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PDGF-regulated miRNA-138 inhibits the osteogenic differentiation of mesenchymal stem cells

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ABSTRACT

Differentiation-specific microRNAs may play a critical role in MSC differentiation, and they can be altered by PDGF signaling. We propose that PDGF modulates MSC differentiation by regulating microRNA expression. Therefore, we investigated whether PDGF treatment could alter the expression profile of miRNAs in MSCs. Furthermore, we assessed the osteoblast phenotype of MSCs after inducing osteogenic differentiation. We found that PDGF treatment significantly inhibits the osteogenic differentiation of MSCs and that miR-138 gene transcription is controlled by PDGF signaling. Our results confirm that miR-138 inhibits the osteogenic differentiation of MSCs and suppresses the phosphorylation of FAK, ERK1/2, and Runx2. Furthermore, our study clearly demonstrates that downregulation of Runx2 by miR-138 is critical for the PDGF-mediated inhibition of osteogenic differentiation of MSCs. These findings indicate that inhibition of miR-138 function in MSCs, either by treatment with anti-miR-138 or by overexpression of the miR-138 target sequence (miRNA sponge), could represent a potential therapeutic strategy for the treatment of bone homeostasis disorders caused by activation of the PDGF pathway.

1. Introduction

Bone homeostasis requires a balance between bone formation by osteoblasts and bone absorption by osteoclasts to maintain a constant bone volume. This balance is altered dramatically in physiological and pathological conditions, including injury and metabolic diseases. Multipotent mesenchymal stromal cells (MSCs) in the bone marrow are important for bone homeostasis because they actively proliferate, migrate, and undergo osteogenic differentiation in response to different stimuli [1,2]. MSCs residing within their native environment—the bone marrow stem cell niche—receive biochemical stimuli from many different cytokines and growth factors that likely influence the differentiation of MSCs into bone cells [1–3]. Among the growth factor receptors, membrane-bound tyrosine kinase-type receptors for growth factors such as platelet-derived growth factor (PDGF) activate diverse signaling molecules that include mitogen-activated protein kinases, signal transducer and activator of transcription proteins, and Smads (Sma- and Mad-related proteins). Increasing evidence has shown that these signaling molecules strongly affect the bone morphogenetic protein signaling pathways that induce the osteogenic differentiation of MSCs [3,4].

By activating its cognate receptor, PDGF can regulate many cellular processes, including cell proliferation, differentiation, transformation, migration, and metastasis [5,6]. There are five different PDGF isoforms that activate cellular responses through two different receptors. The PDGF family consists of four ligands: PDGF A, B, C, and D. These ligands mainly form homodimers, although the heterodimer AB can also form [7]. The known receptors for these ligands include the alpha (PDGFRA) and beta (PDGFRB) receptors [6]. PDGF ligands and receptors are upregulated during tissue remodeling in bone fractures; PDGF-A and PDGF-B, which are synthesized by osteoblasts, chondrocytes, and MSCs, are assumed to accelerate the repair process by recruiting MSCs to lesion sites [5]. Recently, PDGF has been found to play important roles in the osteogenic differentiation of MSCs [3]. Treatment of MSCs cultured under mineralizing conditions with purified PDGF-BB protein resulted in significant inhibition of the expression of the mature osteoblast marker proteins alkaline phosphatase (ALP), osteocalcin, and type I collagen, while it inhibited the mineralization of pre-osteocytic cell lines [6,8–12]. However, more in-depth mechanistic studies are required to understand how PDGF regulates the processes involved in the osteogenic differentiation of MSCs.

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Emerging evidence suggests that microRNAs (miRNAs), which cause either translational repression or degradation of their target miRNAs, regulate the expression of genes that are fundamental to the osteogenic differentiation of MSCs [13,14]. Recent studies have shown that miR-138 plays a pivotal role in bone formation *in vivo* by negatively regulating osteogenic differentiation in human mesenchymal stem cells (hMSCs), thereby reducing ectopic bone formation [15]. In undifferentiated MSCs, miR-138 expression suppresses focal adhesion kinase (FAK) translation, thereby decreasing the phosphorylation of FAK and its downstream target ERK1/2. Inhibition of this signaling cascade results in decreased phosphorylation of Runt-related transcription factor 2 (Runx2), decreased expression of osteocalcin, and subsequent suppression of MSC differentiation into osteoblasts. Recently, PDGF signaling has been found to alter the expression profile of miRNAs, thereby causing the reversal of osteogenic differentiation [16].

