European Review for Medical and Pharmacological Sciences 2016; 20: 3911-3919

MicroRNA-100 inhibits bone morphogenetic protein-induced osteoblast differentiation by targeting *Smad1*

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) act as key regulators of diverse cellular activities by regulating the expression of protein-coding genes. Osteoblast differentiation, a fundamental step in skeletal development, involves the activation of several signaling pathways, including transforming growth factor β (TGF-β), bone morphogenetic protein (BMP), and Wnt signaling pathways.

MATERIALS AND METHODS: miRNA expression was measured using TaqManRT-PCR. Western blot was used to detect the protein expression of Smad1. Luciferase reporter assay was used to measure the luciferase activity.

RESULTS: In this study, we found that miR-100 was expressed in mesenchymal progenitor cell lines; furthermore, its expression was reduced during osteoblast differentiation. Retroviral overexpression of miR-100 decreased Smad1 protein levels, whereas miR-100 inhibition had the opposite effect, suggesting that miR-100 acts as an endogenous attenuator of Smad1 in osteoblast differentiation.

CONCLUSIONS: Together, our data demonstrate that miR-100 acts as an important endogenous negative regulator of BMP-induced osteoblast differentiation.

Key Words:
MicroRNA, Osteoblast differentiation, miR-100, Smad1.

Introduction

MicroRNAs (miRNAs) are a growing class of small single-stranded noncoding RNAs found in diverse organisms[1]. They negatively modulate translation or transcription of specific mRNAs by partial or full base pairing to complementary sequences in target mRNAs[2-4]. Although the biological functions of most miRNAs are not yet fully understood, they could play critical roles in the regulation of various biological processes, including developmental events, cellular differentiation, proliferation, apoptosis, tumorigenesis, and the metabolism of glucose and lipids[5,6].

Recent studies[7,8] suggest that miRNAs are also important players during osteoblast differentiation. For instance, miR-2861 is transcribed in ST2 stromal cells during bone morphogenetic protein 2-induced (BMP2-induced) osteogenesis. Furthermore, overexpression of miR-2861 enhances BMP2-induced osteoblastogenesis, whereas inhibition of miR-2861 attenuates it. Importantly, miR-2861 is conserved in humans, and a homozygous mutation in pre-miR-2861 blocked miR-2861 expression and was shown to cause primary osteoporosis in two related adolescents[9]. Therefore, identification of these miRNAs and their functions could help to understand the mechanisms of osteoblast differentiation and bone formation.

Previous reports have demonstrated that miR-100 plays a key role in cancer initiation and progression. It also promotes apoptosis by targeting mTOR in human esophageal squamous cell carcinoma[10]. Reduced expression of miR-100 leads to an unfavorable prognosis in bladder cancer patients[11]. Additionally, miR-100 is a potential molecular marker of non-small cell lung cancer and functions as a tumor suppressor by targeting polo-like kinase 1[12]. It also suppresses IGF2 and inhibits breast tumorigenesis by interfering with proliferation and survival signaling[13]. In the present work, we determined for the first time the role of miR-100 in osteoblast differentiation.

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Materials and Methods

Cell Culture

MC3T3-E1 cells were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and were maintained in a modified Eagle's minimal essential medium (MEM, Invitrogen, Shanghai, China) containing 100 units/mL penicillin and 100 µg/mL streptomycin. For some experiments, mouse bone marrow mesenchymal stem cells (MSCs) were isolated and harvested from femur and tibia bone marrow of 10-week-old male C57B/L6 mice.

Analysis of miRNA Expression Using TaqManRT-PCR

Total RNA was extracted using the miRNA Isolation Kit (Ambion, Austin, TX, USA). Expression of mature miRNAs was assayed using a TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA, USA) specific for mus-miR-100. Briefly, 10 ng of total RNA was reverse transcribed to cDNA with specific stem-loop RT primers. Quantitative real-time PCR was performed using an Applied Biosystems 7900 Real-time PCR System together with a TaqMan Universal PCR Master Mix. All the primers were obtained from the TaqMan miRNA Assays. The small nuclear U6 snRNA (Applied Biosystems, Foster City, CA, USA) was used as an internal control.

