MiR-17-5p modulates osteoblastic differentiation and cell proliferation by targeting SMAD7 in non-traumatic osteonecrosis

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MicroRNAs (miRNAs) have recently been recognized to have a role in human orthopedic disorders. The objective of our study was to explore the expression profile and biological function of miRNA-17-5p (miR-17-5p), which is well known to be related to cancer cell proliferation and invasion, in osteoblastic differentiation and in cell proliferation. The expression levels of miR-17-5p in the femoral head mesenchymal stem cells of 20 patients with non-traumatic osteonecrosis (ON) and 10 patients with osteoarthritis (OA) were examined by quantitative reverse transcription-PCR (qRT–PCR). Furthermore, the interaction between miR-17-5p and SMAD7 was observed. We found that in non-traumatic ON samples the level of mature miR-17-5p was significantly lower than that of OA samples (P = 0.0002). By targeting SMAD7, miR-17-5p promoted nuclear translocation of β-catenin, enhanced expression of COL1A1 and finally facilitated the proliferation and differentiation of HMSC-bm cells. We also demonstrated that restoring expression of SMAD7 in HMSC-bm cells partially reversed the function of miR-17-5p. Together, our data suggested a theory that dysfunction of a network containing miR-17-5p, SMAD7 and β-catenin could contribute to ON pathogenesis. The present study prompts the potential clinical value of miR-17-5p in non-traumatic ON.

INTRODUCTION
Osteonecrosis (ON) of the femoral head is a common disease that regularly affects patients aged 30–50 years and is characterized by destruction of the blood supply of the femoral head.¹,² The causes of non-traumatic ON are complex.³ One study indicated that altered osteogenic differentiation capability of mesenchymal stem cells (MSCs) is associated with non-traumatic ON, and the differentiation potential of MSCs in patients with non-traumatic ON differs according to the risk factor.³ This finding implies one possible theory, that is, the decreased replication capacity of MSCs or decreased MSC activity in the bone marrow may have an important role in non-traumatic ON.⁴

MicroRNAs (miRNAs) are small, noncoding RNAs that potentially regulate thousands of genes.⁵–⁸ It is believed that miRNAs manipulate 30% of the human genome, primarily by binding to the 3'-untranslated region (UTR) of their target mRNAs, resulting in translational repression or mRNA degradation.⁹–¹¹ Although miRNAs are known to behave as regulators in many diseases, there have been few reports that expound on osteoblastic genesis-related miRNAs.⁷,¹² MiRNA-17-5p (miR-17-5p) has been proven to regulate cell proliferation and differentiation in lung, breast and colorectal cancer cell lines.¹³–¹⁵ Therefore, miR-17-5p is one of the most representative miRNAs that can impact proliferation and differentiation. However, the relationships between miR-17-5p, MSCs and non-traumatic ON remain unclear.

In the present study, we showed a discrepancy in the expression levels of miR-17-5p in MSCs of non-traumatic ON patients and hip osteoarthritis (OA) patients. To reveal the potential etiology of non-traumatic ON, we performed a functional study and identified that miR-17-5p could promote the osteogenesis of MSCs through directly targeting and
downregulating SMAD7. Furthermore, it has been reported that forced expression of SMAD7 could retard osteoblastic cell proliferation stimulated by TGF-β1,16 suggesting that miR-17-5p might regulate osteoblastic cell proliferation in part by restraining SMAD7 expression. Therefore, miR-17-5p might be used as a potential biomarker and therapeutic target of non-traumatic ON in the future.