Although the role of miRNAs in MSCs has been documented, few studies have reported the cellular consequences of targeted re-expression of specific miRNAs. Therefore, we hypothesized that PDGF signaling might inhibit the osteogenic differentiation of MSCs via regulation of miRNA biogenesis. In this study, we investigated whether PDGF treatment could alter the expression profile of miRNAs in hMSCs, and we assessed the cellular consequences of this treatment. Our data demonstrate that PDGF exerts its effects on the osteogenic differentiation of MSCs by inducing miR-138 expression, which results in decreased Runx2 phosphorylation.

2. Materials and methods

2.1. Cell culture and induction of osteogenic differentiation

Telomerase-immortalized bone marrow-derived hMSCs were cultured in regular growth medium (minimum essential medium (MEM), 10% FBS, antibiotics, and glutamax I; GIBCO Invitrogen Corporation) as previously described [17]. Osteogenesis was induced using differentiation medium (growth medium supplemented with 10^{-8} M dexamethasone, 0.2 mM L-ascorbic acid, 10 mM β-glycerophosphate, and 10 mM 1,25-dihydroxyvitamin D3). The osteoblast phenotype was evaluated by ALP and Alizarin Red staining as previously described [18].

2.2. Reagents and antibodies

Recombinant human PDGF-BB was purchased from R&D Systems. Chemically synthesized miRNA inhibitors, mimics, and scrambled controls were obtained from Dharmacon or Ambion. Cells were seeded at a density of 100,000 cells/ml and transfected using Oligofectamine (Invitrogen) at the indicated concentrations. Antibodies against alkaline phosphatase, osteocalcin, and RUNX2 were purchased from Santa Cruz. Antibodies against p-RUNX2 and GAPDH were acquired from Abcam. The FITC-conjugated anti-mouse IgG antibody used for osteocalcin and RUNX2 staining was purchased from Santa Cruz. The miRNA isolation kit and Taqman miRNA assays were purchased from Ambion and Applied Biosystems, respectively.

2.3. Transfection of miRNA mimics and specific anti-miRNAs

All hMSCs were seeded at a density of 1×10^5 cells per well in six-well plates and transfected with miR-138 mimics or scrambled miRNA controls at a final concentration of 30 nM using Oligofectamine (Invitrogen). The hMSCs were transfected with anti-miR-138 (miRNA inhibitor) or an anti-miRNA control at a final concentration of 100 nM using the Dharma FECT3 transfection reagent (Dharmacon). Three days after the initial transfection, the cells were split and subsequently transfected every 3 days with miR-138, miRNA inhibitors, or controls for the indicated times.

2.4. Intracellular ALP activity

Intracellular ALP activity was measured colorimetrically with an alkaline phosphatase colorimetric assay kit (Abcam, Cambridge, UK) that employs p-nitrophenyl phosphate (pNPP) as a phosphatase substrate. Cells were lysed in deionized distilled water using three freeze–thaw cycles. A 30-μL aliquot of each lysate was added to a 96-well plate with 50 μL of assay buffer and 50 μL of pNPP. The samples were shielded from direct light and incubated at room temperature for 1 h. Subsequently, 20 μL of stop solution (3 N NaOH) was added to the wells, and the plate was read at 405 nm using a microplate reader (Wallac Victor3 1420 Multilabel Counter). The DNA content of the cell lysates was measured using the fluorescent dye bisbenzimide H 33258 (Hoechst 33258, Sigma). The ALP activity in each sample was normalized to the corresponding total DNA content.

2.5. Quantitative real-time PCR

RNA was purified using RNeasy (Qiagen) or miRVANA (Ambion) kits with DNase digestion on RNeasy columns. Complementary DNA (cDNA) was generated using the High-Capacity cDNA Reverse Transcription Kit (Roche). An iQ5 PCR machine (Bio-Rad) was used to obtain quantitative measurements of the changes in gene expression levels. The PCR cycling conditions were as follows: 94 °C for 3 min followed by 40 cycles of 94 °C for 15 s, 60 °C for 20 s, and 72 °C for 40 s. Real-time PCR was used to quantify the mRNA expression levels. All reactions were performed in duplicate. The delta-delta cycle threshold (ddCt) values were calculated based on the average normalized expression for each gene. Taqman miRNA assays were used to quantify miR-138 and pri-miR-138 expression levels, and these values were normalized to the average of the values obtained for RNU6B.

