MicroRNA-141 and -200a Are Involved in Bone Morphogenetic Protein-2-induced Mouse Pre-osteoblast Differentiation by Targeting Distal-less Homeobox 5

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MicroRNAs (miRs) are endogenously expressed 18–25-nucleotide RNAs that regulate gene expression through translational repression by binding to a target mRNA. Recently, it was indicated that miRs act as key regulators in cell differentiation, cell growth, and cell death. In osteogenesis, several miRs (for example miR-26a, -125b, -133, and -135) regulate osteoblast cell growth or differentiation in human adipose tissue-derived stem cells, mouse mesenchymal ST2 stem cells, and mouse premyogenic C2C12 cells. Additionally, Smad proteins control Drosha-mediated miR maturation. Therefore, miRs are closely related to osteogenesis. Here we investigated miR expression profile by an miR array and identified the candidate miRs, miR-141 and -200a, as pre-osteoblast differentiation-related miRs. The effects of miR-141 and -200a on pre-osteoblast differentiation were examined by using transfection of murine pre-osteoblastic MC3T3-E1 cells with mature miR-141 or -200a and antisense inhibitor for miR-141 or -200a. It was shown that miR-141 and -200a remarkably modulated the BMP-2-induced pre-osteoblast differentiation through the translational repression of Dlx5, which is a bone-generating transcription factor expressed in pre-osteoblast differentiation. Furthermore, it was indicated that Dlx5 is a common target of miR-141 and -200a by using a luciferase reporter assay. Thus, we have observed for the first time that miR-141 and -200a are involved in pre-osteoblast differentiation in part by regulating the expression of Dlx5.

Recently, Mizuno et al. (14) reported that miR-125b inhibits bone morphogenic protein-4 (BMP-4)-induced osteoblast differentiation in mouse mesenchymal ST2 stem cells. miR-26 has been shown to regulate osteogenic differentiation in human adipose tissue-derived stem cells (15), and miR-223 was reported to regulate osteoclastogenesis in RAW 264.7 cells (16). In the osteoblast differentiation process, BMPs (such as BMP-2, -4, and -7) act as potential differentiators. In this study, the relationship between the expression of miRs and differentiation was investigated by using an miR array in BMP-2-treated mouse pre-osteoblast MC3T3-E1 cells. We have identified miR-141 and -200a as the pre-osteoblast differentiation-related miRs. The transfection of MC3T3-E1 cells with miR-141 or -200a significantly suppressed pre-osteoblast differentiation. Bioinformatic study and Western blot analysis indicated that Dlx5 (Distal-less homeobox 5) was a common target gene of miR-141 and -200a, which was also assessed by luciferase reporter assay. Furthermore, the protein and mRNA expression levels of Osx (Osterix), which is transcriptionally regulated by Dlx5, was also down-regulated in cells transfected with miR-141 or -200a. These findings together provide evidence suggesting that miR-141 and -200a modulate BMP-2-stimulated pre-osteoblast differentiation by targeting Dlx5, which is one of the osteogenic master transcriptional factors.

EXPERIMENTAL PROCEDURES

Materials—Bioactive recombinant human BMP-2 was from BioVision Research Products (Mountain View, CA). Eagle’s α-minimal essential medium (α-MEM), Opti-MEM, fetal bovine serum, and Lipofectamine™ RNAi Max were from Invitrogen. ALP assay kit LabAssay™ALP was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ALP staining kit TRACP & ALP double-stain kit was from Takara-Bio Inc. (Ohtsu, Japan). The antibodies to mouse Dlx5 (Y-20) and Osx were from Santa Cruz Biotechnology.

Cell Culture—Mouse pre-osteoblast cell line MC3T3-E1 was obtained from RIKEN Cell Bank (Tsukuba, Ibaraki, Japan) and was cultured in phenol red-free α-MEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37 °C. BMP-2 dissolved in PBS was added to the culture medium at a concentration of 300 ng/ml.

MicroRNA Array Hybridization—Total RNA was extracted from cells by TRIzol containing phenol/guanidinium isothiocyanate (Invitrogen) with DNase I treatment. We examined the expression profile of miRNAs by using the Genopal®-MIC
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Quantitative RT-PCR—To ascertain the reproducibility of the miR expression profile obtained by the miR array analysis, we examined their expression levels by using a TaqMan® microRNA reverse transcription kit and TaqMan® microRNA assay kit (Applied Biosystems, Foster City, CA). Briefly, after reverse transcription (RT) of 25 ng of total RNA, cDNA was generated. The RT products were subjected to quantitative RT-PCR (qRT-PCR) using a real time-PCR (Roche Applied Science). The expression levels were normalized to U6, an internal control, and measured by comparative Ct (ΔΔCt) method. The qRT-PCR consisted of 50 cycles (95 °C for 10 s, 60 °C for 40 s, and 72 °C for 1 s) after an initial denaturation step (95 °C for 10 min).

