Core-binding factor beta is required for osteoblast differentiation during fibula fracture healing

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Abstract

Background: Growing evidence has implicated core-binding factor beta (Cbfb) as a contributor to the osteoblast differentiation, which plays a key role in fracture healing. Here, we conducted the present study with the main objective to assess whether Cbfb affects osteoblast differentiation after fibula fracture.

Methods: Initially, Cbfb conditional knockout mouse model was established. Immunohistochemical staining was carried out to detect the expression of proliferating cell nuclear antigen (PCNA) and Collagen II in the fracture end. Then osteoblasts were isolated from specific Cbfb conditional knockout mice and cultured. BrdU method, Alkaline phosphatase (ALP) staining and Von Kossa staining were followed to detect osteoblast proliferation, differentiation and mineralization, respectively. Western blot analysis and RT-qPCR were used to detect the expression of osteoblast differentiation-related genes. Cbfb conditional knockout mouse model was successfully constructed.

Results: The mice treated with Cbfb knockout were shown to exhibit significantly decreased expression of PCNA and Collagen II, ALP activity and mineralization, as well as inhibited expression of Runx2, ALP, BglapI, SPPI, Osteocalcin, Atf4 and Osterix. Further, Cbfb knockout showed no effects on osteoblast differentiation.

Conclusion: Overall, these results demonstrated that the Cbfb could potentially promote fibula fracture healing and osteoblast differentiation and thus could comprise a potential means of impeding the progression of fibula fracture.

Background

Fibula fracture is relatively common long bones fractures and this fracture often leads to soft tissue complications and non-union [1]. Fibula fracture represents a recurring injury that often needs surgical stabilization and orthopaedic surgery is a general treatment approach [2]. Fracture healing is a complicated biological process since it refers to specific regenerative patterns and covered the changes of several thousand genes expression [3]. The performance of anatomical reduction and fixation in advance can facilitate the reduction of fibula fracture and lower-extremity alignment restoration [4]. The fractures that cannot be healed or unite quickly or completely is responsible for obvious pain and function loss [5]. Osteoblasts originate from mesodermal progenitors and have been suggested to play a critical role during the bone-forming and mineralization process [6]. Osteoblasts can express receptors for several hormones including estrogen, parathormone (PTH) and glucocorticoids, which all participate in the regulation of osteoblast differentiation [7], whose deficiency will impair fracture healing [8].

Core-binding factor beta (Cbfb), also known as polyomavirus enhancer binding protein 2 beta gene and SL3 enhancer factor 1, is located on 16q22 fuses which can result in a chimeric protein [9]. Cbfb is essential for embryonic bone morphogenesis since it controls the balance of chondrocyte differentiation and proliferation via regulation of indian hedgehog expression and parathyroid hormone-related protein receptor in postnatal cartilage and bone formation [10]. Cbfb knockout mice presented with severely

delayed bone formation, which is indicated by diminished osteoblast differentiation and maturation, suggesting Cbfb to be an essential for skeletal development [10]. A similar finding revealed by Fukuda et al. was that osteoblast differentiation and bone formation are favored by Cbfb [11]. What's more, Cbfb has been implicated in the fracture healing process especially in the bone development [12]. On the basis of aforementioned evidence, it can be subsequently concluded that Cbfb exerts certain effects on fracture healing and osteoblast processes. Therefore, this study was conducted with the main objective of investigating the potential effects of Cbfb on the fibula fracture healing and osteoblast differentiation.

Materials And Methods

Ethics statement

All experimental procedures were strict accordance with the requirements of relevant animal Ethics Committee of Xianyang Central Hospital Extensive efforts were made to ensure minimal suffering of the animals used during the study.