Plasmid Construction and Transfection

For the miR-100 expression plasmid, a miR-100 precursor was cloned into a pSilencer 4.1 (Ambion, Austin, TX, USA). The negative control plasmid consisted of a scrambled sequence (Ambion, Austin, TX, USA). To inhibit miR-100 function, a miRNA inhibitor was used (Ambion, Austin, TX, USA), along with the negative control. For transfection, a complex of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and 25 nM miRs were prepared following the manufacturer's instructions.

Western Blot

Cells were harvested and lysed with ice-cold lysis buffer (50 mMTris-HCl, pH 6.8, 100 mM2-ME, 2% w/v sodium dodecyl sulfate [SDS], 10% glycerol). After centrifugation at 20000× g for 10 min at 4 °C, proteins in the supernatants were quantified and separated by a 10% SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred to a nitrocellulose membrane (Amersham Bioscience, Buckinghamshire, UK). After blocking with 10% nonfat milk diluted in phosphate buffered saline (PBS), membranes were immunoblotted with primary antibodies as indicated, followed by horseradish peroxidase (HRP)-linked secondary antibodies (Cell Signaling, Beverly, MA, USA). Signals were detected using the SuperSignal West Pico Chemiluminescent SubsCell signaling trate kit (Pierce, Rockford, IL, USA) in accordance with the manufacturer's instructions. Anti-Smad1, Smad3, Smad4, Sma7, and Runx2 primary antibodies were purchased from (Beverly, MA, USA). Protein levels were normalized to total GAPDH, using a mouse anti-GAPDH antibody (Abcam, Cambridge, MA, USA).

Luciferase Reporter Assay

Total cDNA isolated from MC3T3-E1 cells was used to amplify the 3 UTR of Smad1 by PCR. The Smad1 3 UTR was cloned into a pMir-Report (Ambion, Austin, TX, USA), to obtain pMir-Report-Cyr61. Mutations were introduced to potential miR-100 binding sites using the QuikChange site-directed mutagenesis Kit (Stratagene, La Jolla, CA, USA). Cells were transfected with pMir-Report vectors containing the 3 -UTR variants and the miR-100 precursor, (control plasmids) for 36 h. The pRL-TK vector (Promega, Madison, WI, USA) carrying the Renilla luciferase gene was used as an internal control to normalize the transfection efficiency. Luciferase values were determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical Analysis

Data are expressed as the mean ± SEM from at least four separate experiments. Differences between groups were determined using a Student's *t*-test or One-Way ANOVA analysis. A value of $p < 0.05$ was considered statistically significant.

Results

miR-100 Expression Is Altered During Osteoblast Differentiation in MC3T3-E1 Cells

To determine whether miR-30 family members had a role in osteogenesis, their kinetics were examined in MC3T3-E1 cells following BMP2 treatment. Expression levels of miR-100 were

down-regulated and reached a minimum at 16 h (Figure 1), suggesting that miR-100 could be involved in the BMP2-induced osteoblast differentiation of MC3T3-E1 cells.

miR-100 Family Members Affect Osteoblast Differentiation in MC3T3-E1 Cells

BMP2 treatment up-regulates the expression and activity of ALP in MC3T3-E1 cells, a representative marker of osteogenesis[14]. To determine whether miR-100 could affect osteoblast differentiation, miR-100 mimics or inhibitors were transfected into MC3T3-E1 cells, followed by BMP2 treatment for 8 days. Then, ALP activity was measured in the transfected cells. As shown in Figure 2A, ALP activity in miR-100 overexpressing cells was significantly lower than that in miR-NC transfected cells. In contrast, miR-100 knockdown increased ALP activity (Figure 2B). We also observed that miR-100 negatively affected ALP mRNA and protein levels (Figure 2C and D). These results suggest that miR-100 acts as a negative regulator of BMP2-induced osteogenesis.