In addition, to determine the levels of Dlx5 and Osx mRNA, we prepared cDNA from total RNA samples using a PrimeScript™ reagent kit (Takara). qRT-PCR was performed (Takara cycler Dice® TP800) using a SYBER® Premix Taq™ II Kit (Takara) and the following primer sets: Dlx5 sense, 5’-GCTCTAGAGCTAGATGGGCTACTTTCTCT-3’, and Dlx5 antisense, 5’-GCTCTAGAGCGTTCAAACATCCCCGTATGA-3’; Osx sense, 5’-TCTCCATCTGGCATCTCCTT-3’, and Osx antisense, 5’-ACGTATGCTTCTTTGTGC-3’. The cDNA of glyceraldehyde phosphate dehydrogenase was used as an internal control. Semi-quantitative RT-PCR consisted of 20 cycles (94 °C for 30 s, 57.5 °C for 30 s, and 72 °C for 30 s) after an initial denaturation step (94 °C for 4 min). The PCR products were analyzed by electrophoresis on 2% agarose gels.

Transfection of MC3T3-E1 Cells with miRNAs—MC3T3-E1 cells were seeded in 6-well plates at a concentration of 1 × 10⁵ cells/ml/well on the day before the transfection. The mature types of miR-141 and -200a (Applied Biosystems; sequence miR-141, UAACACUGUCUGGAAGUUGG; miR-200a, UAACACUGUCUGGAACGAGU) and their antisense inhibitors (Applied Biosystems), which were designed to bind to endogenous specific miRNAs when introduced into cells and inhibit their activities, were transfected by using cationic liposomes (RNAiMAX) according to the manufacturer’s lipofection protocol. The transfection efficiency was evaluated by the transfection of the cells with an Alexa Fluor 488 (Molecular Probes), and it was more than 80% (data not shown). Nonspecific control miR (Applied Biosystems) was used as a control for nonspecific effects. The culture medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin was changed at 12 h after the transfection. Transfected cells were cultured for 3 days and then reseeded at a concentration of 1 × 10⁵ cells/ml.

Activity and Staining of ALP—The control nontransfected and transfected MC3T3-E1 cells were inoculated into 24-well plates (1 × 10⁵ cells/ml, 500 µl/well; Nunc, Roskilde, Denmark) and cultured with or without BMP-2 for 3 days. After incubation, the treated cells were washed twice with PBS, and 200 µl of lysis buffer was added to the cell layer and kept on ice for 5 min.

Increased ALP activity after BMP-2 treatment in MC3T3-E1 cells. MC3T3-E1 cells were seeded into 24-well plated (1 × 10⁵ cells/ml, 500 µl/well) and cultured with or without BMP-2 for 3 days. ALP activity was examined by a spectrophotometric method using a LabAssay™ ALP kit. The values of each group were expressed as mean ± S.E. of three separate experiments. Values are significantly different as indicated. *p < 0.01 by Student’s t test. p < 0.05 is significant.

The cell lystate was sonicated for 1 min and centrifuged at 1,000 × g, 4 °C, for 10 min. ALP activity was assayed by a spectrophotometric method using a LabAssay™ ALP kit.
(Wako Pure Chemical Industries, Ltd., Osaka, Japan). The absorbance at 405 nm of each well was measured with the microplate reader (Immuno-Mini NJ-2300, Nalge Nunc International K.K., Tokyo, Japan). ALP activity was also estimated by using the TRACP & ALP double-stain kit (Takara) according to the manufacturer’s protocol.

**Alizarin Red Staining**—For detection of calcification during differentiation, BMP-2-treated or nontreated MC3T3-E1 cells were washed twice with PBS and fixed with 500 μl of ice-cold 70% ethanol for 10 min. The fixed cells were stained with 500 μl of Alizarin red solution (Sigma).