2.6. Western blot analyses

Western blot analyses were performed using total cell lysates, which were obtained by lysing the cells in RIPA buffer containing 50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, and 1 mM protease inhibitor cocktail. Total cell lysates were separated on SDS–PAGE gels, transferred to PVDF membranes (Millipore), immunoblotted with antibodies, and visualized using an enhanced chemiluminescence detection system (Amersham Biosciences). Protein expression was quantified by densitometry using ImageJ gel analysis software (rsbweb.nih.gov/ij). All values were normalized to GAPDH.

2.7. Immunofluorescence microscopy

Briefly, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked with 10% goat serum. The cells were then incubated for 1 h with antibodies against RUNX2 (1:50) or osteocalcin (1:50) in 5% goat serum. After this incubation, the cells were stained for 1 h with a FITC-conjugated secondary antibody (1:100). The cells were subsequently viewed by fluorescence microscopy, and captured images were analyzed using Advanced Spot software (Diagnostic Instruments, Sterling Heights, MI).

2.8. Statistical analyses

The data presented are the average (with standard error) of at least three experiments performed in triplicate. We performed an analysis of variance followed by Tukey's multiple comparison test or Student's *t* test using SPSS 15.0. *P* values of 0.05 were considered significant and are indicated with asterisks.

3. Results

3.1. PDGF inhibits the osteogenic differentiation of MSCs

We first examined whether PDGF inhibits the osteogenic differentiation of MSCs. The hMSCs were treated with vehicle or PDGF-BB (50 ng/ml), and standard osteoblast induction medium was used to induce osteogenic differentiation. The osteoblast phenotype was confirmed by alkaline phosphatase and Alizarin Red staining (Fig. 1A). The results indicate that PDGF significantly inhibits the course of osteogenic differentiation after induction. We subsequently confirmed these results using quantitative analyses of alkaline phosphatase activity 5, 10, and 15 days after inducing osteogenesis. As shown in Fig. 1B, the ALP activity in PDGF-treated cells was significantly suppressed compared to that observed in vehicle-treated cells. Using real-time PCR (Fig. 1C), we also found that PDGF treatment significantly downregulated the expression of genes associated with osteogenic differentiation in hMSCs, including ALP, osteocalcin, and RUNX2, 15 days after osteoblast induction. Specifically, PDGF decreased the level of RUNX2 gene expression by 78% compared to vehicle-treated controls. This change in expression was concomitant with the loss of RUNX2 expression as observed by immunofluorescence staining (Fig. 1A). These results suggest that PDGF inhibits the osteogenic differentiation of MSCs.

3.2. Regulation of miRNA-138 expression by PDGF treatment in MSCs

Recent studies have shown that miR-138 plays a pivotal role in bone formation *in vivo* by negatively regulating osteogenic differentiation in hMSCs and thereby reducing ectopic bone formation [15]. Real-time PCR was used to determine whether PDGF treatment could alter miRNA expression [16] in MSCs after osteoblast induction. We found that PDGF-BB treatment led to the enrichment of several miRNAs in MSCs 15 days after the induction of osteogenesis. Of these miRNAs, miR-138 was the most significantly enriched (Fig. 2A), suggesting that PDGF signaling may activate miR-138 expression during osteoblast induction. The expression of miR-138 was examined after PDGF-BB treatment in MSCs between 0 and 15 days after osteogenesis was induced. After 2 days of treatment with PDGF, miR-138 was induced 4.9-fold (Fig. 2B). We also observed a robust induction of pri-miR-138 as early as 2 h after PDGF treatment (Fig. 2D), suggesting that PDGF signaling likely induces miR-138 transcription. Importantly, blocking PDGF signaling with the receptor tyrosine kinase inhibitor Sunitinib, indeed restored the expression of miR-138 after PDGF treatment (Fig. 2C), suggesting that PDGF signaling likely activates miR-138.

