Analysis of Effects of PTEN-Mediated TGF-β/Smad2 Pathway on Osteogenic Differentiation in Osteoporotic Tibial Fracture Rats and Bone Marrow Mesenchymal Stem Cell under Tension

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Purpose. To discuss effects of phosphatase and tensin homolog protein (PTEN)-mediated transforming growth factor-β (TGF-β)/Smad homologue 2 (Smad2) pathway on osteogenic differentiation in osteoporotic (OP) tibial fracture rats and bone marrow mesenchymal stem cell (BMSC) under tension. Methods. A tibial fracture model was established. The rats were divided into sham-operated group and model group, and tibia tissue was collected. Purchase well-grown cultured rat BMSC, and use the Flexercell in vitro cell mechanics loading device to apply tension. The expression of PTEN was detected by qRT-PCR. After the BMSCs were transfected with si-PTEN and oe-PTEN, the force was applied to detect cell differentiation. The expression of TGF-β and Smad2 protein was detected by Western blot. The formation of calcium nodules in BMSC was detected by alkaline phosphatase (ALP) staining and alizarin red (AR) staining.

Results. The expression of PTEN was higher in the model group and tension MSC group, and the expression of TGF-β and Smad2 protein was lower. The expression of TGF-β and Smad2 protein in oe-PTEN group was lower than the oe-NC group and control group. The expression of TGF-β and Smad2 protein in si-PTEN group was higher than the si-NC group and control group. The results of ALP staining and AR staining also confirmed the above results.

Conclusion. PTEN-mediated TGF-β/Smad2 pathway may play a key role in the osteogenic differentiation of OP tibial fracture rats. Downregulation of PTEN and upregulation of TGF-β/Smad2 signal can promote the osteogenic differentiation of BMSC under tension.

1. Introduction

Osteoporosis (OP) is a progressive disease of systemic bone metabolism disorder, which is characterized by degradation of bone microstructure, accelerated bone loss, and destruction of bone microstructure, and will increase the risk of fracture [1, 2]. Tibial fracture, as the most serious consequence of OP, will bring severe living burden and economic burden to patients. At present, OP-induced fractures are affecting more and more people, especially middle-aged and elderly patients, and people pay more and more attention to them.

Bone marrow mesenchymal stem cell (BMSC) can differentiate into osteoblasts and adipose cells in bone marrow [3]. OP leads to the impairment of the function and differentiation ability of BMSC in patients with tibial fracture, and then the imbalance between osteogenic differentiation and adipogenic differentiation occurs, resulting in the decrease of osteoblast formation in bone marrow and the increase of adipose tissue formation [4]. Previous studies have shown that BMSC is one of the most sensitive cells to mechanical stress, and mechanical tensile stress can have a certain influence on the osteogenic differentiation of BMSC [5]. Mechanical tension is divided into physiological tension
and pathological tension, and its regulation is closely related to the size, frequency, and duration of mechanical stimulation. Low-level tension is not enough to maintain bone formation; proper tension can provide effective physiological stimulation for the maintenance of bone tissue; when the tension is too large, the speed of bone tissue destruction is greatly accelerated, which is pathological distraction tension.

Phosphatase and tensin homolog protein (PTEN) is a factor with the activities of lipid phosphatase and protein phosphatase, it participates in the process of cell DNA repair, apoptosis and proliferation [6]. The deletion of the PTEN gene will lead to cancer, nervous system diseases, metabolic diseases, and immune system diseases [7]. Transforming growth factor-β (TGF-β) is one of the most important factors involved in process of bone remodeling [8]. Smads family is an important new gene family in TGF-β pathway in vertebrates. Smad homologue 2 (Smad2) is a receptor-activated Smads protein, which participates in TGF-β or activin signal transduction [9]. Some experts found that TGF-β/Smad2 pathway of BMSC can promote its proliferation and osteogenic differentiation, regulate the late osteogenic differentiation, and participate in collagen secretion and calcium salt deposition [10].

However, at present, there are few research on the relationship between TGF-β/Smad2 pathway, PTEN, and osteogenic differentiation of BMSC under tension. In this study, the author observed the effects of PTEN-mediated TGF-β/Smad2 pathway on the osteogenic differentiation of BMSC in OP tibial fracture rats under tension, in order to provide theoretical basis for bone tissue engineering research.

2. Methods

8 clean grade rats aged 4-5 months (260-300 g) were selected, and all rats were fed with normal diet under the same conditions. The experimental rats were randomly divided into two groups according to body weight: sham-operated group and model group. In the model group, the rats were anesthetized by intramuscular injection of 20% urethane, and the bilateral ovaries of the rats were removed from both sides of the lumbar spine of the rats. In the sham-operated group, the ovaries were exposed in the same way, but the ovaries were not removed.

