European Review for Medical and Pharmacological Sciences 2022; 26: 5413-5421

The effect of BMP2/Smads pathway mediating platelet-rich fibrin on rat bone mesenchymal stem cells

L.-L. WU, Y.-M. JIA, Y.-X. SUI, S. AO, Y. WANG, Y. LIU, W.-D. XU, H.-B. ZHANG, H. ZHANG, J.-H. MAO, X.-T. YANG, H. LENG

Department of Spine Surgery, Chifeng Municipal Hospital, Hongshan District, Chifeng City, Inner Mongolia, China

Leilei Wu and Yiming Jia contributes equally to this work

Abstract. – OBJECTIVE: We explored the influences on platelet-rich fibrin (PRF) to rat Bone Mesenchymal Stem Cells (BMSCs), as well as the role of bone morphogenetic protein 2 (BMP2)/maternal signal protein homolog (Smads) pathway.

MATERIALS AND METHODS: The proposed research is approved by the ethics board of the Second Affiliated Hospital of Harbin Medical University. The BMSCs were isolated and purified. The BMSCs were assigned to a control group arbitrarily, PRF group, BMP activator group and BMP inhibitor group (hereinafter referred to as activator group and inhibitor group). Each group of BMSCs in the logarithmic growth phase was detected for the alkaline phosphatase (ALP) activity since 3 days and 14 days of culture; CCK-8 assay was conducted for detection of the proliferation of BMSCs; Real time PCR was conducted for detection of the osteogenic differentiation marker collagen I (COL-I), BMP2, Runt-related transcription factor 2(RUNX2), osteocalcin (OCN) mRNA relative expression levels; Western-Blot detection of BMP2, OCN, P-SMAD1/5/8, relative expression level of RUNX2 protein.

RESULTS: In contrast to the control group, BMSCs' the ALP activity of the PRF group, activator group, as well as inhibitor group increased for 3 days and 14 days, and the activator group>PRF group>inhibitor group ($p \leq 0.05$). ALP activity in each group was elevated with the increase in culture time, the ALP activity of the control group, PRF group, activator group and inhibitor group increased ($p \leq 0.05$). In comparison to the control group, the relevant expression levels of COL-I, BMP-2, RUNX2 and OCN in the PRF group, activator group, and inhibitor group increased, and the activator group>PRF group>inhibitor group ($p \leq 0.05$). The relative expression levels of BMP2, OCN, p-SMAD1/5/8 and RUNX2 protein in each group were statistically different, the activator group>PRF group>control group>inhibitor group ($p \leq 0.05$).

CONCLUSIONS: PRF can promote the proliferation and osteogenic differentiation of BMSCs by activating the BMP2/Smads signaling pathway.

Key Words:
Platelet-rich fibrin, BMSCs, Osteogenic differentiation, Bone morphogenetic protein 2/maternal signal protein homolog pathway.

Introduction

Osteoporosis (OP) has been a systematic illness defined by decreased bone density and microstructure deterioration, leading to greater brittleness and fracturing risk. Skeletal reconstruction is achieved by constantly accumulating old bone and forming new bone in order to maintain its structural integrity. Inadequate bone formation and excessive bone absorption can lead to OP[1]. Mesenchymal stem cells (MSCs) in membranes and cartilage differentiate into osteoblasts. In osteogenic processes, the differentiation mechanism of osteoblasts is basically the same, namely the differentiation of BMSCs[2]. A number of studies[1-3] have shown BMSCs' capacity to differentiate into osteoblasts had been reduced in OP patients.

BMSCs are the most widely used in cartilage tissue engineering. Studies[4] have confirmed that it has the potential to differentiate into bone, cartilage, fat, tendon, and spirit meridian cells under different induction conditions. BMSCs can construct cartilage tissue *in vivo* and *in vitro* and repair cartilage defects successfully. BMSCs are also perfect seed cells in tissue engineering[5].