**Western Blotting**—For preparation of cell lysate, MC3T3-E1 cells were washed twice with PBS and harvested. The cell pellet was resuspended in RIPA buffer containing 25× Complete® and Phosphatase Inhibitor Mixture® (Roche Applied Science). Protein content was measured with a DC protein assay kit (Bio-Rad). Each whole cell lysate was resuspended in SDS-PAGE buffer containing 2% 2-mercaptoethanol and boiled at 98 °C for 5 min. Twenty micrograms of protein from each cell lysate was separated by SDS-PAGE on 12% polyacrylamide gel and then electroblotted onto a polyvinylidene difluoride membrane (PerkinElmer Life Sciences). After blockage of nonspecific binding sites for 1 h by 5% nonfat milk in TPBS (PBS and 0.1% Tween 20), the membrane was incubated overnight at 4 °C with anti-Dlx5 or anti-Osx antibody. The membrane was then washed three times with TPBS, incubated further with alkaline phosphatase-conjugated goat anti-mouse antibody or anti-rabbit antibody at room temperature, and then washed three times with TPBS. Protein bands were detected with enhanced ECL kit and chemiluminescence detector (LAS-1000, Fuji, Japan).

**Luciferase Assay**—To determine the common target region of miR-141 and -200a in Dlx5, we constructed four kinds of pGL3-Dlx5/
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Expression Profile of miRs in BMP-2-induced Pre-osteoblast MC3T3-E1 Cell Differentiation—To investigate the expression profiles of miRs in BMP-2-treated MC3T3-E1 cells, we subjected their total RNA extracted from BMP-2-treated (24 h) or nontreated cells to Genopal®-MICH chips. The expression levels of 9 miRs (log ratio, <1.5) were distinct between BMP-2-treated and nontreated cells. Among them, we focused on the down-regulation of miR-141 and -200a, because the nucleotide sequences of miR-141 and -200a were identical except for two nucleotides as shown in Fig. 1A, and the target genes of both miRs were possibly both from the miRBase Target version 5 data base. To confirm the results from miRNA array analysis, we examined the expression levels of miR-141 and -200a in BMP-2-treated and nontreated cells by TaqMan® assay using a real-time PCR. As expected, the expression levels of both miRs in BMP-2-stimulated cells were significantly down-regulated following BMP-2 treatment up to 72 h, as compared with those in control nontreated cells (Fig. 1B).

Effects of miR-141 and -200a on Differentiation in MC3T3-E1 Cells—ALP activity as an osteoblast differentiation marker increased time-dependently after BMP-2 treatment (Fig. 2). To investigate the role of miR-141 or -200a, we measured ALP activities in MC3T3-E1 cells transfected with miR-141 or -200a with and without BMP-2 treatment. As shown in Fig. 2A and B, the ALP activities in the transfected cells were significantly suppressed, which were compatible with the results of Alizarin red staining (Fig. 2A, right panel). There was no significant difference between co-transfection with both miRs and single transfection. On the other hand, the ALP activities in the transfected cells with the antisense inhibitor for miR-141 or -200a were significantly enhanced in a time-dependent manner at each dose of BMP-2 tested (Fig. 2C). These results suggested that miR-141 and -200a act as osteogenesis modulators in pre-osteoblast differentiation by BMP-2 stimulation.

Expression of Osteogenic Master Transcription Factor Dlx5 Is Regulated by MicroRNA-141 and -200a—To disclose the target gene of miR-141 and -200a, we have searched for the candidate genes by using an miR Base Target version 5 data base. Either of the miRs targets about 1,000 genes, and osteogenic master transcription factor Dlx5 is included as a common target gene (Fig. 1A). BMP-2 stimulation caused a considerable increase in the expression levels of the Dlx5 protein up to 48 h (Fig. 4A), whereas the levels of the Dlx5 mRNA appeared to be unchanged from 24 to 48 h (Fig. 4B). This finding led us to assume that the increase of Dlx5 expression from 24 to 48 h may be due to the decrease in the levels of miR-141 and -200a (Fig. 1B). Ulsamer et al. (17) have reported that Oxs transcription activity was controlled by direct phosphorylation of Dlx5 at Ser-34/217 by p38 MAPK. We examined the expression levels of protein and mRNA of Oxs during BMP-2-induced pre-osteoblast differentiation. As shown in Fig. 4A, the levels of Oxs were also increased concurrent with Dlx5 expression (Fig. 4B). However, transfection with either miR-141 or -200a reduced the expression levels of Dlx5 and Oxs to almost the same levels.

