MicroRNA-186-5p mediates osteoblastic differentiation and cell viability by targeting CXCL13 in non-traumatic osteonecrosis

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Abstract. MicroRNAs (miRs) serve varying and important roles in the pathogenesis of non-traumatic osteonecrosis (ON). However, the role miR-186-5p serves in the pathogenesis of osteonecrosis remains unknown and the clinical outcome of ON is still uncertain. The aim of the present study was to determine the expression characteristics, biological function and molecular mechanisms of miR-186-5p, which is associated with cancer development and progression, in osteoblastic differentiation and cell viability. The results of the present study showed that the expression levels of miR‑186‑5p were significantly higher in clinical non‑traumatic ON compared with osteoarthritis samples (P=0.0001). An inverse association was observed between miR-186-5p and CXCL13 expression levels. Furthermore, miR-186-5p inhibited phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT) signaling, downregulated osteoblast-specific markers and reduced the viability and differentiation of human mesenchymal stem cells from bone marrow (HMSC-bm) through targeting cXcl13. Increasing expression of cXcl13 in HMSC-bm cells partially restored miR-186-5p-mediated inhibition. In conclusion, abrogation of PI3K/AKT signaling triggered by miR-186-5p/CXCL13 may contribute to ON pathogenesis. These results highlight the possible clinical value of miR-186-5p in treatment for non-traumatic ON.

Introduction

Non-traumatic osteonecrosis (ON) arises from increased intraosseous pressure caused by fat cell hypertrophy and abnormal adipogenesis in the bone marrow, which subsequently results in ischemia which leads to osteocyte and bone marrow cell (BMC) death (1). The incidence of ON has been increasing, especially in men between 30 and 50 years of age (2-4). The underlying pathogenesis of ON is multifactorial, including adipogenesis or fat hypertrophy, imbalance of osteoblasts, angiogenesis inhibition and apoptosis of endothelial cells (5-7). ON therapy primarily includes conventional core decompression and joint replacement surgery. However, this treatment only achieves a moderate decrease in intra-medullary pressure, with few clinical benefits and a high expense (8).

Based on the theory that mesenchymal stem cells (MSCs) are pluripotent cells which can differentiate into a range of cell types, transplantation of MSCs is used as a novel treatment strategy. For example, implantation of autologous BMcs has been successfully applied in treating early stages of ON (9,10). However, several studies indicate that decreased osteogenic differentiation and replication capacity of MSCs in the bone marrow also have crucial roles in non-traumatic ON (11), and abnormal MSCs are observed after transplantation of MSCs (12,13), thus limiting its application.

MicroRNAs (miRNAs/miRs) are small, endogenous, noncoding RNAs that potentially regulate a large number of genes (14-16). miRNAs are thought to modulate 30% of the human genome through base-pairing with 3′untranslated regions (UTRs) of their target mRNAs and ultimately cleaving mRNA or repressing translation mRNAs (14-17). Although miRNAs serve as regulators in a number of biological processes, including morphogenesis, differentiation, proliferation and tumorigenesis (18), there are relatively fewer reports examining the role of miRNA in the pathogenesis of ON (19,20).

miR-186 has been reported to serve as an inhibitor of tumor cell growth and migration in various types of cancer such as lung cancer (21,22) and colorectal cancer (23). In the present study, the role of miR-186-5p in osteoblastic differentiation and cell growth of ON patients was determined. miR-186-5p expression was relatively higher in MSCs of patients with non-traumatic ON and inhibited the viability and differentiation of osteoblastic cells in human mesenchymal stem cells.
(HMSCs). CXCL13 was a functional target of miR-186-5p in HMSCs. In addition, miR-186-5p modulated protein kinase B (AKT) signaling in HMSCs. These findings suggest that miR-186-5p may be a potential molecular target for treating patients with non-traumatic ON.

Materials and methods

Clinical samples. The present study was approved by the Ethics Committee of Wuhan Union Hospital, bone marrow samples were obtained with informed consent from 15 ON patients and 15 osteoarthritis (OA) patients receiving surgery between October 2010 and July 2013 at Wuhan Union Hospital, Huazhong University of Science and Technology. Patient characteristics are shown in Table I.