MATERIALS AND METHODS

Subjects

Upon approval from the ethics committee of Union Hospital and obtaining written informed consents, bone marrow samples were obtained from non-traumatic ON patients (n = 20) and OA patients (n = 10) undergoing surgery between April 2008 and July 2010 at Wuhu Union Hospital, Huazhong University of Science and Technology. Bone marrow samples (5 ml) were obtained from the proximal end of the femurs while inserting a tapered awl into the femoral canal during total hip arthroplasty. Non-traumatic ON was diagnosed based on radiographic and magnetic resonance imaging. OA was confirmed using radiographic images, and the diagnostic criteria were satisfied for all patients. Of the 20 patients with non-traumatic ON, 11 patients were treated with corticosteroids, 7 patients suffered from alcoholism and 2 patients were diagnosed with idiopathic ON. The clinical characteristics of all patients are shown in Table 1. An ALP staining kit (Beyotime Institute of Biotechnology, Nanjing, Jiangsu, China) was used according to the procedures of the manufacturer and of a previous report.17

Regulation of the miR-17-5p levels

To alter miR-17-5p expression, cells were transiently transfected with has-miR-17-5p mimics (final concentration, 100 nM) or has-miR-17-5p inhibitor (final concentration, 100 nM) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). For osteoblastic differentiation, the medium was removed 6 h after transfection and substituted for fresh medium containing 10% FBS and 100 ng ml⁻¹ BMP-2 (R&D Systems, Minneapolis, MN, USA).

Quantitative real-time PCR

Total RNA of cells or tissues was extracted using the Trizol reagent (Invitrogen). qRT–PCR reactions were carried out with the Power SYBR Green PCR Master Mix and 7900HT thermocycler (Applied Biosystems, Carlsbad, CA, USA). Data were analyzed using the SDS Relative Quantification Software version 2.2 (Applied Biosystems), with the automatic threshold cycle (Ct) settings at baseline and the threshold for ΔCt determination; expression of the U6 small nuclear RNA gene was used as an internal control. The results are presented as the comparative Ct for each miRNA. Each qRT–PCR was performed in triplicate. The 20-μl qRT–PCR reaction contained the following reagents: 9 μl SYBR Green Mix, 2 μl miRNA reverse transcription (RT) product, 2 μl Bulge-Loop miRNA Forward Primer (5 μM), 2 μl Bulge-Loop miRNA Reverse Primer (5 μM; RiboBio Co., Ltd, Guangzhou, Guangdong, China) and RNase-free H₂O in a final volume of 20 μl. The reactions were incubated at 95°C for 20 s, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s and 70°C for 10 s and finally 95°C for 10 s, 60°C for 20 s and 95°C for 10 s.

miRNA target prediction and luciferase reporter assay

TargetScan version 6.2 (http://www.targetscan.org/vert_50) and PicTar (http://picTar.mdc-berlin.de/) were used to predict the miR-17-5p targets. Information on the complete computational protocols are available at the corresponding websites. The miR-17-5p target region of the SMAD7-3′UTR (sequence containing 5′-AAAUAAGAAAA-GAUGCCAUUG-3′, part of the NCBi ReSeq ID of NM_005904) was inserted into the pmir-RB-Report vector and named SMAD7-3′UTR WT (RiboBio Co., Ltd). As a control, the plasmid (sequence 5′-AAAUAAGAAAAAGUGAAG-3′) containing the mutant sequence of the miR-17-5p target region of the SMAD7-3′UTR was also inserted into the pmir-RB-Report vector and named SMAD7-3′UTR MT. Co-transfection of 200 ng SMAD7-3′UTR WT or SMAD7-3′UTR MT with 100 nM of miR-17-5p mimic or miR-17-5p inhibitor and their negative controls was carried out using jetPRIME (Polyplus-transfection Inc., New York, NY, USA) in 48-well plates, respectively. Each experiment was repeated four times.

Preparation of cytoplasmic and nuclear fractions

The NE-PER kit ( Pierce, Waltham, MA, USA) was used to prepare cytoplasmic and nuclear fractions.18 Cell membranes were destroyed to deliver the cytoplasmic contents. Integrated nuclei were retrieved from the cytoplasmic extracts by centrifugation, and the nuclei were washed with PBS and disrupted to harvest the nuclear extract. Blots were visualized using an Odyssey IR scanner (Li-Cor, Lincoln, NE, USA) with the Odyssey imaging software 3.0 (Li-Cor).