Tissue engineering and regenerative medicine are rapidly evolving fields, how to apply the

Corresponding Author: Hui Leng, MD; e-mail: cflh33388@163.com

achievements in this field to clinical practice and become an alternative therapy of traditional medicine is a hot topic at present. In the process of *in vitro* culture of seed cells, if the treatment can improve the proliferation and migration of stem cells, it will provide technical support for better construction of tissue engineering tissues. However, how to select seed cells and improve their proliferation ability through effective means is still the focus of current research.

In 1984, Assoian et al[6] were the first to extract platelet-rich plasma (PRP) from human plasma. Subsequently, research[7] revealed that PRP is rich in platelets, and platelets degranulated can release a large number of growth factors to promote the healing and regeneration of hard and soft tissues. Initially, PRP was mainly applied in plastic surgery and trauma surgery[8,9]. In 1997, PRP was first applied in oral clinical research by Whitman et al[10] and achieved good results. Subsequently, PRP technology became a hot topic in oral medicine research. Since the 1990s, PRP has been widely applied in animal model studies and clinical application studies[11-14], and the research results show that PRP has a strong ability to promote the healing of hard and soft tissues. However, recent studies[15-18] have shown that PRP does not significantly promote repair of hard and soft tissues. Meanwhile, the application of PRP has been controversial due to the risk of immunological rejection and the transmission of infectious illnesses due to the addition of heterologous thrombin and anticoagulant in the preparation process. In 2000, Choukroun et al[19] prepared and extracted a latest type of platelet concentrate, namely Choukroun's platelet-rich fibrin (Choukroun's PRF) for the first time. It continues the advantages of PRP, platelet concentrates of the previous generation, and has the advantages of strong osteogenic ability, simple preparation process, completely derived from autologous blood, without adding any biological agents, etc., avoiding ethical disputes and the risk of blood cross infection.

Choukroun's PRF was examined for its influence on the growth and difference of human bone marrow mesenchymal stem cells, as well as its dosage dependency by David et al[20]. The results showed that Choukroun's PRF may greatly increase the growth and differentiation of BMSCs into osteoblasts in a dose-reliant fashion. Chang et al[21] showed that Choukroun's PRF can promote osteoblast proliferation and at the same time activate phosphorylated extracellular signal-regulated protein kinase and osteoprotectin to promote bone formation.

As an important extracellular signaling molecule, BMP-2 is the greatest significant factor in stimulating the activation and differentiation of osteoblasts, and the BMP-2/SMAD signaling pathway is critical for BMSC development into osteoblasts and extracellular matrix. Bmp-2 attaches to cell membrane receptors and triggers intracellular SMAD1/5/8 phosphorylation. SMAD that has been triggered may be transported from the cytoplasm to the nucleus or act on downstream genes to activate transcription of specific target genes. However, there are still few reports on the the pathway's function in the differentiating rat BMSCs into osteoblasts induced by Choukroun's PRF. The impact of Choukroun's PRF on BMSCs and its mechanism were studied in this work.

Materials and Methods

BMSCs Isolation

In this study, BMSCs were extracted from the femur of Wistar rats at the age of 4 weeks. Every animal assay was completed as per the National Institutes of Health Guide for the Welfare and Utilization of Lab Animals and accepted by the regional ethical board. After the rats were sacrificed by cervical dislocation, the two sides of femur and tibia were disinfected in 75% alcohol, the bone marrow was removed, washed, trypsin digested, supernatant was centrifuged, and the cells were suspended for further culture. The primary cell growth and fusion reached 90% when the passage. The BMSCs of the third generation of rats were cultured in culture plates or culture plates with osteogenic induction medium (dMEM-F12 medium containing 10% fetal bovine serum, 10-8 dexamethasone, 10 mmol/Lβ-sodium glycerophosphate and 50 μg/ mL ascorbic acid).