3.3. miRNA-138 is essential for the PDGF-induced inhibition of osteogenic differentiation of MSCs

We transfected MSCs with synthetic miR-138 (miR-138 mimic) or a control mimic (containing a sequence from GFP) and examined

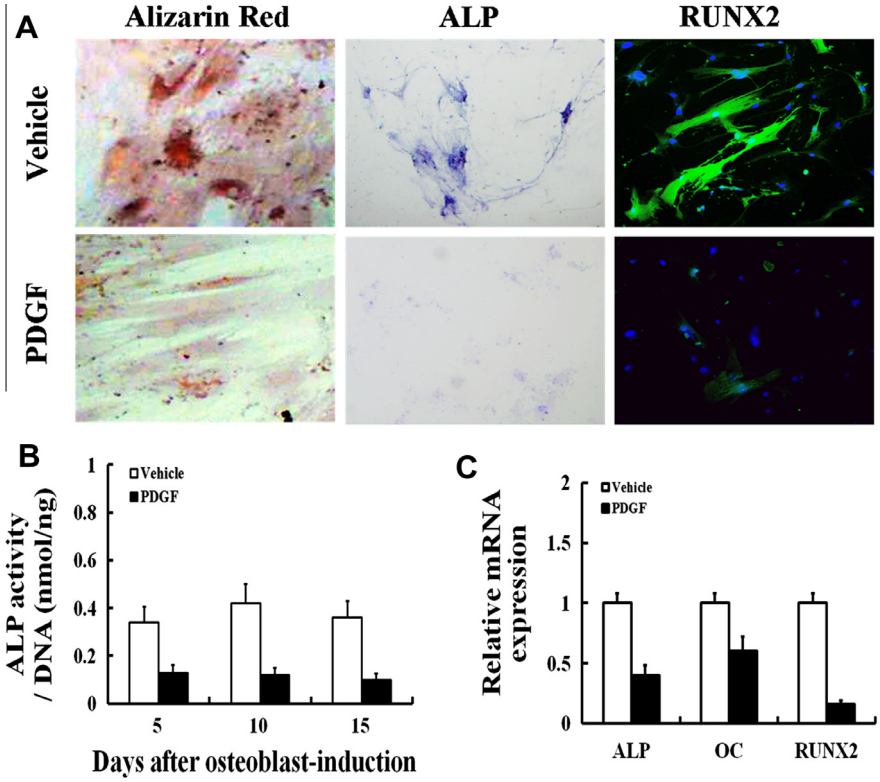

Fig. 1. PDGF inhibits the osteogenic differentiation of MSCs. MSCs incubated with vehicle or PDGF-BB (50 ng/ml) were subjected to alkaline phosphatase staining, Alizarin Red staining, and immunofluorescence staining for RUNX2 (A) 15 days after osteoblast induction. Original magnification, 200×. Quantitative analyses of alkaline phosphatase activity (B) 5, 10, and 15 days after osteoblast induction and detection of alkaline phosphatase (ALP), osteocalcin (OC), and RUNX2 mRNA levels by real-time PCR (C) 15 days after osteogenic induction. The relative mRNA expression levels of ALP, OC, and RUNX2 in PDGF-induced MSCs were quantified and are displayed relative to their levels in the vehicle-treated group.