FIGURE 3. Effects of transfection with mature and antisense inhibitor for miR-141 or -200a on differentiation in MC3T3-E1 cells. A, differentiation and mineralization in MC3T3-E1 cells transfected with miR-141 or -200a were observed by ALP and Alizarin red staining. Negative control miR-NC (miR-NC) was designed to have no significant sequence similarity to mouse, rat, or human transcript sequences. B, increased expression of miR-141 or -200a by transfection significantly suppressed the ALP enzyme activity. Cells were treated with BMP-2 (300 ng/ml) for 3 days. The values of each group were expressed as mean ± S.E. of three separate experiments. Values are significantly different as indicated. *, p < 0.01 by Student’s t test. p < 0.05 is significant. C, transfection of the cells with antisense inhibitor for miRNA-141 or -200a significantly increased the ALP activity. The values of each group were expressed as mean ± S.E. of three separate experiments. Significant differences are shown as follows: *, p < 0.1; **, p < 0.05; and ***, p < 0.01 by Student’s t test. p < 0.05 is significant.
Expression of Dlx5 is translationally regulated by miR-141 and -200a in osteoblast differentiation.

A, time course of Dlx5 and Osx expressions in BMP-2-treated MC3T3-E1 cells (20 μg of protein/lane).

B, time course of Dlx5 and Osx mRNA expressions in BMP-2-treated MC3T3-E1 cells.

C, transition of Dlx5 and Osx expressions in MC3T3-E1 cells transfected with miR-141 or -200a (treatment time: 72 h, 20 μg of protein/lane).

D, changes of Dlx5 (panel i) and Osx (panel ii) mRNA expression in the transfected cells were examined by qRT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Panel iii, relative levels of Dlx5 mRNA in the transfected cells were normalized to glyceraldehyde-3-phosphate dehydrogenase.

Lane 1, BMP-2 (+); lane 2, BMP-2 (+); lane 3, BMP-2 (+) (300 ng/ml) and mir-negative control; lane 4, BMP-2 (+) and mir-141 (40 nM); lane 5, BMP-2 (+) and mir-200a (40 nM).

E, changes of Dlx5 and Osx protein expression in MC3T3-E1 cells transfected with antisense inhibitor mir-141 or -200a (treatment time: 72 h, 20 μg of protein/lane).
as those in the control cells without BMP-2 stimulation at the indicated times (Fig. 4C). These results suggested that miR-141 and -200a participated in the BMP-2-induced pre-osteoblast differentiation through the down-regulation of Dlx5. The levels of Dlx5 mRNA in cells transfected with miR-141 or -200a were unchanged compared with BMP-2-stimulated cells examined by qRT-PCR using a real time-PCR (Fig. 4D, panel ii). On the other hand, Osx mRNA expression level was decreased in cells transfected with miR-141 or -200a concurrent with the decrease of Dlx5 protein expression (Fig. 4D, panel ii). These results further supported that the levels of Osx are transcriptionally controlled by Dlx5 via BMP signaling. Furthermore, it was shown that the expression levels of Dlx5 and Osx in the cells transfected with antisense inhibitors for miR-141 or -200a were up-regulated compared with those in BMP-2-treated control cells (Fig. 4E). Taken together, these findings suggested that the miR-141 and -200a regulate the expression of Dlx5 at the translational level and indirectly affect the transcriptional expression of Osx.

Dlx5 Is a Common Target of miR-141 and -200a—To determine the target region of miR-141 and -200a for Dlx5 mRNA, we constructed four types (A–D) of luciferase reporter plasmids examined, including the 3′-UTR of Dlx5 mRNA (Fig. 5A). B. Luciferase activities were measured by the using the luciferase assay system (Promega) according to the manufacturer’s protocol. The luciferase activity per 1 μg of protein was measured, and the relative luciferase activities were expressed as the ratio of that in treated cells to that in the control cells. The activity value of miR-NC (nonspecific control) is expressed as 100% in each group. The values of each group were expressed as mean ± S.E. of three separate experiments. Values are significantly different as indicated. *, p < 0.01 by Student’s t test. p < 0.05 is significant. C, mutation within or without the region corresponding to the seed sequence of miR-141 and -200a was made in each mutant from pGL3-Dlx5/miR-141 and -200a sensor-B. D, MC3T3-E1 cells were co-transfected with the pGL3-Dlx5/miR-141 and -200a sensor-B or mutated pGL3-Dlx5/miR-141 and -200a sensor-B (Mut1–3) and miR-141, -200a, or miR-NC. The activity of miR-NC (nonspecific control) is expressed as 100% in each group. The values of each group were expressed as mean ± S.E. of three separate experiments. Values are significantly different as indicated. *, p < 0.01 by Student’s t test. p < 0.05 is significant.

