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FAK and BMP-9 synergistically trigger osteogenic differentiation and bone formation of adipose derived stem cells through enhancing Wnt-β-catenin signaling

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ABSTRACT

Backgrounds: Adipose derived stem cells (ADSCs) could undergo osteogenesis via focal adhesion kinase (FAK) and bone morphogenetic protein (BMP) 9 signals, both of which could affect Wnt-β-catenin signal, a signal pathway closely related to ADSCs osteogenesis. It's still enigma whether FAK and BMP-9 contribute to osteogenesis. Here, we examined the effect of FAK on BMP9-inducedosteogenic differentiation, unveiled the possible molecular mechanism underling this process.

Methods: In the present study, ADSCs were isolated and purified, and cells of passage 3 underwent virus mediated transfection to prepare ADSCs with stable FAK shRNA expression. Cell viability and migration were detected by MTT and transwell assay, respectively. Expression of osteogenic gene, phosphorylation of FAK and GSK were detected by western blot. Osteogenic potential was evaluated by activity of alkaline phosphatase (ALP) and calcium deposition by ALP staining and Alizarin Red S staining.

Results: BMP-9 administration promoted ADSCs osteogenesis. Knocking down FAK attenuated this process, inhibited osteogenic proteins expression through Wnt-β-catenin signal. BMP-9 also triggered ADSCs proliferation and migration, and shFAK antagonized such effects too. Although Wnt signal is affected by FAK shRNA, Smad signal remains intact in ADSCs with shFAK.

Conclusion: FAK and BMP-9 could cross talk on Wnt signal pathway and promote ADSCs osteogenesis. FAK could participate in BMP-9 induced ADSCs osteogenesis via Wnt signal pathway other than Smads signals (see in graph).

1. Introduction

Human adipose tissue contains a sub-type of cells with potential to differentiate into multiple lineages. These cells are described as adipose derived stem cells (ADSCs) [1,2]. ADSCs were considered similar to mesenchymal stem cells (MSCs). Under proper stimulation, ADSCs could exert osteogenic differentiation and promote bone regeneration [1]. Thus studying osteogenic regulation of ADSCs is important.

Focal adhesion kinase (FAK), also known as protein tyrosine kinase2 (PTK2), is a tyrosine kinase and vital member in integrin-mediated signaling pathways [3]. In response to integrin involvement, FAK would be phosphorylated. It was reported that FAK is involved in cellular adhesion and spreading [4]. Besides FAK is also an signal adaptor, which could cross talk with multiple other signal transduction pathways, and participate in regulation of various cellular processes including apoptosis, cell mobility and migration [5,6]. It was also reported that FAK could promote osteogenesis of ADSCs via Wnt-β-catenin signal [3,7].

BMP-9, also named as growth differentiation factor2 (GDF-2), was first identified in mice liver tissues [8]. BMP-9 could promote MSCs differentiation towards adipose, blood veins and cartolare [8,9]. Among all these effects mentioned above, osteogenesis and adipogenesis effects of BMP-9 were mostly valued [9,10]. BMP-9 could also enhance Wnt-β-catenin signal, and promote osteogenesis [11]. Although other BMP family members such as BMP2/4/6/7 were described to possess similar functions, yet BMP-9 is much stronger [8,12].

Although both of FAK and BMP-9 could be implicated in osteogenesis regulation via Wnt-β-catenin signal pathway, the interplay

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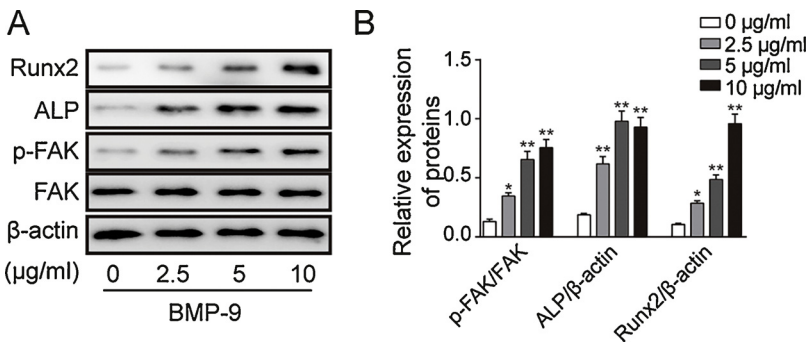

Fig. 1. BMP-9 induces p-FAK, ALP and Runx2 expression in ADSCs.

A. ADSCs were isolated and cultured into in medium supplied with increasing amount of BMP-9 as indicated. Cells were passaged 3 times and detected for p-FAK, ALP, Runx2 expression by Western blot.

B. Statistical analysis of relative intensity of proteins detected in A. Intensity of p-FAK was normalized to FAK, intensities of other proteins were normalized to β-actin.

between FAK and BMP-9 remains enigma.

This study aims to reveal the existence and unveil the mechanism of FAK-BMP-9 cross talk during their osteogenic regulation through Wnt-β-catenin signaling pathway, and provide theological support for bone regeneration studies.