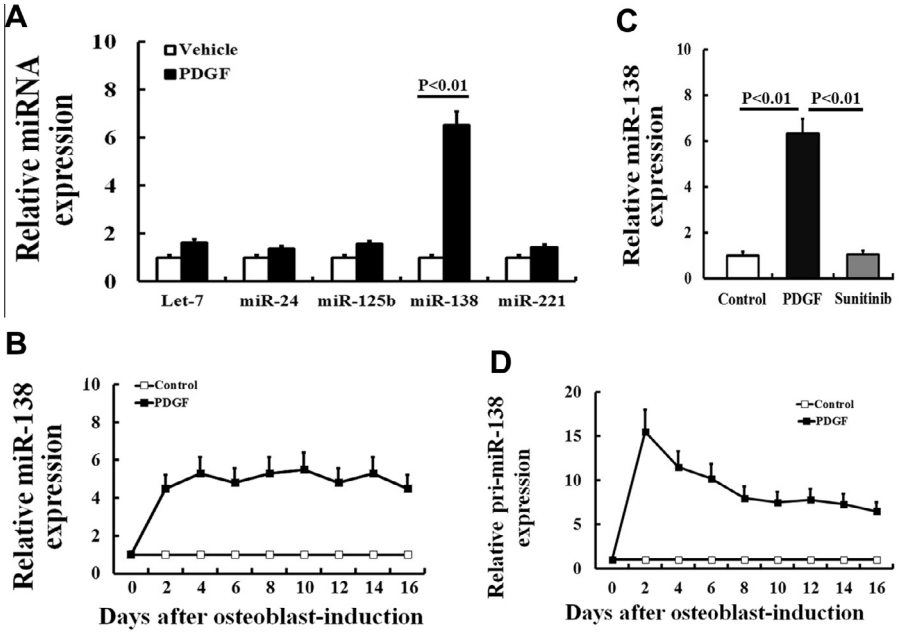

Fig. 2. The PDGF-BB signaling pathway regulates miR-138 expression in MSCs. Real-time PCR was used to determine the relative miRNA expression levels in MSCs incubated with vehicle or PDGF-BB (50 ng/ml) (A) 15 days or (B) over a time-course after osteogenic induction. MSCs were incubated with the PDGF receptor tyrosine kinase inhibitor sunitinib or a negative control and subsequently treated with PDGF-BB. The expression of miR-138 was determined by qRT-PCR 15 days after osteoblast induction (C). The expression of pri-miR-138 was determined by qRT-PCR over a time-course after osteoblast induction (D). The relative miRNA expression level in PDGF-induced MSCs was quantified and is displayed relative to its level in the vehicle-treated group.

the osteoblast phenotype after inducing osteogenesis. Similar to PDGF treatment, exogenous expression of miR-138 significantly suppressed ALP activity (Fig. 3A) and downregulated the mRNA and protein expression of ALP, osteocalcin, and RUNX2 (Fig. 3B and C). Next, RNA oligonucleotides targeted against miR-138 (anti-miR-138) were transfected into MSCs to inhibit the function of miR-138. Transfection of anti-miR-138 reduced both the basal and PDGF-induced expression of miR-138 (Fig. 3F). When