Construction of Cbfb conditional knockout mouse model

Loxp was inserted into both ends of the Cbfb allele to construct Cbfb fluorine and oxygen (Flox) mice (Jackson Lab, California, USA). Then the Cbfb Flox mice were hybridized with the Dermo promoter Cre (Cyclization Recombination Enzyme) mice (Jackson Lab, California, USA), and the first filial generation Cbfbf/f; Dermo-Cre mice were obtained. Afterwards, the first filial generation mice were hybridized with Cbfb Flox mice, consequently, the second generation experimental group (Cbfbf/f; Dermo-Cre mice following specific knockdown of Cbfb in mesenchymal stem cells) obtained. The Cbfbf/f and Cbfbf/f + mice were taken as the control of wild type (WT) mice.

Identification of Cbfb conditional knockout in mouse genotype

Then mice of 2–3 weeks old after birth were taken out and their tails were then cut off (2–3 mm). The tails were put in 0.2 mL mouse tail digestive juice, then added with 4 μ L proteinase K solution (Yeasen Biotechnology Co., Ltd., Shanghai, China) and incubated overnight at 55 °C by shaking. Next, the samples were added with 0.1 mL 6 M NaOH solution (Guangzhou Laiyu Chemical Co., Ltd, Guangzhou, China), shaken drastically and centrifuged. Subsequently, the supernatant was collected into 0.5 mL of 95% ethanol (Sinopharm Chemical Reagent Co., Ltd., Beijing China), shaken up and down for 35 times and allowed to stand at room temperature for 10 min, followed by centrifugation for 5 min. Then the precipitation was washed using 1 mL of 75% ethanol for 15 s. After the precipitation was dried, 0.1 mL 0.1 TE solution (pH = 8.0; Shanghai Haling Biotechnology Co., Ltd., Shanghai, China) was added and shaken at 220 rpm for 1 h at 65 °C to dissolve DNA. The forward sequence of Cbfb identification primers was 5'-TGTCTGAAGACAACTACAGTGTAC-3', and the reverse sequence was 5'-CTCTCTGAACACTATATCAGTTCC-3'. The forward sequence of CRE (Cyclization Recombination Enzyme) identification primers was 5'-CCTGGAAAATGCTTCTGTCCGTTTGCC-3, and the reverse sequence was 5'-

GAGTTGATAGCTGGCTGGTGGCAGATG-3'. The reaction conditions of reverse transcription quantitative polymerase chain reaction (RT-qPCR) consisted of pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 45 s, annealing at 54 °C (CRE) and 57 °C (Cbfb) for 45 s, extension at 72 °C for 45 s, with a total of 30 cycles, and elongation at 72 °C for 5 min. The PCR was performed on a 2720 thermal cycler (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA, USA). The PCR products were separated using 1.0% agarose gel electrophoresis and photographed under ultraviolet imaging system. The position of PCR product bands can be used to judge the size of the product fragments and then determine the genotypes of mice.

Establishment of fracture healing model

Our study enrolled 30 mice aged 7 weeks in the experiment group and the control group (15 mice per group) as experimental subjects. The mice were fixed on a homemade autopsy table. An incision about 5 mm long was made along the proximal part of right lateral leg to bone nodule of about 12 mm. The soft tissues and muscles surrounding fibula were separated by blunt dissection, staunched and then the fibula was exposed, followed by transverse shearing. Next, the incision was sutured layer by layer and the experimental model was established. Before the mice woke up, X-ray imaging system was used to photograph the right lower limb at 23 KV and 13 s. The mice with deformity of fracture and failing to meet the model requirements were excluded. Then routine X-ray photograph was conducted every week to observe the fracture healing. Five mice for each group were subsequently euthanized after injury at the 1st, 14th, 28th day respectively. At last, the fracture end and the surrounding tissues were taken for subsequent experiments.