Smad1 is a Direct Target of miR-100

To identify miR-100 targets in osteogenesis, we used the miRNA target prediction database (miRWalk) to select candidate genes. The search predicted that miR-100 could target Smad1 (Figure 3A), a key downstream mediator of BMP signaling during osteogenesis[15-19].

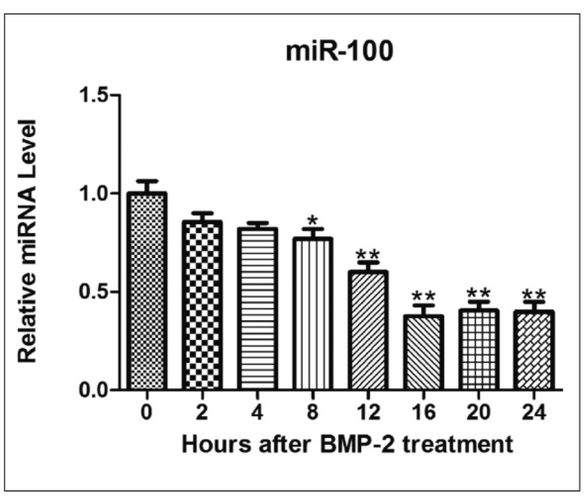

Figure 1. ***A,*** Changes in miR-100 expression during BMP2-induced osteoblast differentiation. MC3T3-E1 cells were treated with BMP2 (200 ng/mL) for 0, 2, 4, 8, 12, 16, 20, and 24 h to determine the relative levels of miR-100 by TaqMan MicroRNA expression assays. Values are represented as mean ± SEM of each group of cells from four separate experiments. The data point at 0 h was designated as the control group. $*p < 0.05$, $**p < 0.01$.

To test whether Smad1 could be regulated by miR-100, MC3T3-E1 cells were transfected with miR-100 mimics or inhibitors. Results showed that following transfection, Smad1 protein levels decreased, whereas miR-100 levels increased (Figure 3B). In contrast, Smad1 expression increased after miR-100 knockdown (Figure 3C). However, other members of the Smad pathway, including Smad3, Smad4, Smad7, and Runx2 were not altered by either miR-100 overexpression or inhibition (Figure 3B-3C), suggesting that miR-100 could specifically regulate Smad1 expression.

To examine whether miR-30 could directly regulate Smad1 expression, MC3T3-E1 cells were transfected with a luciferase reporter construct containing a wild-type Smad1 3 -UTR, in combination with miR-100 mimics or inhibitors. Evidently, renilla luciferase activity was lower in miR-30-overexpressing cells, and higher in miR-30 knockdown cells, when compared to control cells (Figure 3E-3F).

Then, the predicted target sites in the Smad1 3 UTR were mutated (Figure 3A). As expected, miR-100 significantly inhibited the activity of the wild-type reporter gene, whereas mutations in the binding sites abolished the miR-100-mediated repression of reporter gene activity (Figure 3G).

Smad1 Overexpression Reversed the Inhibitory Role of miR-100

To further elucidate the role of Smad1, MC3T3-E1 cells were transfected with retroviruses expressing Smad1 in combination with miR-100 (Figure 4A). As shown in Figure 4B and 4C, Smad1 overexpression reversed the inhibitory effect of miR-100 on both ALP activity and mRNA levels, suggesting that Smad1 restoration could rescue the miR-100 overexpression phenotype. Therefore, our results confirmed that Smad1 lies downstream of miR-100.

miR-100 Inhibits Osteogenic Differentiation of Primary Mouse Bone Marrow MSCs

Finally, we addressed miR-100 function in primary mouse bone marrow MSCs. miR-100 mimics or antisense were transfected into mouse MSCs. As a result, ALP activity and expression