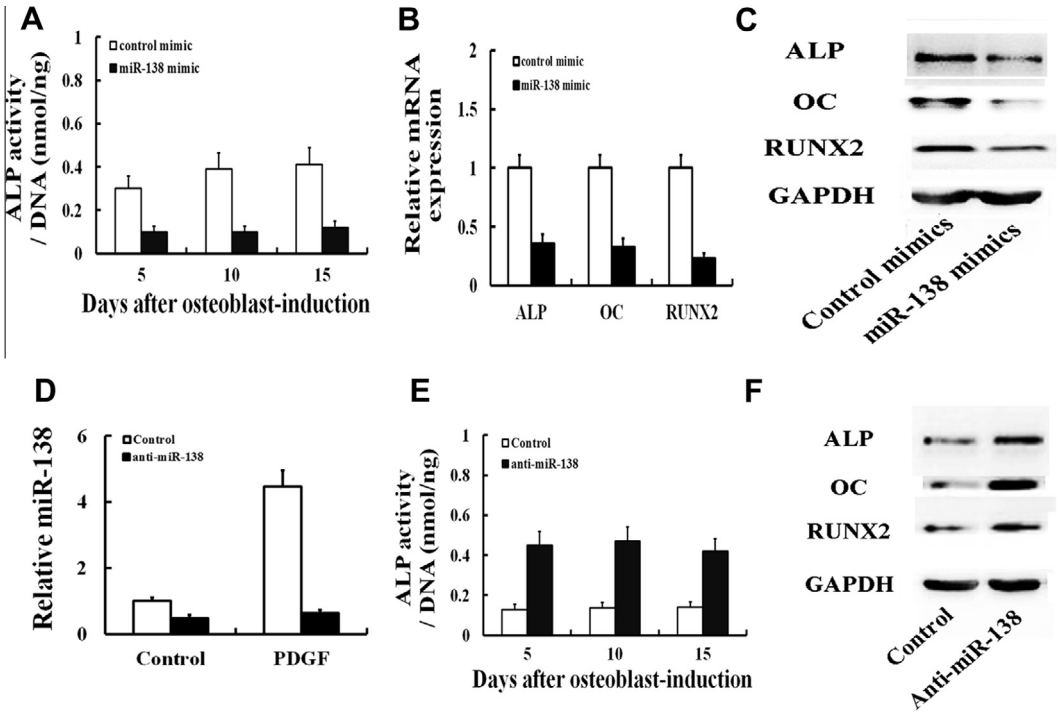

Fig. 3. miR-138 is critical for PDGF-mediated osteogenic differentiation of MSCs. MSCs were transfected with the miR-138 mimic or a negative control and subsequently incubated with PDGF-BB (50 ng/ml). Quantitative analyses of alkaline phosphatase activity were performed (A) 5, 10, and 15 days after osteoblast induction. The expression of ALP, OC, and RUNX2 was determined by real-time PCR (B) and Western blot analyses (C) 15 days after osteogenic induction. MSCs were transfected with anti-miR-138 or a negative control and subsequently incubated with PDGF-BB (50 ng/ml). The expression of miR-138 was determined by real-time PCR (D) 15 days after osteogenic induction. Quantitative analyses of alkaline phosphatase activity were performed (E) 5, 10, and 15 days after osteogenic induction, while the expression levels of ALP, OC, and RUNX2 were determined by Western blot analyses (F) 15 days after osteogenic induction.

miR-138 function was blocked by anti-miR-138, PDGF did not alter the activity level of ALP (Fig. 3E) or the expression levels of ALP, osteocalcin, and RUNX2 after osteogenic induction (Fig. 3F). Conversely, in MSCs transfected with the miR-138 mimic alone, significant inhibition of osteogenic differentiation that was comparable to that observed in untransfected cells treated with PDGF was observed after osteogenic induction (Figs. 1B, C and 3A, B). These results indicate that PDGF-dependent induction of miR-138 is essential for inhibiting osteogenic differentiation. This result raises the question of whether miR-138 might dispatch signals through multiple functionally and physically distinct targets. miR-138 is expressed in undifferentiated MSCs and suppresses the translation of FAK and its downstream target ERK1/2 [14]. Inhibition of this signaling cascade results in decreased phosphorylation of Runx2, decreased expression of OSX, and subsequent suppression of the osteogenic differentiation of MSCs. Our results also show that exogenous expression of miR-138 suppresses the phosphorylation of FAK (Fig. 4A and B), ERK1/2 (Fig. 4A and B), and Runx2 (Fig. 4A and B) after osteogenic induction in MSCs.

3.4. PDGF-mediated downregulation of Runx2 by miR-138 inhibits osteogenic differentiation

Runx2 is the first transcription factor required for commitment to the osteoblast lineage, while Sp7 and the canonical Wnt signaling pathway further direct the differentiation of mesenchymal cells into osteoblasts by blocking their ability to differentiate into chondrocytes [19]. Moreover, we found that PDGF treatment significantly downregulated RUNX2 gene expression in hMSCs cells after osteogenic induction (Fig. 1C). Previous studies have shown that miR-138 inhibits Runx2 expression and osteogenic differentiation of MSCs [15]. Therefore, we hypothesized that PDGF-induced inhibition of osteogenic differentiation of MSCs is affected by miR-138-mediated downregulation of Runx2. To test the role of miR-138 in the PDGF-mediated downregulation of Runx2, we used anti-miR-138 to reduce the endogenous expression levels of miR-138. Runx2 expression levels were measured over a 15-day time course after osteogenic induction. The results show that transfection of anti-miR-138 significantly increased

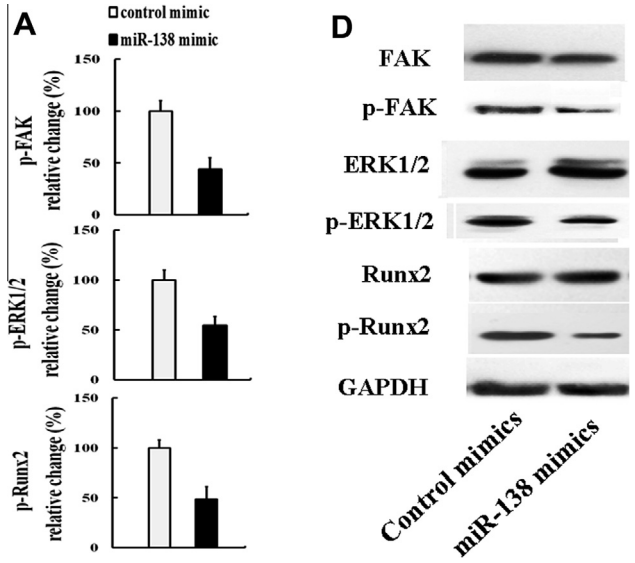

Fig. 4. miR-138 suppresses the phosphorylation of FAK, ERK1/2, and Runx2 after osteogenic induction in MSCs. MSCs were transfected with the miR-138 mimic or a negative control. FAK (A and D), ERK1/2 (B and D), and Runx2 (C and D) expression levels and phosphorylation were determined by Western blot analyses 15 days after osteogenic induction.