Table 1 Characteristics of patients with osteoarthritis and osteonecrosis

| No. of patients | Age (years) | Gender (F/M) |
|----------------|-------------|--------------|
| Osteoarthritis | 10          | 53 ± 7       | 7/3          |
| Osteonecrosis  |             |              |              |
| Alcohol        | 7           | 52 ± 14      | 1/6          |
| Steroid        | 11          | 49 ± 15      | 5/6          |
| Idiopathic     | 2           | 52 ± 8       | 2/0          |

Abbreviations: F, female; M, male.
Western blotting

Extracts of cell lysate and tissue were analyzed by western blotting using the IRDye 800CW-conjugated antibody and LI-COR imaging system (LI-COR Biosciences, Lincoln, NE, USA) according to the manufacturer’s instructions. Membranes were scanned and analyzed using an Odyssey IR scanner and Odyssey imaging software 3.0.

Osteoblastic differentiation

Osteoblastic differentiation was achieved by administering media containing 10% FBS and 100 ng ml\(^{-1}\) BMP-2 (R&D Systems).

Statistics

Experimental data were formulated as the means ± s.d. of triplicate independent samples. As most of the variables were small, nonparametric tests were performed as the statistical method. Statistical significance for two groups was analyzed using the Mann–Whitney test. All other data were analyzed using Kruskal–Wallis analysis of variance, and the Dunn’s multiple comparison test was applied as a posttest. To access the correlation between the miR-17-5p and SMAD7 expression levels, the Pearson correlation coefficient was used. Statistical analyses were performed with SPSS 13.0 (SPSS Inc., Chicago, IL, USA) for Windows (Microsoft Corp., Seattle, WA, USA), and statistical significance was defined as \(P<0.05\).

RESULTS

Differential expression of miR-17-5p between MSCs of non-traumatic ON patients and OA patients

To study the expression of miR-17-5p in non-traumatic ON, we first evaluated the expression of miR-17-5p in all MSC samples from non-traumatic ON patients and OA patients using quantitative PCR (Figure 1a). The expression levels of mature miR-17-5p were lower in the MSCs from the non-traumatic ON group compared with the OA group. All samples were categorized by etiology, and we found a significant difference in the level of miR-17-5p between the two groups (\(P=0.0002\)).

The elevation of expression of miR-17-5p is indispensable for BMP-2-induced osteoblastic differentiation

To determine the potential roles of miR-17-5p during proliferation and osteoblastic differentiation of MSCs, the expression pattern of miR-17-5p was analyzed in the stromal cell line HMSC-bm, which was derived from bone marrow. The results are summarized in Figure 1b. miR-17-5p expression was decreased after a temporary rise in the number of cells that proliferated spontaneously, but had no osteoblastic-inductive BMP-2, over 7 days, which indicated that miR-17-5p may positively regulate cell proliferation during MSC differentiation.

When HMSC-bm cells were treated with BMP-2, the drop in the level of miR-17-5p receded compared with that in cells with no additional osteoblastic-inductive material. We observed that the BMP-2-treated HMSC-bm cells maintained growth, even after long treatment duration. Taken together, these findings suggest that, upon BMP-2 treatment, cell proliferation is maintained by increasing the effect of miR-17-5p, that miR-17-5p may positively influence osteoblastic differentiation and that miR-17-5p should be low during osteoblastic differentiation and proliferation of MSCs.

SMAD7 is a target and is negatively regulated by miR-17-5p

To explore the mechanisms by which miR-17-5p effects proliferation and osteoblastic differentiation of MSCs, we chose the miRNA target prediction databases Pictar and TargetScan to determine the possible target genes of miR-17-5p. Based on results using both databases, SMAD7, which is involved in BMP-2-induced osteoblastic differentiation and contains a conserved 3'UTR element, was selected for further study (Figure 2a).