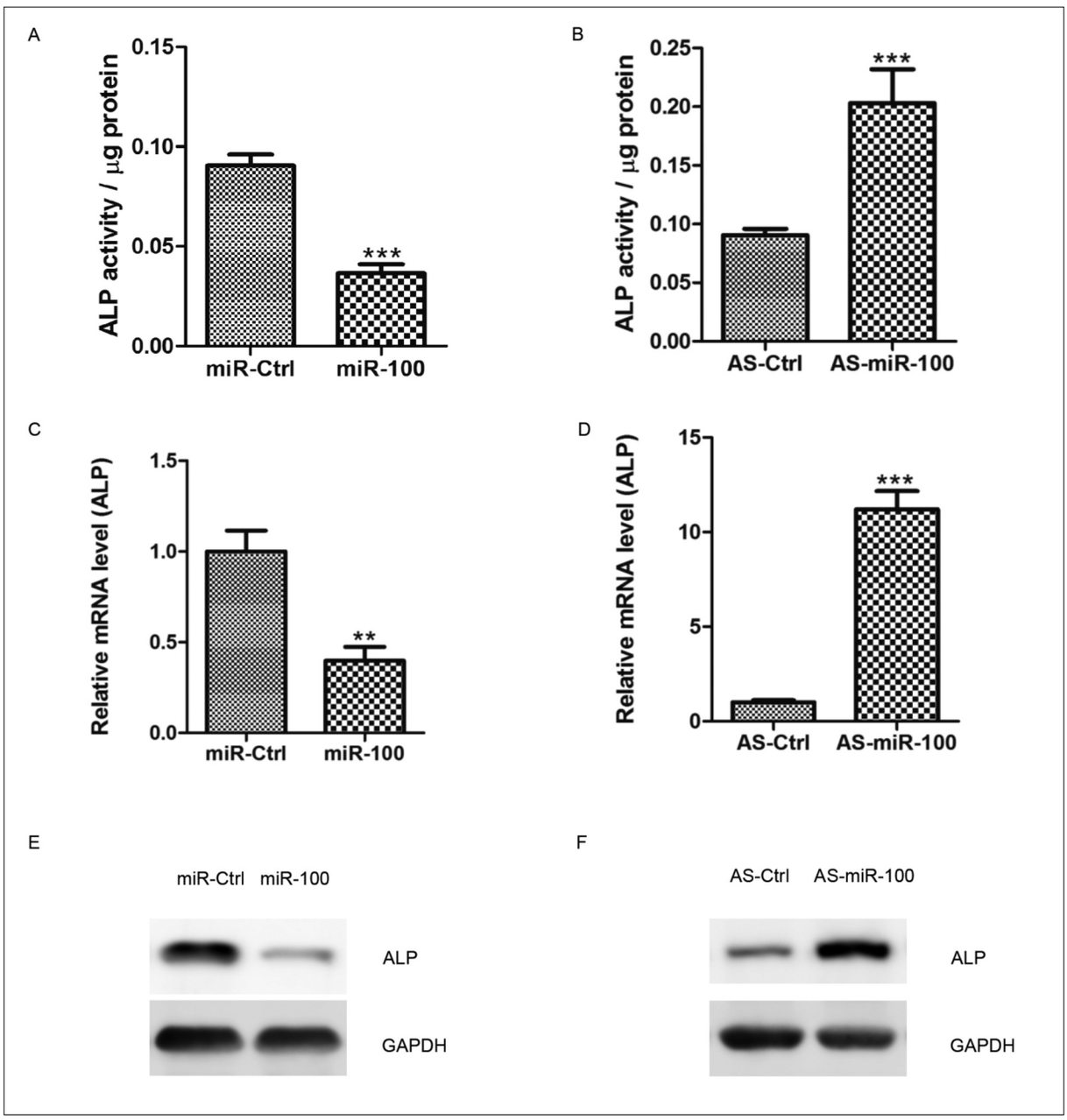

Figure 2. miR-100 negatively regulates osteogenic differentiation. ALP activity ***(A-B)***, mRNA ***(C-D)***, and protein ***(E-F)*** expression were measured in MC3T3-E1 cells. Cells were transfected with 50 nM miR-100 mimics, control (miR-Ctrl), miR-100 antisense (AS), or control oligos for 48 h and then cultivated with or without BMP2 for another 8 d.

levels were significantly suppressed in mice treated with miR-100 mimics, or enhanced in antisense-transfected cells (Figure 5A-5F). In addition, Smad1 expression was inhibited by miR-100 in MSCs (Figure 5G-5H). Therefore, these results suggest that miR-100 negatively regulates osteogenic differentiation of mouse bone marrow MSCs.

