Krüppel-like factor 4 promotes the proliferation and osteogenic differentiation of BMSCs through SOX2/IGF2 pathway

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Objectives: Human bone marrow mesenchymal stem cells (BMSCs) have multi-lineage differentiation potential and have been widely researched in regenerative medicine. The purpose of this research was to explore whether Krüppel-like factor 4 (KLF4) can regulate the osteogenic differentiation of BMSCs. Methods: We transfected human BMSCs with KLF4 overexpression plasmid and si-KLF4 to study the effects of KLF4. We performed cell proliferation assay, flow cytometry and Alizarin Red staining on BMSCs. Quantitative real-time PCR and western blot was performed to determined mRNA and protein expression of osteogenic differentiation markers, KLF4, SOX2 and IGF2. Bone defect animal model was created and the adenovirus containing KLF4 overexpression or knockdown plasmid was injected. Finally, HE staining was performed on tibia to assess the bone regeneration. Results: Our results showed that KLF4 promotes not only the growth of BMSCs, but also their osteogenic differentiation. Also, it mediated these effects through SOX2/IGF2 signaling pathway. In addition, KLF4 overexpression could increase the bone regeneration in in-vivo model, whereas KLF4 knockdown decreased the bone regeneration. Conclusions: KLF4 regulates BMSC’s osteogenic differentiation via SOX2/IGF2 pathway.

Keywords: Krüppel-like factor 4; human bone marrow mesenchymal stem cells; SOX2; IGF2; osteogenic differentiation.

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Purpose of this research was to explore if KLF4 could promote the proliferation and osteogenic differentiation of BMSCs through SOX2/IGF2 pathway. Our results showed that KLF4 promotes not only the growth of BMSCs, but also their osteogenic differentiation. Also, it mediated these effects through SOX2/IGF2 signaling pathway. In addition, KLF4 overexpression could increase the bone regeneration in in-vivo model, whereas KLF4 knockdown decreased the bone regeneration. Therefore, we assumed that KLF4 might play a role in the osteogenic differentiation of BMSCs.

INTRODUCTION

Mesenchymal stem cells in bone marrow have the potential to differentiate into various kinds of cells, for example endothelial cells, adipocytes, osteoblasts, myocytes and chondroblasts (Kolf et al., 2007). This characteristic of BMSCs has attracted much attention as they might be used for cell therapy or tissue engineering. They could be infuse to treat graft-versus-host disease, liver cirrhosis and neurodegenerative diseases and even be engineered into vessels (Rady et al., 2020; Sadapoor et al., 2020). Recent decades have seen the growing demand for bone grafting procedures mainly due to trauma and orthopedic surgery (Brydelle et al., 2010). However, the complications of bone grafting remain, such as pain, infection and even disability (Dimitriou et al., 2011). It is worth mentioning that mesenchymal stem cells serve to maintain osteogenesis and repair fracture (Griffin et al., 2011). Thus, BMSCs have been explored for bone repair and regeneration (Griffin et al., 2011).

Many studies have revealed multiple mechanisms of directing BMSCs into osteocytes. For instance, shear stress, bone morphogenetic proteins (BMPs), calcium phosphate surfaces and collagen scaffolds can induce osteogenic differentiation (Donzelli et al., 2007; Majesky et al., 2017; Müller et al., 2008; Yourek et al., 2010). Krüppel-like factor 4 (KLF4) is a conserved zinc-finger-containing transcription factor and it could modulate differentiation of stem cells (Ghaleb & Yang, 2017). It regulates the differentiation of monocytes, smooth muscle cells, mast cells and goblet cells (Feinberg et al., 2007; Nishimura et al., 2010; Zheng et al., 2009). Moreover, KLF4 has been proven to regulate the osteogenic differentiation and bone regeneration (Akouch et al., 2019; Xu et al., 2018). Therefore, we assumed that KLF4 might play a part in the osteogenic differentiation of BMSCs.

Research has revealed that there is a direct interaction between KLF4 and SOX2 (Wei et al., 2009). SOX2 is key to determining the osteogenic lineage of mesenchymal stem cells (Park et al., 2012). Also, SOX2 can regulate YAP1 to maintain stemness and to determine the differential direction (Seo et al., 2013). Yet, the underlying mechanism of how SOX2 induces osteogenic differentiation remains unclear. SOX2 has been reported to induce insulin-like growth factor-2 (IGF2) signaling pathway in bladder cancer (Chiu et al., 2020), and IGF2 signaling pathway can trigger human BMSCs to undergo osteogenic differentiation (Hamidouche et al., 2010). Thus, we guessed that SOX2 might regulate the osteogenic differentiation in BMSCs via IGF2 signaling pathway.