Bone marrow samples of 5-10 ml from the proximal end of the femur of each patient were taken by inserting a tapered awl into the femoral canal during total hip arthroplasty. OA was diagnosed using radiographic images, and the diagnostic criteria were satisfied for all patients. Non-traumatic ON was confirmed based on radiographic and magnetic resonance imaging. Of the 15 patients with non-traumatic ON, nine were treated with corticosteroids, five suffered from alcoholism and one was diagnosed as idiopathic ON. The clinical characteristics of patients are shown in Table I. To obtain mesenchymal stem cells (MSCs), mononuclear cells from the bone marrow were obtained using Ficoll-Hypaque gradient centrifugation (Fresenius Kabi in orgea G), suspended in low-glucose Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences) containing 10% fetal bovine serum (FBS; Gibco: Thermo Fisher Scientific, Inc.) and seeded at a density of 1x10⁶ cells per cm².

Cell culture. 293T cells and HMSC-bm cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences and cultured in MSC growth medium consisting of low-glucose DMEM supplemented with 10% FBS and maintained in a cell incubator at 37°C with 5% CO₂. Cells were cultured in flasks and upon reaching 90% confluency, cells were passaged at 1:2 dilution. Cells between the 4 and 7th passages were used for the following experiments. Osteoblastic differentiation was accomplished using medium containing 100 ng/ml BMP-2 (R&D Systems China, Co., Ltd.) and 20 nM of the RNA s mentioned above were prepared for transfection.

RNA-seq and data analysis. RNA sequencing was performed at Guangzhou RiboBio Co., Ltd., using an Illumina HiSeq 2500. RNA-seq data was aligned to the Ensembl transcript annotations (GenBank Assembly ID GCA_000001405.25) using bowtie and RSEM as previously described (24). Cufflinks (version 2.2.1) (25) was used to estimate gene expression abundance (number of fragments per kb of each gene per million mapped reads). Differentially expressed genes were detected using DESeq (version 1.16.0) (26) and a false discovery rate <0.01 and a fold-change >1.25 were used as thresholds to define significantly differentially expressed genes. Other bioinformatics analyses were performed using gBASE (27) and additional; details are provided in Fig. S1 and Table SIII.

MTT assays. For the cell viability assay, cells were seeded into a 96-well plate (1x10⁵ cells/well) and used to perform MTT assays (Sigma-Aldrich; Merck KGaA). MTT diluted in PBS (5 mg/ml) was added to each well (20 µl per well) and incubated for 4 h. Formazan was then dissolved in DMSO (200 µl per well) and the absorbance value at 490 nm (optical density, 490 nm) was determined.

Reverse transcription-quantitative PCR (RT-qPCR). The total RNA of samples or cells was extracted using a miRNeasy mini kit or RNeasy kit (Qiagen China Co., Ltd.). RNA was reverse transcribed using Transcriptor (Roche Diagnostics) with an oligo-dT primer or the Bulge-loop® miRNA qRT-PCR Primer Sets specific for miR-miR-186-5p or U6 (Guangzhou Ribobio Co., Ltd.) according to the manufacturer's protocol. Briefly, the reactions were incubated for 60 min at 42°C and 10 min at 70°C and were then stored at -20°C. RT-qPCR analysis was performed using a LightCycler 96 System (Roche Diagnostics) with SYBR Green PCR Master Mix (Takara Bio, Inc.). In brief, the reactions were incubated at 95°C for 20 sec, followed by 40 cycles of 95°C for 10 sec and 60°C for 35 sec. A dissociation stage was performed at the end of the amplification procedure to determine potential non-specific amplifications. U6 and GAPDH were used as the internal control. Relative gene expression in functional assay was analyzed using the 2−ΔΔCq method (28) and present with fold-changes. Relative gene expression in results of clinical samples was present with -ΔCq [ΔCq=Cq (Gene)-Cq (U6)]. Primer used for RT-qPCR were listed in Table SI.

Transfection. To determine the effects of miR-186-5p on cellular function, a miR-186-5p mimic, miRNA mimic control, anti-miR-186-5p, non-specific anti-miRNA control and small interfering (si) RNAs targeting human CXCL13 (si-CXCL13) were all synthesized by Guangzhou RiboBio Co., Ltd. For transfection, INTERFERin® (Polyplus-transfection SA) and 20 nM of the RNAs mentioned above were prepared according to the manufacturer's protocol and mixed with cells in 24-well cell culture plates when the cells were at 50% confluency. The medium was replaced with fresh medium 12 h after transfection.