Figure 1 Differential expression of microRNA-17-5p (miR-17-5p) in patients and HMSC-bm cell lines. (a) Quantitative reverse transcription-PCR analysis of the expression of mature miR-17-5p in the primary culture cells obtained from OA and non-traumatic ON patients. The expression of mature miR-17-5p is significantly higher in cells from osteonecrosis (non-traumatic ON) compared with those from hip osteoarthritis (OA) patients (\(P=0.0002\)). (b) MiR-17-5p expression in HMSC-bm cells stimulated by BMP-2. Cells were harvested at the indicated times, and the endogenous expression level of miR-17-5p was measured. Solid and white bars indicate samples treated with and without BMP-2, respectively. *\(P<0.05\).
downregulate the luciferase activity of the SMAD7-3'UTR-WT plasmid, but not that of SMAD7-3'UTR-MT in comparison with the negative-control miRNAs (Figure 2b). Figure 2c demonstrates that the mRNA level of SMAD7 was markedly decreased or elevated after the administration of miR-17-5p mimics or miR-17-5p inhibitor, respectively, in HMSC-bm cells compared with control cells, and the protein level of SMAD7 displayed the same trend as that of the mRNA (Figure 2d). As expected, the negative correlation between miR-17-5p and SMAD7 in ON tissues of 20 patients was proven (Figure 2e, $R^2 = 0.5440$, $P < 0.001$). Furthermore, it has been reported that SMAD7 might act as a suppressor of bone formation, so we examined the enrichment of SMAD7 during osteoblast differentiation. Interestingly, SMAD7 expression was steadily downregulated during bone formation after administration of BMP-2, which was the opposite of the expression pattern of miR-17-5p (Figure 2f).

**MiR-17-5p has a key role in HMSC-bm osteoblastic differentiation and growth**

To investigate the effect of miR-17-5p on osteoblastic differentiation of HMSC-bm cells, we altered the miR-17-5p level in the cells. After transfection of miR-17-5p mimics or
miR-17-5p inhibitor, qRT–PCR analysis showed that the level of miR-17-5p was increased by the miR-17-5p mimics and decreased by the miR-17-5p inhibitor (Figure 3a), which showed that the cellular level of miR-17-5p in this system could be regulated by transfecting these reagents. Then, HMSC-bm cells were transfected with miR-17-5p mimics or miR-17-5p inhibitor accompanied by the induction of osteoblastic differentiation through the addition of BMP-2. The ALP activity and expression level of the RUNX2, COL1A1 and osteocalcin transcripts were measured to determine bone formation. As expected, compared with transfection of the negative control, the expression of RUNX2 and COL1A1 was increased by transfection of miR-17-5p mimics (Figure 3b). ALP staining was also promoted by transfection of miR-17-5p mimics (Figure 3c). Conversely, the expression of RUNX2 and ALP staining were both retarded by transfection of the miR-17-5p inhibitor (Figures 3b and c). Yet, late-stage osteoblastic markers, such as the absence of osteocalcin gene expression (the Ct value by qRT–PCR was greater than 35), were not observed in the cultures. Furthermore, CCK8 experiments showed that miR-17-5p mimics could boost, whereas miR-17-5p inhibitor impeded, the proliferation of HMSC-bm cells (Figure 3d). In conclusion, our data demonstrated that miR-17-5p has a potential role in facilitating osteoblastic differentiation and proliferation in cells in vitro.