The present research aimed to explore if KLF4 could play a role in the osteogenic differentiation of BMSCs. The following hypotheses were evaluated: (1) KLF4 could promote the osteogenic differentiation in BMSCs; (2) KLF2 mediates its differentiation induction effects via SOX2/IGF2 axis; (3) KLF4 could stimulate osteogenesis in transgenic mice.

MATERIALS AND METHODS

Cells and cell culture

Human bone marrow mesenchymal stem cells (BMSCs) were bought from American Type Culture Collection, USA. These cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, USA). The culture medium was added with 10% fetal bovine serum (Gibco, USA). Cells were incubated at 37°C in a humid atmosphere with 5% CO₂ and would be passaged or used for
Transfecting cells with plasmids, siRNAs and adeno virus

The following products were bought from Genomeditech (Shanghai, China): empty vectors (pcDNA3), KLF4-overexpressing vector (pcDNA3-KLF4), SOX2-overexpressing vector (pcDNA3-SOX2), KLF4 siRNA (si-KLF4), SOX2 siRNA (si-SOX2), IGF2 siRNA (si-IGF2), scrambled siRNAs for KLF4, SOX2 and IGF2 (si-NC). Recombinant plasmids containing human KLF4 fragment (hKLF4) were devised by Genomeditech and recombinant adeno viruses containing hKLF4 were constructed. Ad-hKLF4 plaque forming units (PFUs) were 1×10^10 per milliliter. Lipofectamine 3000 (Genomeditech, Shanghai, China) was employed as the transfection reagent.

Quantitative real-time PCR (qRT-PCR)

We used Trizol reagent (Sigma-Aldrich, USA) to obtain total RNA, and miRNA was obtained by using Molpore Cell/Tissue miRNA Kit (Yeasen, Shanghai, China). In addition, mRNA was transcribed by using Hifair III One Step RT-qPCR Probe Kit (Yeasen, Shanghai, China), yet we used TaqMan MicroRNA Reverse Transcription Kit (Invitrogen, USA) to transcribe miRNA. SYBR green was from Roche, Switzerland. qRT-PCR was performed using the Applied Biosystems 7900HT Fast Real-Time PCR System. The endogenous control for other primers was GAPDH. The primers in our experiments were listed in Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Primer 5'-3'</th>
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<tbody>
<tr>
<td>KLF4</td>
<td>Human</td>
<td>F: CACGCTCACCTGATGAGG</td>
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<tr>
<td></td>
<td></td>
<td>R: GACTCCCTGCAATGAGG</td>
</tr>
<tr>
<td>KLF4</td>
<td>Mouse</td>
<td>F: ACGCTTGATGAGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GACCTCGATGAGG</td>
</tr>
<tr>
<td>SOX2</td>
<td>Human</td>
<td>F: TGAGAGCTGAGTCTGAGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CGAGTAGGACATGCTGAGT</td>
</tr>
<tr>
<td>SOX2</td>
<td>Mouse</td>
<td>F: CCGCACAgATGCAACCC</td>
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<td></td>
<td></td>
<td>R: CGGTCTAGGTAGTCTGCG</td>
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<tr>
<td>IGF2</td>
<td>Human</td>
<td>F: GTCAGCATGTCAGAGAGTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CACGCTCCCTCGAGCCTG</td>
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<tr>
<td>IGF2</td>
<td>Mouse</td>
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<tr>
<td></td>
<td></td>
<td>R: AGCGCTCTCAGGACTG</td>
</tr>
<tr>
<td>RUNX2</td>
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<td></td>
<td></td>
<td>R: TCAAGCATCGGTAGTTG</td>
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<tr>
<td>Osteocalcin</td>
<td>Human</td>
<td>F: GGGCGTACCTGATCAATG</td>
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<td></td>
<td></td>
<td>R: GTGGTCAGCAAATCTGCA</td>
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<td>Osteocalcin</td>
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<tr>
<td>Collagen 1</td>
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<td></td>
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<tr>
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<td>GAPDH</td>
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<td>R: GCCATCAGCCACAGTTT</td>
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<td>Mouse</td>
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<tr>
<td></td>
<td></td>
<td>R: CTTCCACATCTGGGCTT</td>
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Finally, protein bands would be visualized by ECL kits (Millipore, USA). The primary antibodies against KLF4 (dilution: 1:1000, Abcam, UK; ab92256), osteocalcin (dilution: 1:1000, Abcam, UK; ab93876), IGFBP1 (dilution: 1:1000, Abcam, UK; ab231254), collagen I (dilution: 1:1000, Abcam, UK; ab34710) and GAPDH (dilution: 1:1000, Beyotechnology, China; AF1186) were used in this research, and GAPDH was used as the internal control.