MicroRNA target prediction and luciferase reporter assays. TargetScan version 7.2 (http://www.targetscan.org/vert_72/) was used to predict the targets of miR-186-5p. The complete computational protocol is available at the above website. The miR-186-5p target region containing the CXCL13 3′UTR (5′-GGACUCUGGUAUCUAUIUUCUUA-3′) was inserted into the pmirGLO luciferase reporter vector (Promega Corporation). As a control, a plasmid containing mutant sites (sequence 5′-GGACUCUGGUAUCUAUGAAGAA-3′) of the miR-186-5p target region in the CXCL13 3′UTR was also inserted into the pmirGLO luciferase reporter vector. Co-transfection of 200 ng wild type (WT) or mutant (mut) CXCL13 3′UTR with 100 nM of miR-186-5p mimic or anti-miR-186-5p and their respective negative controls into
Table I. Clinical characteristics of patients.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of patients</th>
<th>Age, years</th>
<th>Sex, F/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteoarthritis</td>
<td>15</td>
<td>52±6</td>
<td>8/7</td>
</tr>
<tr>
<td>Osteonecrosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol</td>
<td>5</td>
<td>53±10</td>
<td>1/4</td>
</tr>
<tr>
<td>Steroid</td>
<td>9</td>
<td>46±14</td>
<td>4/5</td>
</tr>
<tr>
<td>Idiopathic</td>
<td>1</td>
<td>52</td>
<td>1/0</td>
</tr>
</tbody>
</table>

F, female; M, male.

Results

miR-186-5p expression is significantly increased in ON of formal head (ONFH) and modulates MSC growth and differentiation. To identify novel functional miRNAs in ONFH progression, miR-186-5p expression levels were determined in HMSCs from patients with OA and non-traumatic ON. RT-qPCR analysis showed that miR-186-5p levels were significantly increased in HMSCs from the ON group compared with the patients with OA (P<0.0001; Fig. 1A). To determine the underlying roles of miR-186-5p during osteoblastic differentiation of HMSCs, the expression of miR-186-5p was analyzed in HMSC-bm. As shown in Fig. 1B, compared with MSCs from the negative control group, miR-186-5p expression levels increased at a slower rate when treated with BMP-2 for 6 days, suggesting that miR-186-5p may affect cell viability during MSC differentiation. These results also indicated that during growth and differentiation of HMSCs, the expression of miR-186-5p was elevated and cell differentiation and viability was negatively regulated by miR-186-5p. When the cells reached confluence, miR-186-5p expression levels decreased. Consistent with a previous study (21) showing that miR-186-5p negatively modulates cell proliferation, overexpression of miR-186-5p significantly suppressed HMSC-bm viability (P<0.05; Fig. 1C-E) and osteoblastic differentiation (Fig. 1F and G), whereas inhibition of miR-186-5p exerted the opposite effects (Fig. 1C-G). Taken together, these results demonstrate that miR-186-5p participates in the disease course of OA through negatively regulating viability and differentiation of MSCs.

CXCL13 is a direct target of miR-186-5p in HMSCs. To determine the mechanism of how miR-186-5p suppressed differentiation of MSCs, the targets of miR-186-5p in HMSCs were determined. Bioinformatics analysis predicted that the 3’UTR of CXCL13 mRNA was a potential target of miR-186-5p (Fig. 2A and Table SII). Furthermore, RNA-seq was performed on MSCs from three patients with ON and three patients with OA patients. The potential functions of these differentially expressed mRNAs were identified using KEGG pathway analyses. The differentially abundant genes between OA and ON patients were significantly associated with Cytokine-cytokine receptor interaction pathways (Fig. S2). Specifically, expression of CXCL13 was downregulated in patients with ON (Fig. 2B; Table SIII). In addition, miR-186-5p expression was negatively correlated with CXCL13 mRNA levels in bone marrow samples from patients with ON patients (Fig. 2C). The dual-luciferase reporter assays suggested that transfection with miR-186-5p mimic or anti-miR-186-5p significantly reduced or increased, respectively the luciferase activity of WT CXCL13 3’UTR (P<0.05), whereas neither
had any significant effects on the mut 3'UTR (Fig. 2D). These results suggest that miR-186-5p directly targets the 3'UTR of CXCL13. Transfection of miR-186-5p mimics or anti-miR-186-5p mimics into HMSCs cells or primary MSCs from patients with OA resulted in both mRNA and protein expression levels of CXCL13 being significantly reduced or increased, respectively (P<0.05; Fig. 2E-G). These results suggested that miR-186-5p directly targeted and abrogated CXCL13 expression in HMSCs.