Streptavidin-peroxidase (SP) assays

The specimens were taken out, rinsed using normal saline and then fixed in 4% paraformaldehyde (pH = 7.4, Beijing Cellchip Biotechnology Co., Ltd., Beijing, China) at 4 °C for 24–48 h. Next, decalcification was conducted with the use of 200 g/L ethylenediamine tetraacetic acid (EDTA) (Baoding Kaiyue Chemical Co., Ltd., Hebei, China) for 4 weeks, with the decalcification fluid replaced once every 3 days. Thereafter, the specimens were dehydrated with gradient ethanol, cleared with xylene (Changsha Tang Hua Chemical Trading Co., Ltd., Changsha, China) and vertically embedded into paraffin. The paraffin-embedded specimens were put in a 4 °C refrigerator, after which the specimens were sectioned and stained using Safranin-O/Fast Green. Then the fracture healing condition was observed under a microscope. The immunohistochemical staining was carried out to detect the expression of proliferating cell nuclear antigen (PCNA) and Collagen II in the fracture end. The primary antibody PCNA and Collagen II was purchased from Abcam Inc. (Cambridge, MA, USA) and the secondary antibody was purchased from Shanghai MICROTEK Biotechnology Co., Ltd. (Shanghai, China).

Cell culture and detection of cell proliferation and differentiation

Osteoblast precursor cells were isolated and then inoculated into a cell culture dish (165) with the density of 3×10^3 cells/cm². Upon reaching confluence, the cells were added with induction medium of

osteoblasts: BGJB medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS), 50 µg/mL L-ascorbic acid (A4544, Sigma-Aldrich Chemical Company, St Louis, MO, USA) and 5 µg β-glycerolphosphate (G9891, Sigma-Aldrich Chemical Company, St Louis, MO, USA). Then 5-bromo-2'-deoxyuridine (BrdU) method was used to measure osteoblast proliferation. Next, the cells were colored using 3, 3'-diaminobenzidine (DAB) and the color condition was observed under a microscope, The coloration was subsequently terminated when positive cells presenting with brownish yellow appeared. At the 14th day, alkaline phosphatase (ALP) staining (A2356, Sigma-Aldrich Chemical Company, St Louis, MO, USA) was adopted to detect cell differentiation. At the 21st day, Von Kossa staining (Thermo Fisher Scientific Inc., Waltham, MA, USA) was applied to test the mineralization of osteoblasts.

RNA isolation and quantitation

Total RNA was extracted from primary osteoblasts using Trizol. Then reverse transcription was conducted using Invitrogen SuperScript VILO Master Mix kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Then RT-qPCR was performed on a PCR instrument (Applied Bio-systems, Foster City, CA, USA) using SYBR Green (Thermo Fisher Scientific Inc., Waltham, MA, USA). The primers used are shown in Table 1. Then Westerns blot analysis was performed. In brief, the cells were rinsed twice with phosphate buffered saline (PBS), and then added with loading buffer for 10-min boiling at 95 °C. The protein was separated using 10% polyacrylamide gel electrophoresis (Boster Biological Technology Co. Ltd., Wuhan, Hubei, China) (40 µg/well) and then transferred onto polyvinylidene fluoride (PVDF) membranes using wet transfer method. The membranes were blocked with 5% bovine serum albumin (BSA) at room temperature for 1 h and incubated with primary antibodies diluted at 1: 500-1: 1000: Osteocalcin, (AM0911, Millipore, Billerica, MA, USA), Osterix, (ab22552, Abcam Inc., Cambridge, MA, USA) and ATF4 (abl05383, Abcam Inc., Cambridge, MA, USA) at 4 °C overnight. After being rinsed with Tris-buffered saline Tween-20 (TBST) for 3 times (5 min/time), the membranes were added with corresponding secondary antibody (Abcam Inc., Cambridge, MA, USA) (Shanghai Precision & Scientific Instrument Co., Ltd., Shanghai, China) and incubated at room temperature for 1 h, followed by 3 washes with TBST (5 min/time). The immunocomplexes on the membrane were visualized using enhanced chemiluminescence (ECL) reagent (Shanghai Genmed Gene Pharmaceutical Technology Co., Ltd., Shanghai, China) and band intensities were quantified using a multifunctional imaging system.