Preparation of PRF

PRF was developed utilizing the approach by Li et al[22]. The venous blood collection method was used to collect 5 mL of the experimental animal's autogenous blood immediately and transfer the blood to sterile negative pressure collection vessel quickly. Centrifugation was conducted at 3000 RPM for 10 min. White PRF clots between plasma and blood cells were separated and the excess fluid was discharged. The exudate was centrifuged at 3000 RPM for 10 min to obtain hemocyte-free exudate, which was processed through a sterile screen and kept at -80°C until future use.

Grouping and Processing

The BMSCs were separated into four groups arbitrarily, in groups of PRF, activator and inhibitor. BMSCs in control group were cultured in double antibody α-MEM medium containing fetal bovine serum at 10%. In THE PRF group, the volume fraction of PRF was 50% by adding -80°C PRF into the double antibodyα-MEM medium of fetal bovine serum (FBS) at 10%. Activator group as well as inhibitor group were supplemented with Arnicolide C (final concentration of 10 μM) and noggion (final concentration of 5 μM) on the basis of PRF group.

Survival and Proliferation of BMSCs in Each Group Were Analyzed by Cck8 Method

The third generation BMSCs had been digested by 0.25% trypsin and centrifuged in order for the preparation of cell suspension with a concentration of $5\times10^3\cdot mL\text{-}1$. Cultured at 37°C and 5% CO_2 for 24 h, the medium was replaced and platelet-rich fibrin h-DMEM complete medium was added for 1, 3, 5 and 7 days, respectively. Each well received a CCK8 solution, and the culture had been kept going for 4 h at 37°C with 5% CO_2. The culture was terminated, and the supernatant was discarded. The light absorption value (A) of each well was measured by Elisa (λ=450 nm) and recorded. ANOVA has been applied to compare the groups, and a difference of $p < 0.05$ was considered statistically meaningful.

Measurement of Alkaline Phosphatase (ALP) Activities

BMSCs at logarithmic growth stage were cultured in the control group, PRF group, activator group and inhibitor group in the corresponding medium. After 3d-14d culture, Triton X-100 was added and the cells were completely lysed and incubated with ALP substrate mixture for 0.5 h, 0.5 mol/L NaOH terminated the reaction. The optical density (A) at 490 nm had been evaluated through a microplate reader.

The mRNA Expression of COL-I, BMP-2, RUNX2 and OCN Have Been Detected by Real time PCR

BMSCs culture medium suspension was inoculated on 96-well plates, and cells were collected at 3, 7 and 14 days, respectively. TRIzol was used to extract total RNA from the cells after ultrasonic lysis. Reverse transcription of RNA into cDNA according to the instructions of the ReverTra Ace qPCR RT kit. Then, PCR reaction was performed according to the instructions of SYBR Green PCR Master Mix kit, and primer sequences were shown in Table I. Rat GAPDH was used as an internal reference, and $2^{-\Delta\Delta Ct}$ method was used to calculate the mRNA relative expression levels of target genes.

Table I. Primers used for PCR.

cDNA primer	Sequences
collagen I-F	CGGCTCCTGCTCCTCTTAGG
collagen I-R	AGGGACCCTTAGGCCATTGT
BMP-F	CACGAGAATGGACGTGCCC
BMP-R	CACTAGAAGACAGCGGGTC
OCN-F	GCAACTCGGTGCAGACCTA
OCN-R	CTCAGAGTCGCTGGGCTTT
Runx2-F	AACCAAGTGGCCAGGTTCAA
Runx2-R	GGACCGTCCACTGTCACTTT
GAPDH-F	AGTCGCCAGCCTCGTCTCATA
GAPDH-R	AAGAGAAGGCAGCCCTGGTA

The Relative Expression Levels of BMP2, OCN, P-SMAD1/5/8 and RUNX2 Proteins Have Been Detected by Western-blot

BMSCs at logarithmic stage were cultured for 14 d, the cells have been harvested, while the entirety of the total protein was obtained. The cells were added with SDS-loading buffer, and then denaturated in water bath at 100°C. SDS-page gel electrophoresis was performed, and the membrane was transformed, and the cells were sealed at room temperature for 2 h. The relative expression of target protein was calculated.