Discussion

In the present work, we investigated the effects of miR-100 on BMP2-induced osteogenic differentiation, which was determined by overexpression and knockdown experiments performed in both osteoblast cell lines and mouse primary cells. Overexpression of miR-100 led to decreased ALP

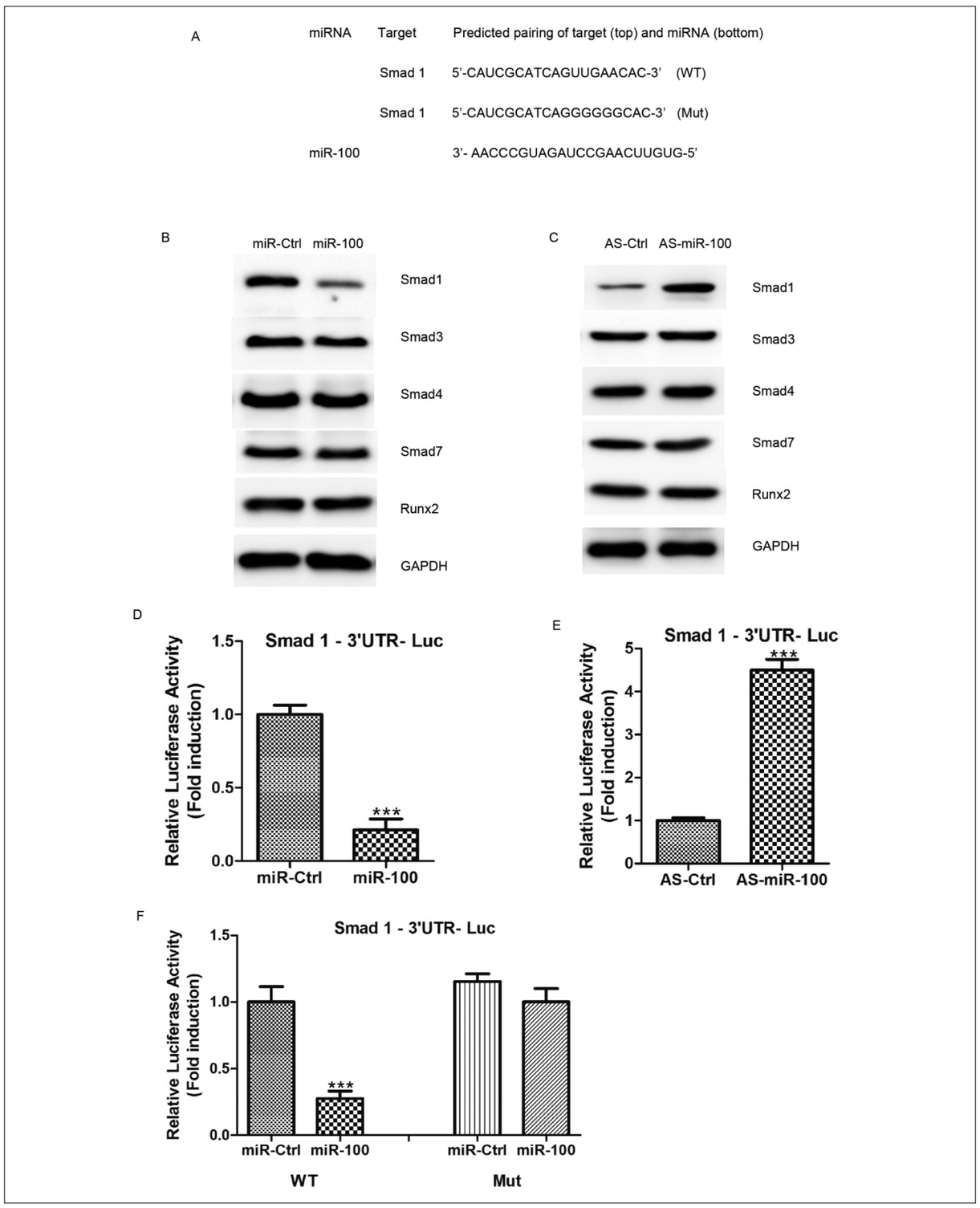

Figure 3. miR-100 inhibits Smad1 expression. ***A,*** Computer prediction of the miR-100 binding sites in the 3′ UTRs of the Smad1 gene. Potential binding sites and point mutations are highlighted. ***(B-C)*** Western blot analysis of Smad1, Smad3, Smad4, Smad7, and Runx2 was measured in MC3T3-E1 cells transfected with 50 nM miR-100 mimics, control (miR-Ctrl), miR-100 antisense (AS), or control oligos. ***D,-E,*** Luciferase reporter assays in MC3T3-E1 cells. Cells were transfected with 100 ng of a 3′-UTR-reporter construct together with 50 nM miR-100 mimics, miR-Ctrl, AS, or control oligos. ***F,*** Luciferase reporter assays in MC3T3-E1 cells. Cells were transfected with 100 ng of the wild-type (WT) 3′-UTR-reporter or mutant (Mut) construct in combination with 50 nM miR-100 mimics or control (miR-Ctrl)