Alizarin Red staining

Cells were initially cultured in 12-well plates for 21 days to undergo the induction of osteogenic differentiation. After that, they were fixed in 90% ethanol for about 20 min, then they were stained with alizarin red staining for 5 min. Next, cells were washed gently, and they were observed. The cells that were stained with Alizarin Red staining were cultured with 10% cetylpyridinium chloride for 30 min and then matrix mineraliza-
tion was quantified. The absorbance of the supernatant was read at 562 nm.

Animal bone defect model

Our animal experiments were approved by the Ethical Committee of Funing People's Hospital, and they were performed under the guidelines published by the committee.

Briefly, 9 BALB/c nude mice (4–6 weeks old, male, 18–20 g) were purchased from our animal center and kept at animal rooms (10-h light/14-h dark cycle, 22–27°C, relative humidity: 40–60%) under sterile environment. In this model, we first made an incision in the skin and then gradually exposed the tibia. Near the medial side of tibia, a dental machine was employed to make a 2-mm bone defect that was 1 mm away from the growth plate. We also exposed the bone marrow cavity. The defect was closed in layers. After the operation, each rat received 400,000 units of penicillin and then twice a day 3 days after the operation. These animals were randomly divided into 4 groups: (1) the blank control group (n=3); (2) bone defect surgery (Sham) control group (n=3); (3) the negative control group that received 5 μl of adenovirus solution via injecting it into rat's tibia during the operation and on the second postoperative day (n=3); (4) the ad-hKLF4 treatment group that received 5 μl of ad-hKLF4 solution via injecting it into rat's tibia during the operation and on the second postoperative day (n=3). 21 Days after the operation, mice were sacrificed by cervical dislocation, and tibia was collected for further research.

RESULTS

KLF4 promotes the proliferation and osteogenic differentiation of BMSCs

To confirm that cells we collected were BMSCs, flow cytometry was conducted, and the results indicated that these cells were CD29+, CD90+, CD34- and CD45- (Fig. 1A). These results suggested that these cells were BMSCs. BMSCs were then transfected with KLF4-overexpressing plasmid or si-KLF4, and the expression of KLF4 in BMSCs using qRT-PCR and Western blotting was determined. The results showed that overexpression of KLF4 significantly increased the expression of KLF4 at both mRNA and pro-

HE staining

In brief, tibia tissues were sectioned at the thickness of 5 μm, and then embedded in paraffin. After that, sections were stained with hematoxylin and eosin. Next, images were taken and the ratio of new bone area to the entire bone area was determined.

Statistical methods

Our data was analyzed by operating on GraphPad Prism 8.0 and was displayed in the form of mean ± standard deviation (S.D.). Two-tailed \( P \text{<} 0.05 \) was considered as carrying statistical significance. All our experiments were performed five times independently. Student's t-test and one-way analysis of variance (ANOVA, Bonferroni post hoc test) were adopted in our analysis, depending on the experiment.

Figure 1. KLF4 promotes the proliferation and osteogenic differentiation of BMSCs.

(A) The cell surface antigen markers CD29, CD34, CD45 and CD90 were detected in isolated BMSCs by flow cytometry. (B) KLF4 mRNA expression in BMSCs after transfection of KLF4 overexpression plasmid and si-KLF4. (C) KLF4 protein expression in BMSCs after transfection of KLF4 overexpression plasmid and si-KLF4. (D) Cell proliferation assay using CCK-8 to detect the growth rate of BMSCs at different time intervals. (E) Cell cycle activity was examined in BMSCs by flow cytometry. (F) Western blots for RUNX2, osteocalcin and collagen I in BMSCs after transfection of KLF4 overexpression plasmid and si-KLF4. (H) Alizarin red staining in BMSCs after transfection of KLF4 overexpression plasmid and si-KLF4. NS, non-significant; \(* P \text{<} 0.05; \text{**} P \text{<} 0.001 \) by two-sided Student's t-test, versus the control group.
tein level, whereas si-KLF4 significantly reduced the expression of KLF4 as compared to their respective NCs (Fig. 1B and 1C). Meanwhile, we used CCK-8 assay to determine the growth rate and proportion of viable cells of BMSCs. It was found that the proliferation of BMSCs was accelerated by the overexpression of KLF4, but their growth was inhibited with KLF4 silencing at 24, 48 and 72 h (Fig. 1D). Furthermore, the cell cycle analysis exhibited that increased number of BMSCs were shifted from G1 phase to S and G2 phases following KLF4 overexpression (Fig. 1E). However, silencing of KLF4 increased the BMSCs transition into G1 phase (Fig. 1E). To further examine the role of KLF4 in the osteogenic differentiation, osteogenic markers were investigated in BMSCs. It was found that the protein expression levels of RUNX2, osteocalcin and collagen I were increased following the silencing of KLF4 (Fig. 1F). Finally, BMSCs underwent Alizarin red staining after 28 days of incubation which could exhibit the mineralization features. The results indicated that KLF4 overexpression could promote the mineralization in BMSCs, but its knockdown could reduce this (Fig. 1G). This data indicated that KLF4 regulates the growth and osteogenic differentiation of BMSCs.