**FIGURE 5. Identification of the target gene of miRNA-141/-200a by luciferase reporter assay. A**, schematic representation of the sensor vectors used in the luciferase assay for identification of target region in Dlx5 for miR-141 and -200a. Construction of four kinds (A–D) of luciferase reporter plasmids is shown, including the 3′-UTR of Dlx5 mRNA. B, luciferase activities were measured by the using the luciferase assay system (Promega) according to the manufacturer’s protocol. The luciferase activity per 1 μg of protein was measured, and the relative luciferase activities were expressed as the ratio of that in treated cells to that in the control cells. The activity value of miR-NC (nonspecific control) is expressed as 100% in each group. The values of each group were expressed as mean ± S.E. of three separate experiments. Values are significantly different as indicated. *, p < 0.01 by Student’s t test. p < 0.05 is significant.
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counter, the level of luciferase activity in cells transfected with the mutant of the non-seed region was significantly suppressed compared with that in the nonspecific control sensor vector. From these results, it was strongly suggested that the target region of Dlx5 is the common binding site for miR-141 and -200a.

**DISCUSSION**

Several osteogenic differentiation-related miRNAs, such as miR-26a, -133, and -135, have been reported to be involved in the differentiation from mesenchymal cell to immature osteoblast (15, 18). In this study, to search for the miRNAs related to BMP-stimulated differentiation from pre-osteoblast to mature osteoblast, we used mouse pre-osteoblast MC3T3-E1 cells. Based on the analysis of differential miRNA arrays, we have selected and focused on miR-141 and -200a. Transfection experiments with miR-141 or -200a showed significant suppression of the ALP activity, which is widely accepted as a potential osteoblast differentiation marker. Moreover, we identified Dlx5 as a target gene for miR-141 and -200a by using a sensor luciferase reporter assay. Taken together, we demonstrate for the first time that miR-141 and -200a are involved in BMP-2-induced mouse pre-osteoblast differentiation by targeting Dlx5. It has been reported that BMP-2 up-regulates the expression of osteogenic master transcriptional factors, such as Runx2 (Runt-related transcription factor 2), Osx, and Dlx5 (19–21). Ulsamer et al. (17) and Lee et al. (22–25) reported that Dlx5 modulates BMP-2-stimulated Runx2 and Osx expression. Shirakabe et al. (26) also have shown that Dlx5 and msh homeobox 2 regulate the transcription activity of Runx2. Based on these findings, Dlx5 is considered to play an important role in osteogenic differentiation; therefore, it is of great interest to note that miR-141 and -200a control Dlx5 expression.

Recently, we have obtained the reciprocal expression profiles of miR-141/-200a and Dlx5 protein during BMP-2 treatment in MC3T3-E1 cell differentiation and primary murine osteoblast cell differentiation, i.e. miR-141 and -200a were up-regulated. However, it was thus considered that miR-141 and -200a are involved in the osteogenic differentiation by targeting Dlx5 (supplemental Fig. 1). In osteogenesis from mesenchymal cells, several transcriptional factors, which are spatiotemporally expressed, control the gene expression positively or negatively in osteogenic differentiation (27). Recently, Li et al. (28) reported that the expression of miR-29b was up- and down-regulated depending on the differentiation stages such as proliferation, matrix maturation, and mineralization. Therefore, the difference in miR-141 and -200a expression profiles between BMP-2-treated MC3T3-E1 cells and primary murine osteoblast cells is likely to be due to their different differentiation stage.

Davis et al. (29) have shown that maturation of miR-21 was modulated by interaction between receptor-regulated Smads and RNA helicase p68-DROSHA complex in BMPs and transforming growth factor-β signaling. It was also suggested by Fukuda et al. (30) that the MH2 domain of Smad1 interacts with a subunit of RNA helicase p68 in the miRNA processing pathway. From these studies, we can assume that the BMP-induced transcription factors, such as Smads, possibly regulate the expression of miR-141- and -200a in BMP-2-mediated osteogenic differentiation. Elucidation of the mechanism underlying the modulation of BMP-2-mediated osteoblast differentiation by miR-141 and -200a should await further study.

In conclusion, we have found that miR-141 and -200a play pivotal roles in regulation of BMP-2-mediated differentiation of mouse pre-osteoblasts by targeting Dlx5. It can be reasonably assumed that these miRs may also target human Dlx5 from the nucleotide sequence analysis. Thus, it is conceivable that they can be potent candidates as osteoblast differentiation biomarkers for the development of preventive or therapeutic agents for osteogenic disorders.

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