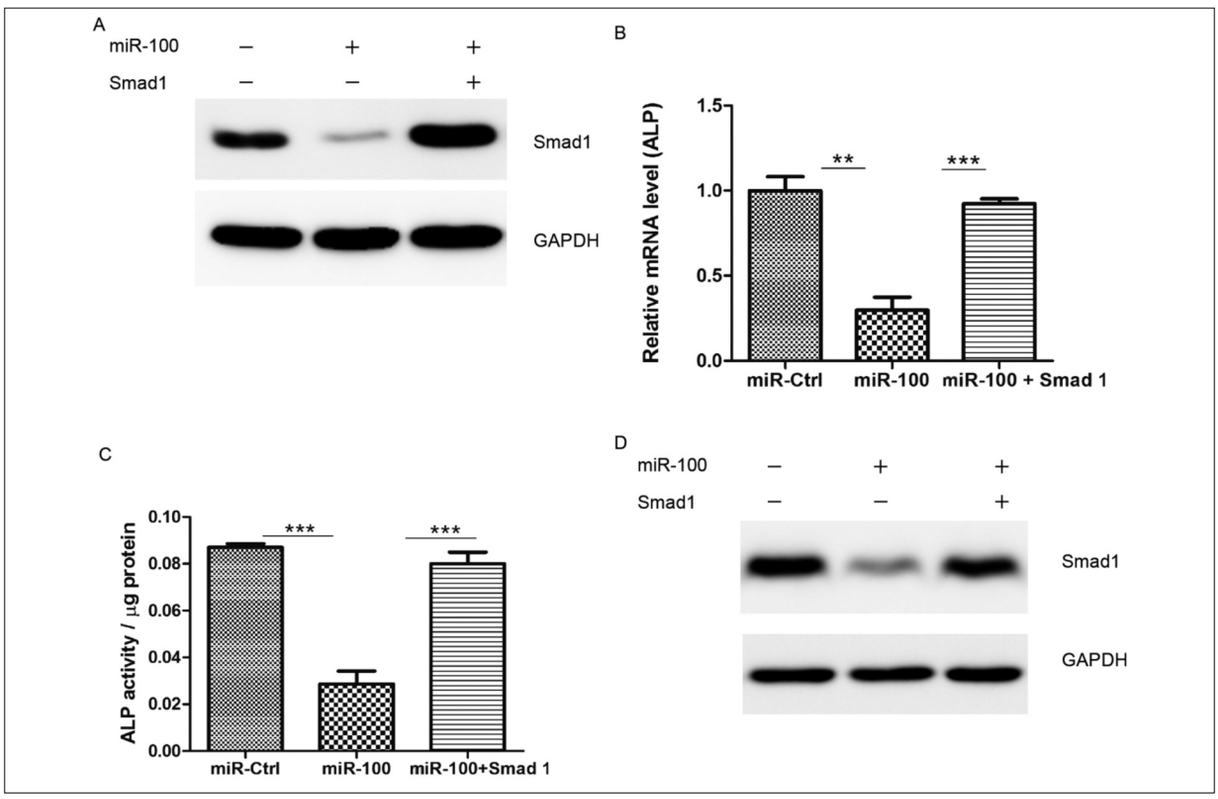

Figure 4. Smad1 overexpression reversed the inhibitory role of miR-100. ***(A)*** Western blot analysis of Smad1 in MC3T3-E1 cells. Cells were co-transfected with a retrovirus expressing Smad 1 or an empty vector (EV) together with miR-100 or control. ALP activity ***(B)***, mRNA ***(C)***, and protein ***(D)*** expression were measured in MC3T3-E1 cells.

activity and expression, whereas miR-100 knockdown increased both the activity and expression levels of ALP, when compared to control cells. Consistent results were observed in mouse bone marrow MSCs after overexpression or inhibition of miR-100. Therefore, our data suggest that miR-100 is a negative regulator of the BMP2-induced osteoblast differentiation. However, it remains to be determined whether miR-100 is expressed in cartilage or osteoclast. Additional researches are still needed to determine the role of miR-100 in osteoblast differentiation *in vivo*. In addition to BMP pathways, members of the Smad family, including Smad1, are also mediators of the transforming growth factor- (TGF-) pathway[15,20,21]; thus, it is possible that miR-100 could be involved in the regulation of the TGF- signaling pathway.

The impaired postnatal bone formation was observed in an osteoblast-specific Smad1 knockout mouse model, suggesting that Smad1 is an immediate downstream transducing molecule of the BMP receptor and that it plays an important role in the BMP signaling pathway[22-24]. Previous investigations have shown that Smad1 expression is tightly regulated by several miRNAs under different physiological conditions. For instance, miR-26a regulated osteogenesis in human adipose tissue-derived stem cells, skeletal muscle differentiation, and regeneration through Smad1[25,26]. It could also induce cell death by inhibiting Smad1 in melanoma[27], suggesting that the miR-26a/Smad1 regulatory pathway is a potential therapeutic strategy for metastatic melanoma. Additionally, miR-199a was shown to negatively regulate early chondrocyte differentiation by directly targeting Smad1[28]. These studies, together with our data, indicate that Smad1 could be regulated by different miRNAs under different physiological conditions.

Conclusions

We demonstrated that miR-100, in response to BMP2, acts as a negative regulator of early osteoblast differentiation through the suppression of Smad1. The present study could provide new insights into BMP/Smad signaling regulation in osteoblast differentiation.

