenic potential of these cells was unaffected by *DLX5*-siRNA, as proteoglycans for chondrogenesis and neutral lipids for adipogenesis were similarly detected by immunohistochemical staining in control cells exposed to inductive conditions. These results indicate that *DLX5* drives the osteogenic differentiation program in MSCs.

Tanshinone IIA is a major active phytochemical that is isolated from the roots of *S. miltiorrhiza* and enhances BMP2-stimulated differentiation of myoblasts into osteoblasts (23). However, there is little research on the effects of tanshinone IIA on the osteogenic differentiation of MSCs. To examine the effects of tanshinone IIA on MSCs, *DLX5* not-expressing cells were cultured with tanshinone IIA. Our data showed that *DLX5* induced by tanshinone IIA activated osteogenic marker genes, including *osterix*, *RUNX2* and *osteopontin*, in cooperation with *BMP2*, with cell morphologies that remained similar to control cells, whereas tanshinone IIA suppressed *BMP7* gene (chondrogenesis) and *C/EBPA* gene (adipogenesis) expression. These results are in line with previous studies showing that *DLX5* plays a role in *BMP2*-induced osteogenesis through upregulation of the *RUNX2* gene, and that it functions as part of the BMP signaling pathway (21,31). In addition, these results suggest that tanshinone IIA is involved in the *BMP2* signaling pathway and *DLX5*-induced osteogenic differentiation. Functional validation with tanshinone IIA was carried out by differentiation assays and PCR analysis. MSCs with strongly upregulated *DLX5*, *RUNX2*, *BMP2* and *osteopontin* genes following tanshinone IIA treatment differentiated into osteoblasts and showed significantly increased calcium deposition compared to *DLX5* not-expressing cells. However,

a higher concentration of tanshinone IIA (6 μM) decreased the osteogenic capacity of MSCs, indicating that osteogenic differentiation following *DLX5* induction in treated cells is tanshinone IIA concentration-dependent (data not shown). Additionally, MSCs treated with tanshinone IIA differentiated into chondrocytes and adipocytes despite inhibition of *BMP7* and *C/EBPA*, indicating that these genes may not be essential factors for differentiation. Furthermore, *DLX5* may play a role as an osteogenesis determinant through the upregulation of *RUNX2* and the downregulation of *BMP7* and *C/EBPA*.

Here, we showed that *DLX5* is a specific target of *BMP2*-induced osteogenesis of MSCs, demonstrating that *DLX5* and *BMP2* can contribute to *RUNX2*-independent regulation of osteogenesis. This indicates that *RUNX2* induction is not mediated by *BMP2* and *DLX5* in MSCs as previously reported (32). Additionally, we confirmed that *RUNX2* is not essential for the induction of an osteogenic lineage of MSCs, indicating that *RUNX2* may function in concert with *DLX5* to induce osteogenic differentiation by regulating the expression of osteogenesis-specific markers such as *osteopontin*. These findings are in agreement with previous results showing that *DLX5* plays an important role in the activation of osteogenesis by regulating BMP-induced *RUNX2* (22). Moreover, we showed that tanshinone IIA is capable of stimulating *DLX5* expression with *BMP2*, resulting in osteogenic differentiation of MSCs. To the best of our knowledge, we showed for the first time that tanshinone IIA can be used in place of *DLX5* to induce differentiate of MSCs into osteoblasts. Fig. 6 shows a schematic model that summarizes the osteogenesis of MSCs by the induction of the *DLX5* gene using tanshinone IIA. Our findings contribute to the development of effective bone regeneration therapies for the treatment of bone diseases. Furthermore, tanshinone IIA is a chemical compound that may be used for the treatment of bone diseases; however,

our *in vitro* results require *in vivo* validation. Additional investigations are required for a deeper understanding of the upstream and downstream signaling pathways related to other osteogenesis-related factors.

In conclusion, our data showed that *DLX5* plays a role as a master transcription factor in osteogenic differentiation, and that tanshinone IIA, coincident with the induction of *BMP2*, synergistically induces osteogenesis by targeting *DLX5*.

Acknowledgements

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