Statistical Analysis

The assessment was carried out using SPSS 23.0 tool (SPSS Inc., Armonk, NY, USA) and the results collected were represented by (average ± SD), with normal distribution, homogeneity of variance, diversity. The measurement data were compared using a one-way ANOVA, and LSD-T test was used for comparison of pinioned samples. $p\leq0.05$ had been determined as statistically meaningful.

Result

Effect of PRF on Proliferation Activity of BMSCs

Results of CCK-8 method are shown in Figure 1. The results showed as follows: after statistical analysis, there had not been any meaningful distinction in absorbance value in groups on day 1 ($p >0.05$); On day 3, 5 and 7, the absorbance value of PRF group was higher than inhibitor group, and

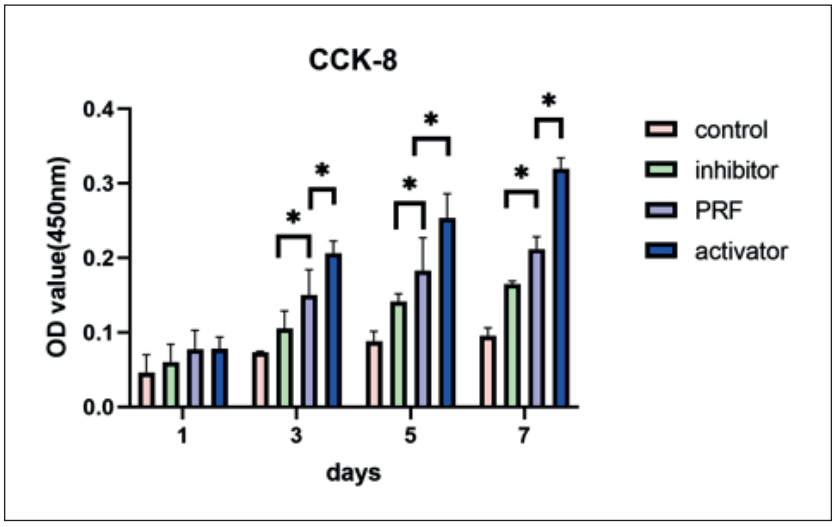

Figure 1. The CCK8 assay of BMSCs (*$p \leq 0.05$).

there was a significant difference after statistical analysis ($p \leq 0.05$), as shown in Figure 1.

ALP Activity Detection of BMSCs

The ALP activity of BMSCs cultured for 3 d, 7 d and 14 d in PRF group, activator group and the inhibitor group had a greater level of inhibition in comparison to the control group. ($p \leq 0.05$). The ALP activity of BMSCs cultured for 3 d, 7 d and 14 d in activator group had been greater to the PRF group, while the ALP activity of BMSCs cultured for 3 d, 7 d and 14 d in inhibitor group which had been lesser to those in PRF group ($p \leq 0.05$). The ALP activity of BMSCs cultured for 3 d, 7 d and 14 d in inhibitor group was lower than that in activator group ($p \leq 0.05$). The ALP activity of control group, PRF group, activator group and inhibitor group also increased with the increase of culture time ($p \leq 0.05$) (Figure 2).

The mRNA Expression of COL-I, BMP-2, RUNX2 and OCN in BMSCs

The mRNA expressions of COL-I, BMP-2, RUNX2 and OCN in PRF group, activator group