2. Materials and methods

1 **ADSCs isolation.** Minced epididymal fat pads were digested by collagenase type1 in krebs-Ringer Bicarbonate buffer, and filtered through 100 μm mesh. SVF was incubated overnight, and non-adherent cells were removed. The medium (DMEM + 10% FBS) were supplied with basic fibroblast growth factor, and ADSCs passaged to 3rd were used for experiments.

2 **Lentivirus infection and stable cell line selection.** FAK shRNA or shNC were cloned into lentivirus system pLKO.1.Virus were packaged following vector providers' instructions(FAK5'CTGTACTTCGG ACAGCGTGA-3′, 5'-ATGTCGTGAGCGCATAGACC-3′). ADSCs were infected with indicated virus and selected with puromycin for 7 days.

3 **MTT Assay.** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conveyed for cell viability detection. Cells seeded on a 96 well plate at a starting concentration at 10^5/ml were cultured as indicated in figure legends. Cells were then rinsed with PBS and administrated with 20 μL 5 mg/ml MTT for 4 h at 37 °C and DMSO was used to dissolve formazan precipitation. Absorbance at 490 nm was detected using a plate reader.

4 **Transwell Assay.** ADSCs treated as indicated were seeded into transwell chamber (Corning) and subjected to transwell assay following manufactures' instructions. Briefly, ADSCs were plated in the transwell inserts, and inserts were placed on 12-well plate with medium adequate to touch the bottom of inserts. After 8 h of incubation, remove the inserts, reverse the chambers and immerse them in PBS gently to remove unattached cells. Cells were then fixed and stained for detection.

5 **Western Blot.** The proteins were extracted with RIPA, quantified, and separated on SDS-PAGE. The membranes were blocked with 5% BSA for 1 h at room temperature, and incubated with primary antibodies at 4 centigrade overnight. Membranes were washed by TBST 3 times and incubated with HRP-conjugated secondary antibodies for another 1 h at room temperature. The blots were analyzed by enhanced chemiluminescence (ECL) western blotting detection system (GE HealthCare Bio-Sciences, Piscataway, NJ, USA).

6 **ALP staining.** Briefly, cells were treated with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate for 5 min. To quantify ALP, Ostase® BAP Immunoenzymetric Assay (Immunodiagnostic Systems Ltd, Scottsdale, AZ, USA) was used following the manufacturer's instructions.

7 **Alizarin Red staining.** Osteogenesis was examined by staining mineralized nodules with Alizarin Red S. Briefly, cells were washed with PBS and soaked in 40 mM Alizarin Red (pH 4.2) for 30 min at 37 °C, then washed with PBS and imaged. Decalcification was performed using 0.1 M HCl overnight at 4 °C. Then, 20 μL of samples were transferred to the test tubes containing 1 mL of methyl thymol blue solution and 1 mL of alkaline solution.

8 **Statistical analysis.** All data values were shown as mean ± standard deviation (SD). Comparison of all other results was performed by one-way analysis of variance (ANOVA) with Tukey's comparison analysis and the statistical significance was analyzed using Student *T* test and analysis of variance. Data was considered significant when $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. The Graphpad Prism 5 was used to compare groups.

3. Results

3.1. BMP-9 up-regulates p-FAK, ALP and Runx2 in ADSCs

To evaluate a role for FAK phosphorylation in stimulating BMP-9 during ADSCs osteogenesis, we isolated primary ADSCs and cultured into 3rd passages. These cells were respectively treated with an increasing concentration of BMP-9, such as 0, 100, 200 and 400 μg/ml. Expression of p-FAK, ALP, Runx2 and FAK were detected by western blot. ALP and Runx2 are both osteogenic marker proteins [13] and β-actin served as loading control.

Intensity of p-FAK increased along with BMP-9 concentration, while total FAK remains stable, indicating that increasing of BMP-9 concentration could lead to stronger FAK phosphorylation. Osteogenesis of ADSCs also became stronger when cells were treated with higher amount of BMP-9, as indicated by increasing ALP and Runx2 expression level (in Fig. 1A).

The relative intensities of protein levels were analyzed and displayed in columns. ALP and Runx2 level were normalized to β-actin and p-FAK was normalized to FAK. All three protein levels exerted positive correlation with BMP-9 concentration.

All these results indicate that BMP-9 could trigger FAK phosphorylation and lead to ADSCs osteogenesis.

3.2. Knocking down FAK inhibited BMP-9 induced ADSC proliferation and migration

Although p-FAK was inceased by BMP-9 administration, we speculated whether it's required for osteogenesis. We therefore constructed FAK-knockdown ADSCs with shRNA, and introduced exogenous BMP-9 into these cells. BMP-9 stimulation could lead to enhanced ADSCs proliferation and migration. We thus detected proliferation of ADSCs with exogenous BMP and FAK-shRNA. Cells with exogenous BMP-9, or BMP-9 with control shRNA both exerted higher viability than control group. And, in cells with FAK shRNA, even exogenous BMP-9 could not rescue its decreased viability (in Fig. 2A).

Migration in another BMP-9 induced response of ADSCs was detected via trans-well assay [14,15]. In consistent with cell viability, migration of ADSCs with exogenous BMP-9 was much stronger than other groups. Knocking down FAK decreased cellular migration to an even lower level than control group, as indicated by decreased cell