Table 1

Primer sequences for reverse transcription quantitative polymerase chain reaction

Gene	Forward sequence	Reverse sequence	Products
Bglap1	CTGACCTCACAGATCCCAAGC	TGGTCTGATAGCTCGTCACAAG	187 bp
ALP	GTGACTACCACTCGGGTGAAC	CTCTGGTGGCATCTCGTTATC	96 bp
Cbfb	GATCATGAGCCCTTTTTCTCC	GGCAAAGCAATCTGGTAGC	175 bp
Runx2	GACTGTGGTTACCGTCATGGC	ACTTGGTTTTTCATAACAGCGGA	84 bp
GAPDH	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA	
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Statistical analysis

Statistical analyses were conducted using SPSS 19.0 statistical software (IBM Corp. Armonk, NY, USA). Measurement data were expressed as mean $\pm$ standard deviation were compared by unpaired t-test between two groups. A $p < 0.05$ demonstrated statistical significance.

Results

Identification of transgenic mice

After the DNA of tails of mice following Cbfb conditional knockout was extracted, we used PCR to identify mouse genotypes, including WT, knocked out fifth exon of Cbfb, Loxp fragments inserted with both ends of the Cbfb allele and the sequence with CRE recombinase. As illustrated in Fig. 1A.

Cbfbf/f; Dermo-Cre mice exhibit delayed fracture healing

Following fracture, the fracture line of Cbfbf/f; Dermo-Cre and WT mice was clearly visible, with no significant difference between the two groups. At the 14th day following fracture, the fracture line of the WT mice turned blurred and the bridge callus appeared. By contrast, the fracture line of Cbfbf/f; Dermo-Cre mice were still clearly visible, with less callus volume and no bridging callus. At the 28th day after fracture, the callus of mice in the two groups was more complete (Fig. 2). We can confer that the callus at the end of fracture in Cbfbf/f; Dermo-Cre mice was less and therefore the fracture healing could be delayed.

Observation of morphological characteristics of Cbfbf/f; Dermo-Cre mice

At the 1st day after fracture, the staining results revealed that most fracture ends of Cbfbf/f; Dermo-Cre mice and WT mice were fibrous tissues. At the 14th day after fracture, chondrocyte at the fracture end of

WT mice exhibited hypertrophy, the number of chondrocyte was decreased significantly and a part of cartilage callus turned bony callus. But the number of chondrocyte of Cbfbf/f; Dermo-Cre mice increased significantly and formed a large amount of cartilage callus. At the 28th day after fracture, the fracture of WT mice was healed, accompanied by regularly distributed bone trabecula. However, the bone trabecula of Cbfbf/f; Dermo-Cre mice was observed to be disordered arrangement and also, the fracture healing was still at the shape stage and a small number of chondrocytes was still existed (Fig. 3).

Cbfb knockout decreases expression of PCNA and Collagen II

Immunohistochemical staining analysis of PCNA revealed that at the 7th day after fracture, the fracture end was mainly composed of undifferentiated fibroblasts and cartilage precursor cells. The positive expression of PCNA was observed to be increased both in the WT and Cbfbf/f; Dermo-Cre groups. But the positive expression of PCNA in the WT group was obviously higher than that in the Cbfbf/f; Dermo-Cre group. On the 14th day after fracture, the number of cells in the proliferative phase of fracture ends was decreased, and the PCNA positive expression in the WT group was reduced. The chondrocyte proliferation in the fracture ends in the Cbfbf/f; Dermo-Cre group was significantly increased (Fig. 4a-d).

Immunohistochemical staining analysis of Collagen II is shown in Fig. 4e-g: on the 7th day after fracture, many chondrocytes in the fracture ends and a large expression of Collagen II were observed in the WT group. Conversely, the Cbfbf/f; Dermo-Cre group has less chondrocytes in the fracture ends and little expression of Collagen II was observed. On the 14th day after fracture, the WT group showed chondrocyte hypertrophy in the fracture ends with the Collagen II expression decreased. However, the Cbfbf/f; Dermo-Cre group great many chondrocytes in the fracture ends, and a large expression of Collagen II was observed.