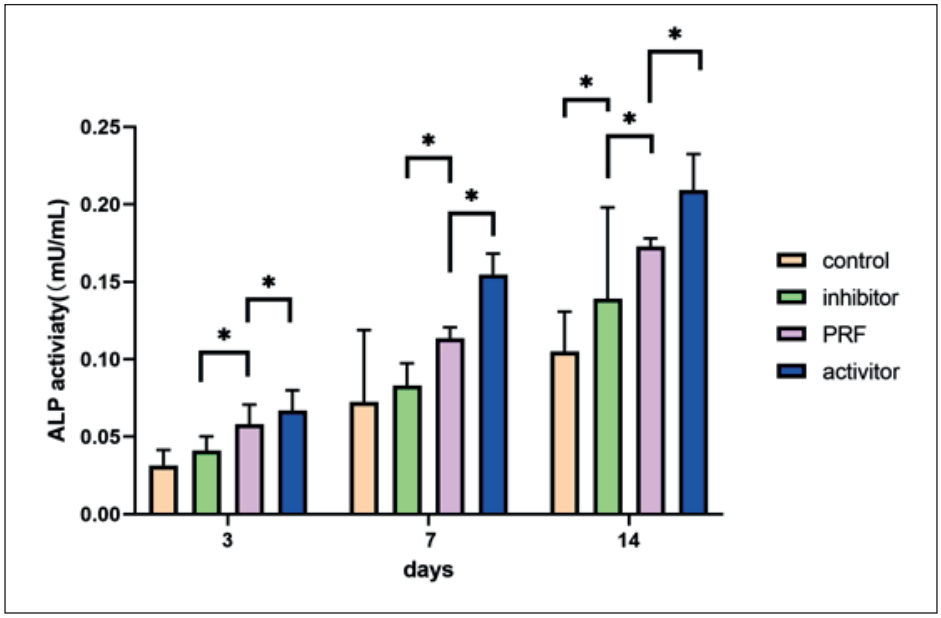

Figure 2. The ALP activity of BMSCs (*$p \leq 0.05$).

and inhibitor group have been greater to those in control group ($p \leq 0.05$). The mRNA expression of COL-I, BMP-2, RUNX2 and OCN in activator group had been greater to the PRF group, and the mRNA expression of COL-I, BMP-2, RUNX2 and OCN in inhibitor group had been lesser to PRF group ($p \leq 0.05$) (Figure 3).

Relative Expression Proteins Levels of BMP-2, P-SMAD1/5/8, OCN and RUNX2

Relative expression proteins levels of BMP-2, P-SMAD1/5/8, OCN and RUNX2 in the PRF group, activator group and inhibitor group have been greater to the control group. The protein expression of BMP-2, P-SMAD1/5/8, OCN and RUNX2 in activator group was higher than that in PRF group, and the protein expression of BMP-2, P-SMAD1/5/8, OCN and RUNX2 in inhibitor group had been lesser to the PRF group (Figure 4).

Discussion

BMSCs are the most studied adult stem cells in clinical and experimental studies. BMSCs are a kind of adult stem cells, which belong to the early stage of mesoderm development, and are another kind of stem cells in bone marrow besides hematopoietic stem cells. They have self-renewal and multidirectional differentiation ability and can differentiate into hematopoietic stromal cells and non-hematopoietic tissues in their fibroblast-like growth. For example, osteoblasts, chondrocytes, myocytes, adipocytes, cardiomyocytes, endothelial cells, pulmonary epithelial cells, etc.[23].

Since BMSCs are derived from adult cells, or even from patients themselves, rather than fetal or embryonic stem cells, there are no ethical and moral issues involved. In addition, BMSCs have the following advantages: (1) they are easy to be cultured *in vitro*, have high amplification ability, and have good gene stability after multiple passages *in vitro*; (2) multi-differentiation potential, differentiated into different tissue cells in the suitable growth microenvironment *in vivo* through different induction conditions; (3) can be combined with a variety of viral vectors, so as to carry out gene transfection, not only maintain the stability of their own genes, but also highly express the transfected genes, so as to produce proteins needed for the repair of damaged sites; (4) homologous BMSCs did not cause immune rejection after entering the body. Because of these advantages, BMSCs have been widely used in experimental and clinical studies in recent years. Bone morphogenetic proteins (BMPs) are a member of the transforming growth factor (TGF) superfamily that is critical for bone cell differentiation and are ectopic osteogenic growth factors. It can transform bone marrow stromal cells into osteoblasts *in vivo* and *in vitro* and keep the number of well-differentiated osteoblasts stable. Currently, more than 40 proteins have been isolated from the BMPs family, among which the most important is BMP-2 protein, which is made up by 396 amino acid residues, and its gene located at 20pl2, is involved in the early development of skeletal system, the formation of accessory bones, and the occurrence of heart, brain and kidney[24].