the expression of Runx2 and promoted the osteogenic differentiation of MSCs treated with PDGF (Fig. 5A–D); the magnitude of this effect was similar to that observed after treatment with the PDGF receptor inhibitor sunitinib (Fig. 5D). When endogenous miR-138 was depleted using anti-miR-138, PDGF treatment did not repress the osteogenic differentiation of MSCs (Fig. 5D). Thus, the PDGF-dependent inhibition of osteogenic differentiation of MSCs appears to be mediated by the activation of a specific target of miR-138 and upregulation of Runx2.

4. Discussion

Recently, PDGF signaling has been found to play important roles in the osteogenic differentiation of MSCs [1–3]. Moreover, it has become increasingly clear that MSCs residing in the bone marrow play key roles in maintaining bone homeostasis because of their ability to actively proliferate, migrate, and undergo osteogenic, chondrogenic, or adipogenic differentiation in response to different stimuli. Therefore, the biology of MSCs is of specific clinical interest for the treatment of various bone diseases. Although PDGF signaling is considered to be important for the differentiation of MSCs, the regulatory mechanisms of MSC differentiation remain to be elucidated. Our current study clearly demonstrates that PDGF-BB treatment inhibits the osteogenic differentiation of MSCs by upregulating miRNA-138 expression and downregulating Runx2 expression.

Furthermore, our data demonstrate that miR-138 gene transcription is regulated by PDGF signaling. The PDGF pathway is a well-known modulator of the expression of various protein-coding genes. Of the miRNA genes found to be regulated by PDGF, miR-138 was the most significantly upregulated. Specifically, we found that miR-138 was induced 5-fold after osteogenic induction and treatment with PDGF, suggesting that miR-138 expression is activated by PDGF signaling. We also observed robust induction of pri-miR-138 as early as 2 h after PDGF treatment. It is possible that PDGF may activate miR-138 transcription through the recruitment of microphthalmia-associated transcription factor or other E-box binding proteins [15]. Importantly, when the receptor tyrosine kinase inhibitor sunitinib was used to block PDGF signaling, PDGF treatment did not alter the level of miR-138, suggesting that miR-138 expression is likely activated by PDGF signaling.

Recent studies have shown that miR-138 plays a pivotal role in bone formation *in vivo* by negatively regulating osteogenic differentiation in hMSCs and causing reduced ectopic bone formation [15]. We found that exogenous expression of miR-138 significantly suppressed the osteogenic differentiation of hMSCs by downregulating ALP activity and reducing the expression of ALP, osteocalcin, and RUNX2. In addition, PDGF signaling has recently been found to alter the expression profile of miRNAs, leading to the reversal of osteogenic differentiation [16]. When miR-138 function was blocked by the expression of anti-miR-138, we found that PDGF did not alter ALP activity or the expression of ALP, osteocalcin, or RUNX2 after osteogenic induction. These results confirm that miR-138 plays an essential role in the PDGF-mediated osteogenic differentiation of MSCs. Our results also confirm that miR-138 inhibits the osteogenic differentiation of MSCs and suppresses the phosphorylation of FAK, ERK1/2, and Runx2.

A critical role for miR-138 in the PDGF-mediated osteogenic differentiation of MSCs has been previously demonstrated [15,16]. Recent studies have shown that miR-138 is expressed in undifferentiated MSCs and suppresses FAK translation, thereby decreasing the phosphorylation of FAK and its downstream target ERK1/2. Inhibition of this cascade results in the decreased phosphorylation of Runx2 and expression of OSX, which subsequently results in suppression of MSC differentiation into osteoblasts. Our