OP model was established, and the levels of alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) in the blood of rats were detected by ALP kit and TRAP kit. The absorbance value can be read on the microplate reader, and the corresponding concentration can be converted from the standard curve according to the absorbance value. When the serum ALP level exceeds 147.25 ± 56.29 IU/mL and the TRAP level exceeds 24.50 ± 1.16 IU/mL, the OP model of rats was qualified, and the tibia fracture model was established. qRT-PCR method was used to detect the expression of PTEN, and Western blot method was used to detect the expression of TGF-β protein and Smad2 protein.

The well-grown cultured rat BMSC were purchased; the cells passed the test for bacteria, fungi, mycoplasma, and endotoxin and passed the test of differentiation ability. After being digested with 0.25% trypsin solution, the cells were inoculated into 6-well culture plates at a density of 1×10⁴/cm². The cells were cultured for 48 h and waited for the cell confluency to reach 80%-90%. Then, Flexercell in vitro cell mechanics loading device was used; the tensile force with a frequency of 1 Hz and a deformation rate of 18% were applied, twice a day for 30 min; and BMSC was harvested on the 5th day of experiment to induce the osteogenic differentiation of BMSC. qRT-PCR was used to detect the expression of PTEN in osteoblast differentiated cells. After BMSC were transfected with si-PTEN and oe-PTEN, the tension was applied, and cell differentiation was detected. The expression of TGF-β/Smad2 protein was detected by Western blot.

2.1. qRT-PCR Analysis. Total RNA was extracted by Trizol method, reverse transcribed into complementary DNA, and qRT-PCR detection was carried out with reference to reagent instructions. The internal reference was GAPDH, and every third compound hole was a sample. The Ct value of each group was obtained, and the relative mRNA expression was calculated by 2^-ΔΔCt.

2.2. Western Blot Analysis. The total protein BCA method was used to quantify the protein and detect TGF-β and Smad2 protein in bone cells. SDS-PAGE gel electrophoresis was performed, membrane was transferred for 1 h in ice bath state, BSA was blocked at room temperature for 2 h, primary antibody was incubated overnight at 4°C, and membrane was washed three times for 10 min each time. Then, incubate the second antibody at room temperature for 1 h, and wash for 3 times, each time for 10 min. The electrochemiluminescence substrate chromogenic solution was added, and the image was taken by gel imager.

ALP staining and alizarin red (AR) staining was used to detect the formation of calcium nodules in BMSC. In ALP staining, follow the instructions of the kit. A few drops of No.1 solution were added to the cell slide, fixed at room temperature for 1 min, rinsed for 2 min, and dried. A few drops of action solution were added, incubated in a wet box at 37°C for 2 h, and rinsed for 2 min. A few drops of No.5 solution were added, counterstained for 5 min, rinsed for 2 min, and dried, and take images with a microscope. In AR staining, the cells were transferred to the glass slide, fixed with 95% ethanol for 10 min, and washed. Then, it was incubated in 0.1% alizarin red staining solution at room temperature for 30 min. Rinse with distilled water, dry, seal, and take pictures with microscope.

2.3. Statistical Methods. With SPSS 22.0 statistical software, the data was expressed as mean ± standard deviation, t test was used for comparison, and P < 0.05 was significant.

3. Results

3.1. Establishment of OP Model Rats. Serum ALP level in model group was higher than the sham-operated group (P < 0.05) (see Figure 1).
3.2. Expression of PTEN and TGF-β/Smad2 Protein in Tibia of OP Model Rats. The expression of PTEN in model group was higher than the sham-operated group ($P < 0.05$). The expression of TGF-β and Smad2 protein in the model group was lower than the sham-operated group (see Figures 2 and 3).

3.3. Expression of PTEN and TGF-β/Smad2 in Osteogenic Differentiation of BMSC under Tension. The expression of PTEN in tension MSC group was higher than MSC group ($P < 0.05$). The expression of TGF-β and Smad2 protein in tension MSC group was lower than MSC group (see Figures 4 and 5).

3.4. Effect of Overexpression of PTEN on Osteogenic Differentiation and TGF-β/Smad2 Protein of BMSC under Tension. In ALP staining, compared with the control group and oe-NC group, the osteogenic differentiation ability of BMSC and ALP activity in oe-PTEN group were obviously weaker (see Figure 6). In the AR staining, compared with the control group and oe-NC group, oe-PTEN group had lower mineralization degree, fewer calcium nodules, and lighter cell staining (see Figure 7). The expression of TGF-β and Smad2 protein in oe-PTEN group was lower than oe-NC group and control group (see Figure 8).

3.5. Effects of Interfering PTEN on Osteogenic Differentiation and TGF-β/Smad2 Protein of BMSC under Tension. In the ALP staining, compared with the control group and si-NC group, the osteogenic differentiation ability of BMSC and ALP activity in si-PTEN group were obviously stronger (see Figure 9). In the AR staining, compared with the control group and si-NC group, the si-PTEN group had higher mineralization degree, more calcium nodules, and deeper cell staining (see Figure 10). The expression of TGF-β and Smad2 protein in si-PTEN group was higher than the si-NC group and control group (see Figure 11).