BMPs perform a vital biological function in bone development control[25]. BMPs signaling pathway can regulate BMSCs proliferation, osteogenic differentiation, maturation and normal function of osteoblasts[26]. It is known that BMPs can induce ALP expression and osteogenic differentiation in BMSCs isolated from rats or mice with different cell lines[27]. BMP-2 is synthesized as a precursor in the cell. Mature BMP-2 protein structures are homotype or heterotype dimers linked by disulfide bonds. This dimer is released out of the cell and binds to corresponding receptors on the surface of target cells throughout the body (not only on the surface of bone cells) to perform its functions[28]. BMPs receptors are mainly type 2, namely type I and type II, and type I are members of the serine/threonine kinase superfamily. Type I receptors are divided into activated receptor-like kinases 1-7 (ALK1-7), in which ALK1, ALK2, ALK3 and ALK6 participate in BMP-2 signal transduction. Both type I and type II BMP receptors are engaged in BMP-2 signaling. When BMP-2 protein acts, it first forms a heterotetramer receptor complex, and then binds to type II receptor to activate it. Then type I receptor binds to BMP-2 protein and is activated by transphosphoric group under the action of activated type II receptor. BMP-2 mainly transmits signals through SMAD proteins. SMAD proteins are classified into three groups, receptor modulating types, including SMADl, SMAD2, SMAD3, SMAD5 and SMAD8, co-mediated SMAD4, and inhibitory SMAD6 and SMAD7. SMAD2 and SMAD3 play a role in TGF-J3 signaling system, but do not participate in BMPs signaling. SMADl, SMAD5, and SMAD8 are involved in BMP-2 signaling: the activated type I receptor rapidly phosphorylates the serine residues of SMADl, SMAD5, or SMAD8. Phosphorylated SMADl, SMAD5,