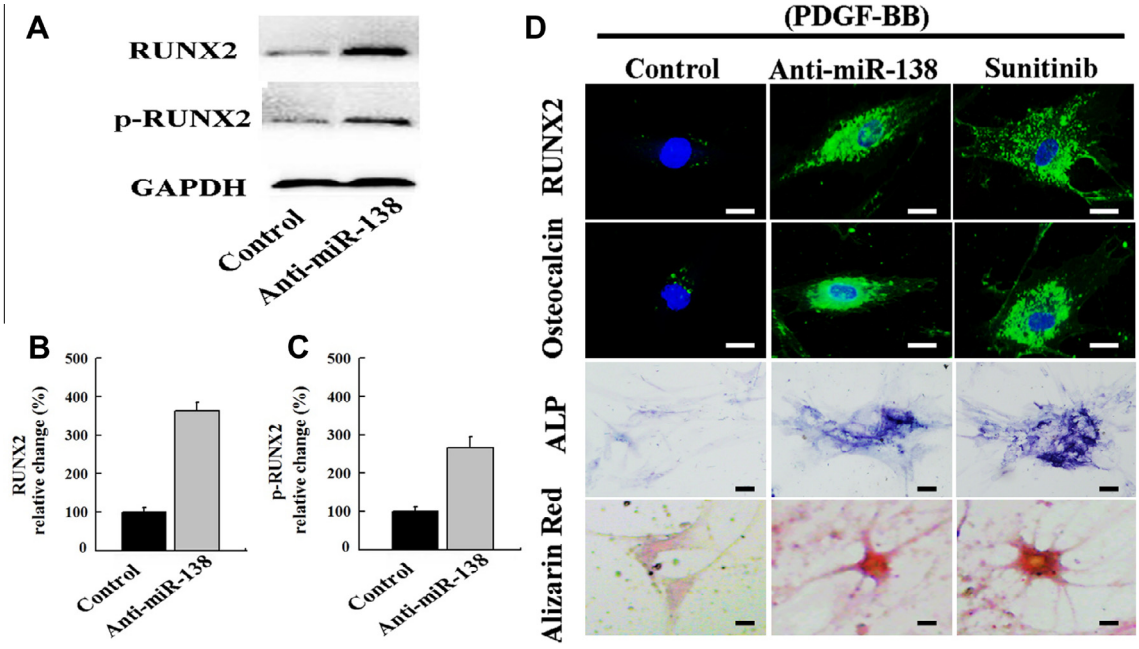

Fig. 5. PDGF-mediated downregulation of Runx2 by miR-138 inhibits osteogenic differentiation. MSCs were transfected with anti-miR-138 or a negative control and subsequently incubated with PDGF-BB (50 ng/ml). Runx2 expression levels and phosphorylation were determined by Western blot analyses (A–C) 15 days after osteogenic induction. (D) MSCs were transfected with a negative control, anti-miR-138, or incubated with the PDGF receptor tyrosine kinase inhibitor sunitinib before incubation with PDGF-BB (50 ng/ml). Alkaline phosphatase staining, Alizarin Red staining, and immuno fluorescence staining for RUNX2 and osteocalcin were performed 15 days after osteogenic induction (scale bars = 10 mm).

results also confirm that miR-138-dependent downregulation of Runx2 inhibits the osteogenic differentiation of MSCs. Runx2 is the first transcription factor required for commitment to the osteoblast lineage, while Sp7 and the canonical Wnt signaling pathway further direct the fate of mesenchymal cells toward the osteoblast lineage by blocking their differentiation into chondrocytes [19]. Therefore, we hypothesized that PDGF-mediated inhibition of the osteogenic differentiation of MSCs is affected by miR-138-mediated downregulation of Runx2. When endogenous miR-138 was depleted by the expression of anti-miR-138, we found that PDGF treatment did not repress the expression of Runx2 or the osteogenic differentiation of MSCs. Thus, PDGF-dependent inhibition of MSC osteogenic differentiation appears to be mediated by the activation of a specific target of miR-138 and upregulation of Runx2.

The results of this study clearly suggest that PDGF exerts its inhibitory effects on the osteogenic differentiation of MSCs by inducing miR-138 expression, thereby downregulating Runx2 expression. Our findings suggest that the inhibition of miR-138 function in MSCs, either by treatment with anti-miR-138 or by overexpression of the miR-138 target sequence (miRNA sponge), represents a potential new therapy for the treatment of bone homeostasis disorders caused by activation of the PDGF pathway.

Conflict of interest

No potential conflicts of interest relevant to this article were reported.

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