KLF4 upregulates SOX2 expression

SOX2 has been reported to regulate the osteogenic differentiation in stem cells and it could also interact with KLF4 [19]. Thus, we assumed KLF4 could mediate its effects through SOX2. It was discovered that KLF4 overexpression upregulated the activity of SOX2, whereas the silencing of KLF4 reduced SOX2 activity at mRNA and protein level (Fig. 2A and 2B). Protein-protein interaction analysis using inBio-Discover tool exhibited that KLF4 could interact with SOX2 directly (Fig. 2C). To further verify the interaction of KLF4 and SOX2 in vitro, we performed coimmunoprecipitation on BMSCs cell-lysate. Results showed that KLF4 immunoprecipitation in BMSCs detected the SOX2 expression (Fig. 2D).

From these results, we exhibited that KLF4 could interact and upregulate the SOX2 expression directly.

SOX2/IGF2 signaling mediates the effects of KLF4 on BMSCs

Initially, BMSCs were transfected with si-SOX2 and si-NC, respectively, and qRT-PCR was used to detect SOX2 expression (Fig. 3A). Also, Western blotting results showed that SOX2 expression was downregulated following the transfection (Fig. 3B). After that, cell proliferation was assessed. It was found that the growth of BMSCs was reduced significantly after SOX2 expression in BMSCs was silenced (Fig. 3C). In addition, after SOX2 expression in BMSCs was downregulated, the protein expression of RUNX2, osteocalcin and collagen I was considerably reduced (Fig. 3D). Interestingly, the co-transfection of KLF4-overexpressing vector and si-SOX2 into BMSCs also decreased the protein expression levels of RUNX2, osteocalcin and collagen I. These results implied that SOX2 is key to mediating the proliferation and osteogenesis in BMSCs. SOX2 has been demonstrated to activate IGF2 signaling, so we assumed that IGF2 signaling pathway might be the downstream signaling of KLF4 to regulate the proliferation and osteogenic differentiation of BMSCs. First, we found that SOX2 overexpression increased IGF2 mRNA expression, whereas SOX2 downregulation reduced its mRNA expression (Fig. 3E). Similarly, the knockdown of KLF4 remarkably downregulated IGF2 mRNA expression (Fig. 3F). In the meantime, we demonstrated that KLF4 could downregulate IGFBP1 expression and activate AKT signaling (Fig. 3G). Upon the activation of IGF2 signaling pathway, AKT would be activated, but IGFBP1 expression would be reduced. Thus, there might be a association between KLF4 and IGF2 signaling pathway.
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IGF2 signaling pathway is important to the proliferation-stimulating and osteogenic induction effects of KLF4

To investigate the role of IGF2 in BMSCs, IGF2 expression was knocked down in BMSCs by transfecting these cells with si-IGF2. Results showed that the transfection of si-IGF2 significantly reduced the expression of IGF2 at mRNA and protein level (Fig. 4A and 4B). Following this transfection, it was discovered that the proliferative ability of BMSCs was significantly decreased, suggesting that IGF2 might be the key to the regulation of BMSCs proliferation (Fig. 4C). Moreover, the protein expression of RUNX2, osteocalcin and collagen I was reduced after IGF2 signaling was suppressed in BMSCs (Fig. 4D). It is noteworthy that the co-transfection of KLF4-overexpressing vector and si-IGF2 also reduced the expression of osteogenic markers RUNX2, collagen I and osteocalcin at protein level (Fig. 4D). Hence, IGF2 signaling is key for KLF4 to stimulate the growth and osteogenic induction in BMSCs.