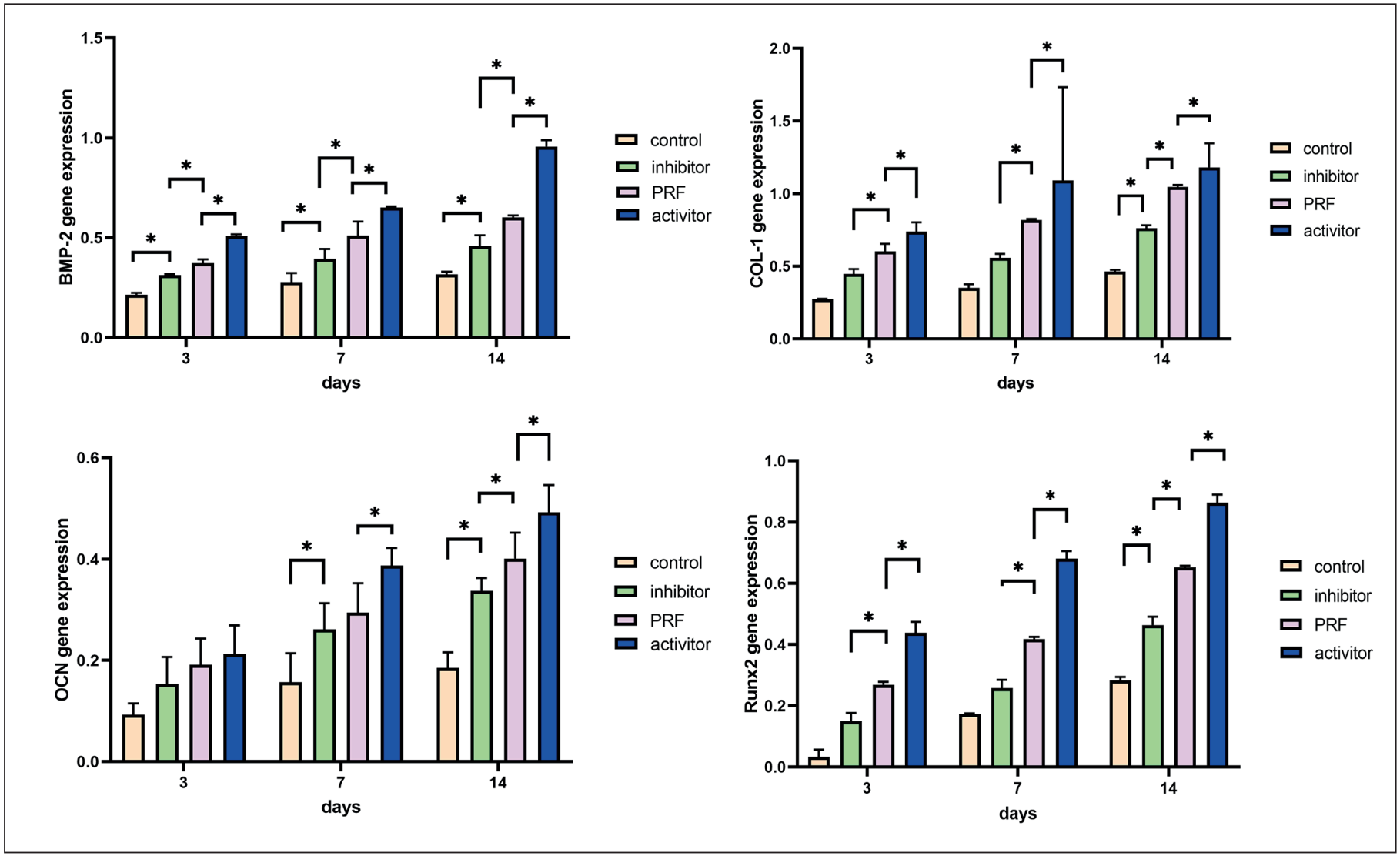

Figure 3. The mRNA of COL-I, BMP2, RUNX2, OCN of BMSCs (*$p \leq 0.05$).

SMAD8 and SMAD4 bind to form complexes and translocate to the nucleus, and act on downstream target genes to activate the transcription of specific target genes by directly binding to specific DNA sequences, or forming complexes with nuclear regulatory factors, or recruiting transcription co-activation or co-inhibition factors.

The core binding factoral (Cbfcl) is a component in the Runt/Cbfa transcription factor family, which was regarded as Runt related gene 2 (Runx2) as well. This transcription factor is highly homologous to the pairing gene RUNT of fruit flies and can trigger the transcription of osteocalcin in straightforward manner, osteopontin, type I collagen and other genes throughout the osteogenic differentiation of bone marrow mesenchymal stem cells[29]. The expression of Runx2 is a marker of osteoblast differentiation and induces BMSCs to develop into osteoblasts or chondrocytes, as a result, this represents the first and greatest exact bone formation marker gene[30]. *In vitro* as well as *in vivo* experiments have shown that Runx2 is a crucial component for bone marrow mesenchymal stem cell osteogenic differentiation and bone development[31].