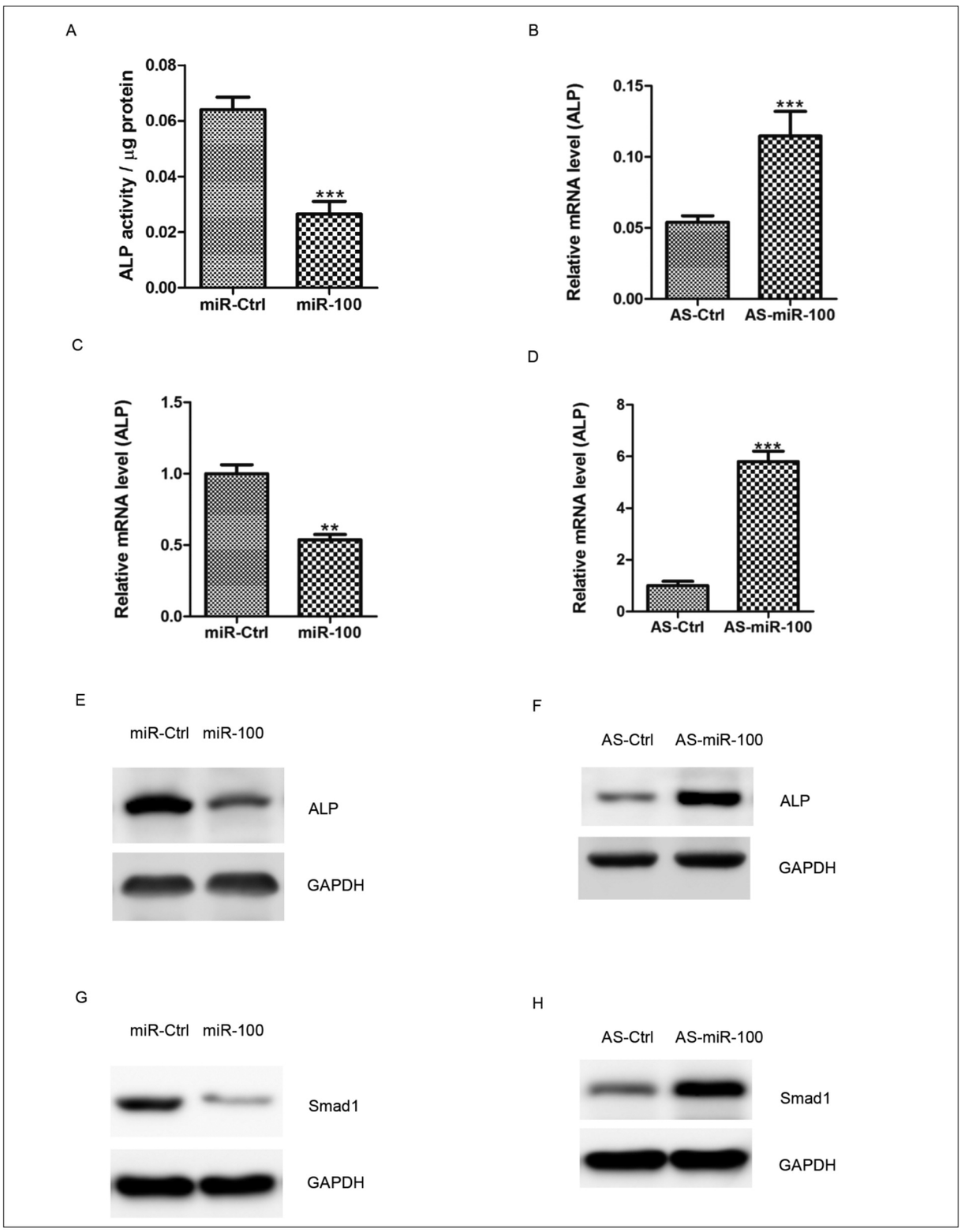

Figure 5. miR-100 inhibits osteogenic differentiation of mouse bone marrow MSCs. ALP activity ***(A-B)***, mRNA ***(C-D)***, and protein ***(E-F)*** expression were measured in mouse primary bone marrow MSCs. Cells were transfected with 50 nM miR-100 mimics, control (miR-Ctrl), miR-100 antisense (AS), or control oligos for 48 h and then cultivated with or without BMP2 for another 8 d. ***(E-F)*** Western blot analysis of Smad1 in mouse primary bone marrow MSCs transfected with 50 nM miR-100 mimics, control (miR-Ctrl), miR-100 antisenses (AS), or control oligos.

Acknowledgements

This research was supported by grants from the Natural Science Foundation of Heilongjiang Province (No. H2013107).

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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