**Negative modulation of SMAD7 expression by miR-17-5p is crucial for miR-17-5p-promoted HMSC-bm cell proliferation and bone formation**

Because miR-17-5p could repress the expression of SMAD7, we wondered if SMAD7 is responsible for the miR-17-5p-promoted HMSC-bm cell proliferation and osteoblastic differentiation. Therefore, we synthesized a SMAD7 expression plasmid using the pcDNA3.1-myc vector (named pcDNA3.1-SMAD7) to study its mechanisms. Figure 4 illustrates that SMAD7 could negatively regulate proliferation and the markers of bone formation; moreover, restoration of SMAD7 blocked the majority of miR-17-5p-induced proliferation (Figures 4a and c) and ALP staining (Figure 4b). In addition, qRT–PCR showed that SMAD7 reversed the upregulation of RUNX2 and COL1A1 that was generated by miR-17-5p (Figure 4c). Taken together, these data suggested that SMAD7 can depress HMSC-bm cell proliferation and bone formation ability, which is indispensable for the pro-osteoblastic differentiation capability of miR-17-5p exposed to BMP-2 stimuli.
The reduction of SMAD7 upon miR-17-5p exposure results in nuclear translocation and transactivation of β-catenin signaling

It has been reported that BMP-2 promotes bone formation by stabilizing β-catenin and increases the transcription of nuclear β-catenin. We wondered whether miR-17-5p facilitates the nuclear translocation of β-catenin by inhibiting the activity of SMAD7. After co-transfecting a β-catenin reporter gene with miR-17-5p mimics or inhibitor, we observed that the activity of β-catenin signaling was increased or decreased, respectively (Figure 5a). Meanwhile, restoration of SMAD7 expression effectively eliminated the miR-17-5p-enhanced, β-catenin reporter gene activity (Figure 5a). Furthermore, transfection of miR-17-5p mimics or inhibitor only was sufficient to enhance or reduce the amount of nuclear β-catenin (Figure 5b, left panel). Similar to the reporter gene assay, overexpression of SMAD7 could abrogate the miR-17-5p-promoted accumulation of β-catenin in the nucleus (Figure 5b, right panel). Taken together, our data suggested that the cytoplasmic-nuclear translocation of β-catenin is responsible for the osteoblastic differentiation and proliferation of HMSC-bm cells that are regulated by miR-17-5p/SMAD7-signaling.

DISCUSSION

As one of the most common diseases observed by orthopedic surgeons, ON is a disorder that is difficult to diagnose because of the lack of early clinical symptoms. As with many other diseases with high morbidity and disability, vigilance and prediction can reveal the patients suffering from the illness but lacking severe symptoms. Most ON results from direct trauma, but non-traumatic ON should be suspected in patients with predilection for the disease. ON may result from direct trauma and non-traumatic causes, such as inflammation and other additive stresses on the anatomic and vascular integrity of the involved region.

In the present study, we reported that miR-17-5p, which is located in the miR-17/92 cluster and has important roles in various cancers, regulated osteoblastic differentiation and cell proliferation through directly targeting SMAD7. We identified miR-17-5p as a potential biomarker by measuring its diverse expression in non-traumatic ON patients and OA patients, but it should be emphasized that further studies with larger prospective cohorts should be conducted to verify the sensitivity and specificity of this miRNA biomarker. MiR-17-5p has been reported to facilitate the cell proliferation of different human cancer cells by inhibiting...
The cytokine BMP-2, which activates a pathway that includes β-catenin protein phosphorylation and nuclear translocation, is highly expressed and stimulates osteoblast differentiation. In our present studies, we observed that the increased level of BMP-2 in HMSC-bm cells is responsible for the induction of miR-17-5p, which may further reinforce β-catenin-induced cell proliferation and osteoblastic differentiation, thus forming a positive-feedback loop.

In summary, BMP-2-induced expression of miR-17-5p has an important role in non-traumatic ON via directly repressing the transcription of SMAD7 and enhancing the activity of the β-catenin signaling pathway, which sheds light on the understanding of miRNA biology for resolving bone diseases such as non-traumatic ON. Illustrating the functions of miRNAs in osteoblast regulation in vivo could further help to find novel mechanisms of bone metabolism and differentiation.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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