PRF is rich in a variety of growth factors related to tissue repair. Domestic and foreign scholars' studies[32] on Choukroun's PRF mainly focus on its effect on the repair of soft and hard tissues. Choukroun's PRF has been confirmed in clinical studies that it can promote soft tissue repair. Choukroun's PRF is of great significance for bone tissue repair. Therefore, studying the effect of PRF on BMSCs osteogenic differentiation is crucial for the development of bone tissue engineering[33,34]. ALP reflects the degree of osteoblast differentiation and promotes the formation of bone matrix mineralization[35,36]. Matsui et al[37] showed that the degree of differentiation of stem cells was directly proportional to ALP activity. Studies have found that the expression of osteogenic markers, for instance, the ALP was significantly upregulated after PRF was added into apical papillary stem cells[38]. In this study, in contrasted to the control group, the activity of ALP in PRF group was significantly increased, suggesting that PRF could induce osteoblast differentiation. COL-I, BMP-2, RUNX2 and OCN can be regarded as markers of osteogenic differentiation of BMSCs. For this research, in comparison to the control group, the expressions of COL-I, BMP-2, RUNX2 and OCN in BMSCs in PRF group were significantly increased, suggesting that PRF can enhance osteogenic differentiation of BMSCs in rats. BMP has been a component of the transforming growth factor-1 (TGF-1) superfamily, which performs a crucial part in regulating growth and development. BMP ligands on the membrane surface bind to BMP type I and type II receptors, which trigger the SMAD-dependent signaling pathway as well as induce high expression of RUNX2 in the nucleus[39,40]. In this study, in comparison to the control group, the expressions of BMP2, P-SMAD1/5/8, OCN and RUNX2 in PRF group were significantly increased, but the expressions of BMP2, P-SMAD1/5, OCN and RUNX2 in PRF group have been lesser to these in activator group and greater to the inhibitor group, suggesting that PRF activated the expression of BMP receptor. It promotes SMAD1/5/8 phosphorylation and induces RUNX2 expression to play a role, thereby promoting BMSCs osteogenic differentiation.

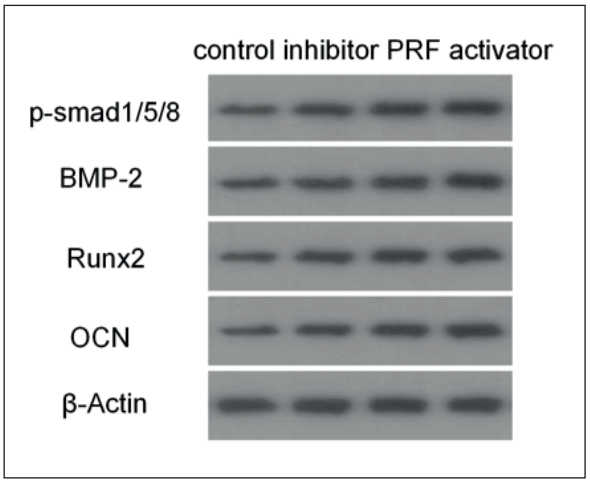

Figure 4. The protein expression of p-SMAD1/5/8, BMP2, RUNX2, OCN of BMSCs (*$p \leq 0.05$).

In conclusion, PRF may promote the osteogenic differentiation of rat BMSCs through triggering the BMP2/Smads signaling pathway, laying the foundation towards bone tissue engineering treatment of osteoporosis.

Conclusions

We showed that platelet-rich fibrin promoted osteogenesis, thus inhibiting bone resorption of osteoclasts. The mechanism of platelet-rich fibrin promotes the entry of SMAD1/5/8 into the nucleus and regulates the differentiation direction of bone mesenchymal stem cells through the BMP-2/SMAD signal pathway, thus promoting bone formation.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Wu LL and Jia YM contributed to the conception of the study; Leng H gave instructions; Wu LL, Jia YM, Sui YX, Ao S, Wang Y, Liu Y, Xu WD, Zhang HB, Zhang H, Mao JH, Yang XT, Leng H performed the experiment, analysis and manuscript preparation; Wu LL and Jia YM revised the manuscript. All authors revised and have given approval to the final version of the manuscript.

Ethics Committee Approval

Every animal assay was completed as per the National Institutes of Health Guide for the Welfare and Utilization of Lab Animals and accepted by the regional ethical board. The proposed research is approved by the ethics board of the Second Affiliated Hospital of Harbin Medical University.

Acknowledgements

The authors are grateful to Animal Experiment Center, the Second Affiliated Hospital of Harbin Medical University for Providing the animal for this experiment.

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