KLF4 overexpression enhances osteogenesis in vivo

We performed a small cut on the knees of rats and then injected KLF4-containing adenovirus into their knees. On the 7th postoperative day, their knees were taken out for further analysis. HE staining showed that there was more new bone formation in the Ad-hKLF4 overexpression group, when compared to the control group (Fig. 5A). Also, in the Ad-hKLF4 knockdown group, there was less new bone formation. In addition, in the Ad-hKLF4 overexpression group, the mRNA expression levels of RUNX2, osteocalcin and collagen I was much higher, in comparison with the control group (Fig. 5B, 5C and 5D). In addition, KLF4 overexpression could result in higher protein levels of RUNX2, collagen I and osteocalcin (Fig. 5E). Therefore, KLF4 could accelerate the recovery of bone defect.

DISCUSSION

To investigate the role of KLF4 in BMSCs, its expression was upregulated or downregulated and then the cell proliferation and osteogenic markers were examined. We found that KLF4 overexpression could not only promote the growth of BMSCs but also stimulate their osteogenic differentiation. KLF4 has the ability to reprogram somatic cells into pluripotent stem cells with the cooperation of OCT4, SOX2 and MYC (Qi & Pei, 2007). Although KLF4 has not been reported to increase the proliferation of stem cells, it can promote the growth of cancer cells. It can interplay with HDAC1 to increase the proliferation of myeloid leukemic cells (Huang et al., 2014). In nasopharyngeal carcinoma, KLF4 can increase the cell growth by activating PI3K/AKT signaling pathway (Tang et al., 2018). Thus, these findings are consistent with our discovery. To examine if KLF4 could induce the osteogenic differentiation we assessed key osteogenic markers such as RUNX2, osteocalcin and collagen I. RUNX2 is considered as a transcription factor regulating the differentiation towards osteoblasts (Fujiita et al., 2004). The secretion of osteocalcin is an early event of osteogenic differentiation of mesenchymal stem cells, and osteocalcin can promote the osteogenic maturation of mesenchymal stem cells and biominerulization (Tsao et al., 2017). Collagen I is an osteogenic differentiation marker and can direct human mesenchymal stem cells to differentiate into osteoblasts (Shi et al., 1996). KLF4 stimulates the mineralization of BMSCs and upregulates the activity of RUNX2, osteocalcin and Collagen I in BMSCs. Also, our animal experiments showed that KLF4 could accelerate the osteogenesis during bone injury. These results demonstrate that KLF4 regulates the growth and osteogenic differentiation of BMSCs.

Furthermore, we found that SOX2 mediates the effects of KLF4 on BMSCs. Because upregulation in KLF4 expression leads to the upregulation of SOX2. Overexpressing SOX2 can activate cell proliferation and direct osteogenic differentiation. The knockdown of SOX2 mitigates the effects of KLF4 on BMSCs. In res-
piratory epithelium, SOX2 is able to increase cell growth and induce differentiation (Tomkins et al., 2011). Thus, in BMSCs, the proliferation and osteogenic differentiation can be enhanced by SOX2. There is interaction between SOX2 and IGFB2 in bladder cancer cells, and our research proves that SOX2 can induce IGFB2 signaling pathway (Chiu et al., 2020). Apart from that, the inactivation of IGFB2 signaling can abrogate the effects of KLF4 on BMSCs. In addition, IGFB2 signaling can trigger the osteogenic proliferation and even the mineralization in BMSCs (Hamidouche et al., 2010). It is noteworthy that KLF4 activates the cell proliferation and osteogenic differentiation of BMSCs via SOX2/IGF2 signaling pathway.

However, there are several limitations in our research work. First, there might be other mechanisms involved in the KLF4-dependent regulation of the osteogenic differentiation and growth in BMSCs. SOX2/IGF2 signaling might be one of the signaling pathways. Second, we needed to use transgenic mice to test the bone repair effects of KLF4 in vivo. At last, the BMSCs cells used in this study have not been studied for population. Therefore, it is unknown if the cells used in the present study were bone marrow stem cells or stromal cells. It has been widely accepted that the population of bone marrow stromal cells is heterogeneous and does not homogenous. As previously mentioned, mesenchymal stem/stromal cells are heterogeneous and have a phenotype that differs in current isolation and cultivation regimes, which often lead to incomparable experimental results (Mo et al., 2016). The study of transcriptomes of cell populations derived from single MSC before and after adipogenic differentiation and before and after thermogenic activation allowed the identification of a minimum of 4 distinct human adipocyte subtypes that can differentiate from single MSC before and after adipogenic differentiation. SOX2/IGF2 signaling links loss of imprinting of IGFB2 to increased cell proliferation and tumor risk. BMC Cell Biol 11: 44. https://10.1186/s12955-011-0044-7

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