Cbfb knockout inhibits ALP activity and mineralization

The results obtained from ALP staining and von Kossa staining indicated that the activity of ALP in osteoblasts in the Cbfbf/f; Dermo-Cre group was decreased and osteoblast differentiation was also observed to be inhibited (Fig. 5A). The calcium nodules of osteoblasts in the Cbfbf/f; Dermo-Cre group was much less than that in the WT group and the mineralization was inhibited (Fig. 5B).

Cbfb knockout has no effects on osteoblast proliferation

Subsequent BrdU results showed that the proliferating cells were yellow brown and there was no statistically significant difference in relation to the proportion of positive cells between the Cbfbf/f; Dermo-Cre group and the WT group ($p > 0.05$) (Fig. 6). It can be concluded that Cbfb knockout did not affect osteoblast proliferation.

Cbfb knockout decreases osteoblast differentiation

RT-qPCR assay was employed to detect the expression of osteoblast differentiation-related genes consisting of ALP, Bglap1, Runx2 and SPPL and the results revealed that the mRNA expression of ALP, Bglap1, Runx2 and SPPL in the Cbfbf/f; Dermo-Cre group was much lower than that in the WT group ($p < 0.05$) (Fig. 7A). Then Western blot analysis was carried out to detect the protein level of osteoblast differentiation-related genes consisting of Osteocalcin, Atf4 and Osterix in osteoblasts. The results displayed that the osteoblast differentiation was inhibited after Cbfb knockout. Compared with the WT group, the protein expression of Osteocalcin, Atf4 and Osterix in the Cbfbf/f; Dermo-Cre group presented with different degrees of decline (Fig. 7B).

Discussion

Fracture healing represents a unique biologic process that begins with an initial inflammatory response, during which bone and the immune system interact with each other closely [13]. A growing number of studies have demonstrated Cbfb to be a critical factor in bone morphogenesis [14]. In this study, we hypothesized that Cbfb is associated with fibula fracture healing and osteoblast differentiation. The main findings of our study indicated that Cbfb could potentially promote fibula fracture healing and osteoblast differentiation.

Initial findings from our study showed that Cbfbf/f; Dermo-Cre mice had marked shortening of limbs and the weight was also lower than that of WT mice. Besides, the callus of the fracture end of the Cbfbf/f; Dermo-Cre mice was less, which acts to result in delayed fracture healing. Fracture healing is considered to be a regenerative process consisted of many phases where each involves the formation of a variety of tissue types [15]. The formation of fracture callus appeared during the secondary bone healing, and it shares an association with fracture mechanical stability [16]. A previous study proved that the mice lacking of epithelial Cbfb exhibited short incisor and marked underdevelopment of the cervical loop and epithelial Fgf9 expression was suppressed, and mesenchymal Fgf3 and Fgf10 expression in the cervical loop [17]. Therefore, Cbfb can be a critical factor in the fracture healing process.

The mice treated with Cbfb knockout were observed to exhibit increased PCNA and Collagen II expression. Collagen II, known to regulate chondrogenesis of mesenchymal stem cells, has been demonstrated to have the potential of facilitating osteogenesis and suppressing adipogenesis during early stage of mesenchymal stem cell differentiation [18]. Stegemann et al. also revealed Collagen II to be a key factor in enhancing chondrogenic differentiation in agarose-based modular microtissues [19]. PCNA is widely accepted to be involved in distinct pathways of DNA postreplication repair [20]. In addition to DNA repair, PCNA also plays a key role in other fundamental cellular processes, such as chromatin remodeling, sister chromatid cohesion and cell cycle control [21]. In the presence of Cbfb knockdown, the differentiation of chondrocytes and osteoblasts is observed to be severely inhibited in vitro [22]. Hence, knockout of Cbfb can result in obviously increased expression of PCNA and Collagen II.