miR-186-5p suppresses cell viability and osteoblastic differentiation by inhibiting CXCL13. Downregulation of CXCL13 mRNA levels was first observed in the RNA-seq data (Fig. 2B) and the results were validated in bone marrow tissues from patients with ONFH.
patients with ON and OA patients using RT-qPCR (Fig. 2H). Compared with MSCs from the negative control group, CXCL13 expression levels increased faster when treated with BMP-2 for 6 days, suggesting that CXCL13 may serve an antagonistic role against miR-186-5p in MSC differentiation (Fig. 2I). Furthermore, the results showed that CXCL13 overexpression promoted cell viability and osteoblastic differentiation of MSCs, and reduction of CXCL13 mRNA expression levels had the opposite effects (Fig. 3A-E). To further investigate whether miR-186-5p modulated CXCL13 expression levels, rescue assays were performed. HMSCs cells were co-transfected with miR-186-5p mimic and a plasmid expressing CXCL13. As shown in Fig. 3, the inhibitory effects of miR-186-5p on CXCL13 were partially reversed after...
transfection with the plasmid (Fig. 3F-I). These results suggest that miR-186-5p targets and regulates CXCL13 in MSCs.

**miR-186-5p/CXCL13 modulates AKT/ERK signaling to regulate cell growth and osteoblastic differentiation.** It has been reported that CXCL13 promotes growth and invasion of colon cancer cells via the PI3K/AKT signaling pathway (31). To determine the underlying mechanism by which the miR-186-5p/CXCL13 axis in ON regulated cell growth and differentiation, the AKT signaling pathway in HMSC-bm cells was examined. Western blot analysis showed that overexpression of miR-186-5p significantly suppressed AKT/ERK signaling (Fig. 4). In addition, when the AKT signaling activator, LiCl, was added to miR-186-5p-overexpressing HMSC-bm cells, the MTT assays suggested that LiCl could restore the negative effects of miR-186-5p on HMSC-bm cell viability (Fig. 4A and B). Furthermore, transfection with the CXCL13 vector also partially rescued the growth of HMSC-bm cells when co-transfected with miR-186-5p (Fig. 4C and D). These data suggest that miR-186-5p/CXCL13 affects MSC growth through modulating PI3K/AKT signaling.

**Discussion**

Accumulating evidence has shown that miRNAs function by modulating various biological processes in human MSCs. In the etiopathogenesis of non-traumatic ON, certain microRNAs, such as miR-17-5p, miR-125 and miR-135 have been identified as
modulators of cell growth and differentiation (32,33), highlighting the importance of miRNAs in osteonecrosis diagnosis and treatment. However, the functional significance of miRNAs is not yet completely understood. In the present study, it was demonstrated that expression of miR-186-5p was increased in the HMSCs of patients with ON and served important roles in regulating cell growth and osteoblastic differentiation through directly targeting CXCL13. The data of the present study demonstrated that the expression of miR-186-5p was altered when CXCL13 expression was increased during osteoblast differentiation of HMSCs, suggesting their involvement in bone development. Furthermore, miR-186-5p overexpression suppressed activation of the AKT/ERK signaling pathway. Specific activation of AKT/ERK signaling rescued the effects of miR-186-5p in HMSCs.

Chemokines are the largest family of cytokines, with at least 47 members which are divided into four major categories, the CX3C, CXC, CC and C subfamilies, based on the spacing and number of conserved cysteine residues in the amino acid sequences. Chemokines bind to seven transmembrane-spanning G-protein-coupled receptors to exert influence on various biological functions (34). CXCL13 directly modulates cellular proliferation and viability induces migration of MSCs to move to the injured area and induces differentiation of osteoblasts (35-37).

Although CXCL13 was considered to be a critical regulator of MSC differentiation, none of characteristics were significantly correlated with CXCL13 expression in the 15 patients enrolled in the present study (Data not shown), given the small sample size. This result should be further explored within a larger cohort.

The RNA-seq analysis results showed that CXCL13 expression was downregulated in HMSCs of patients with ON. In addition, CXCL13 mRNA expression levels were negatively correlated with miR-186-5p expression levels in bone marrow samples in patients with ON, miR-186-5p downregulated mRNA as well as the protein expression levels of CXCL13 in HMSCs and increasing the levels of CXCL13 partially restored miR-186-5p-hindered osteoblastic differentiation, and cell viability. The present study also predicted the underlying functions of differentially abundant mRNAs using KEGG pathway analyses. These findings corroborated the hypothesis that one of the most significantly important pathways in the development of osteonecrosis was cytokine-cytokine receptor interaction, which was also identified by previous studies (38,39). Taken together, the present study highlighted the important role of miRNA-CXCL13 regulation in patients with non-traumatic ON and may serve as potential therapeutic targets for treating patients with ON.
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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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The authors declare that they have no competing interests.

References