4. Discussion

OP is the primary cause of fractures in the elderly in China. The pathogenesis of tibial fractures caused by OP has been highly valued clinically [11]. Bone is composed of bone
matrix and minerals deposited in it, which has the functions of supporting, protecting, and storing calcium and phosphorus for the entire body. The bone can sense and adapt to mechanical tension, maintaining a balance between bone resorption and bone remodeling [12, 13].

TGF-β is a protein polypeptide, which controls bone density by regulating the deposition of osteoblasts and the absorption of osteoclasts, and can maintain bone homeostasis [14]. TGF-β can not only increase the directional migration ability of osteoblasts, but also attract the positioning and movement of osteoblast progenitor cells during bone reconstruction. It can also reduce bone turnover, promote the formation of bone and cartilage, and accelerate osteoclast apoptosis [15]. TGF-β family proteins act on the whole process of osteogenic differentiation. Tu’s team found that reduced TGF-β level may be one of the pathogenic factors of OP, and different doses of TGF-β can regulate the proliferation and differentiation of osteoblasts through different pathways, affecting the absorption and destruction of bone, thus regulating the process of bone transformation [16]. In the early stage, TGF-β can regulate the osteogenic differentiation of BMSC and promote the proliferation of osteoblasts. In the late stage, TGF-β can regulate the collagen secretion and calcium salt deposition of osteoblasts [17]. In addition, Lin’s team found that there is abundant expression of Smad2 protein in normal bone tissue, it is widely expressed in the epiphysis, and it is mainly expressed in osteoblasts on the surface of bone matrix and around trabecular bone [18]. TGF-β/Smad2 signal transduction is a rather complicated process from cell membrane to nucleus; the main steps include activation binding stage, recombination separation stage, and transfer action stage, involving many genes and proteins. In the process of BMSC aggregation differentiation to osteoblasts and osteoblasts’ own proliferation and differentiation, Smads directly combined with DNA as transcription factor or interacted with other transcription factors and activating factors to induce the transcription response to TGF-β signal. TGF-β affects the expression of its downstream osteogenic specific transcription factor Runx2 through the classical Smad signal pathway and regulated the expression of osteogenic related genes [19]. Li’s team research shows that silencing endogenous Smad2 expression in BMSC can enhance bone formation, but inhibit adipogenesis, and miR-10b promotes osteogenic differentiation and bone formation through TGF-β pathway [20]. Yuan’s team believes that after ovariectomy, the expression of TGF-β1 is down-regulated and the expression of Smad2 protein is also significantly reduced and Smad2 protein and its mediated TGF-β1 may play an important role in the formation of postmenopausal OP [21].

PTEN is associated with the differentiation of osteoblasts and osteoclasts. Shen’s team found that the upregulation of PTEN facilitated the osteogenesis of dental pulp mesenchymal stem cells [22]. Cai’s team showed that the addition of PTEN inhibitor partially blocked the process of oxaloacetic acid affecting the differentiation of mouse embryonic osteoblast precursor cells, myoblasts and osteoblasts, indicating that PTEN can inhibit the differentiation of osteoclasts [23]. Qi’s team found that the tensile force of 2 000 μ strain can promote the osteogenic differentiation of ST2 cells, while 10% and 15% strain rate can inhibit the osteogenic differentiation of ST2 cells [24]. We found that the expression of PTEN was higher
in the model group and tension MSC group, and the expression of TGF-β and Smad2 protein was lower. The results showed that PTEN and TGF-β/Smad2 pathways might play a key role in the osteogenic differentiation of BMSC in OP tibial fracture rats, while pathological tension inhibited the differentiation of MSC cells into mature osteoblasts and reduced the expression of osteogenic related genes under the conduction of stress stimulation signals.

In this study, we interfered with PTEN and then gave osteogenic differentiation. The results showed that the expression of TGF-β and Smad2 protein increased in si-PTEN group, but decreased after overexpression of PTEN. This indicates that PTEN plays an important role in the osteogenic differentiation of rat BMSC stimulated by tension and PTEN may regulate the osteogenic differentiation of BMSC by mediating TGF-β/Smad2 pathway. Downregulation of PTEN can upregulate the expression of TGF-β and Smad2 protein and interfere with PTEN to promote osteogenic differentiation of BMSC. Meanwhile, the ALP staining results and AR staining results also confirmed that interfering PTEN can promote the osteogenic differentiation of cells under tension, but the specific mechanism remains to be further explored.

5. Conclusion

To sum up, PTEN-mediated TGF-β/Smad2 pathway may play a key role in the osteogenic differentiation of OP tibial fracture rats. Downregulation of PTEN and upregulation of TGF-β/Smad2 signal can promote the osteogenic differentiation of BMSC under tension, which can be used as a target for bone tissue research.

Data Availability

The data used and/or analyzed during the current study are available from the corresponding author.

Ethical Approval

The animal experiments in this study have been approved by the Animal Ethics Committee of Zhabei Central Hospital.

Conflicts of Interest

The authors declare no conflict of interest, financial, or otherwise.

Authors’ Contributions

Shiyong Ling and Chen Yan are co-first authors.

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