Additionally, our findings indicated that Cbfb knockout can inhibit ALP activity and mineralization of osteoblasts. Extracellular matrix mineralization represents a physiological process in teeth and bones, as

well as in growth plate cartilage during skeletal growth [23]. ALP activity is known to be one of the serum biochemical markers of bone formation and has been verified to be a clinically useful tool in predicting fractures at a nonunion risk and evaluating the progress of healing [24]. Diminished ALP activity and mineralization in MC3T3-E1 osteoblast-like cell line can result in delayed osteoblast differentiation [25]. IL-6, an important factor in the early stages of fracture healing, the mice with IL-6 knockout presented with delayed callus maturity, mineralization, and remodeling when compared to the callus of the WT mice during fracture healing [26].

Cbfb knockout disrupted osteoblast differentiation, which was evidenced by decreased expression of ALP, Bglap, Runx2 and SPPL as well as the inhibited protein expression of Osteocalcin, Atf4 and Osterix in mice treated with Cbfb knockout. Besides, BrdU results showed no effects of Cbfb knockout caused on osteoblast proliferation. Similarly, the expression of chondrocyte maturation markers consisting of Runx2, Osterix and Osteopontin, is significantly reduced in Cbfb knockout mice relative to the WT mice [27]. Another study revealed that microRNA-145 physiologically regulates bone formation and osteoblast differentiation by forming a regulatory microRNA network through Cbfb expression [11]. Moreover, in addition to facilitate chondrocyte differentiation at the skeleton in postnatal mice for the growth and maintenance, Cbfb also promotes osteoblast differentiation [10].

Conclusion

In conclusion, the key findings obtained from the present study provide evidence that Cbfb could potentially stimulate fibula fracture healing and osteoblast differentiation. Furthermore, Cbfb can be a new target for clinical diagnosis and treatment of fibula fracture. Under these experiment conditions, Cbfb conditional knockout may be affected by various factors so that the experimental results may show some deviations. Before the clinical application of this therapeutic approach, further studies are required to fully understand the clinical efficacy of Cbfb conditional knockout.

Abbreviations

core-binding factor beta (Cbfb)

proliferating cell nuclear antigen (PCNA)

Alkaline phosphatase (ALP)

proliferating cell nuclear antigen (PCNA)

Declarations

Authors' contributions

Tuanmao Guo, Zhongning Chen and Yanli Xing wrote the paper and conceived and designed the experiments; Xianhong Wang and Haiyun Zhu analyzed the data; Lan Yang and Yong Yan collected and provided the sample for this study. All authors have read and approved the final submitted manuscript.

Ethics approval and consent to participate

All experimental procedures were strict accordance with the requirements of relevant animal Ethics Committee of Xianyang Central Hospital Extensive efforts were made to ensure minimal suffering of the animals used during the study.

Consent for publication

Not applicable..

Availability of data and materials

The datasets analysed during the current study are available.

Conflicts of interest

The authors have declared that no conflicts of interest exist.

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Figures

Figure 1

Identification of transgenic mice.

Figure 2

Fracture healing of WT mice and *Cbfbf/f; Dermo-Cre* mice at the 1st, 14th and 18th day after fracture.

Figure 3

Observation of morphological characteristics of Cbfbf/f; Dermo-Cre mice ($\times 400$). A, C and E, morphology of healed tissues of WT mice at the 7th, 14th and 18th day following fracture; B, D and F, morphology of healed tissues of Cbfbf/f; Dermo-Cre mice at the 7th, 14th and 18th day following fracture.

Figure 4

Cbfb knockout decreases expression of PCNA ($\times 400$) and collagen II ($\times 400$).

Figure 5

Cbfb knockout inhibits ALP activity and mineralization in osteoblasts. A, ALP staining ($\times 200$) analysis of ALP activity; B, Von Kossa staining ($\times 200$) analysis of mineralization of osteoblasts.

Figure 6

Cbfb knockout exhibits few effects on osteoblast proliferation. A, BrdU assay ($\times 200$) for osteoblast proliferation; B, the proportion of BrdU positive cells at A.

Figure 7

Cbfb knockout represses osteoblast differentiation. A, mRNA expression of ALP, Bglapl, SPPL and Runx2 detected by RT-qPCR; B, Western blot analysis of Osteocalcin, Atf4 and Osterix proteins.