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Fibroblast growth factor 2 inhibits bone morphogenetic protein 9-induced osteogenic differentiation of mesenchymal stem cells by repressing Smads signaling and subsequently reducing Smads dependent up-regulation of ALK1 and ALK2

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ABSTRACT

Understanding the interactions between growth factors and bone morphogenic proteins (BMPs) signaling remains a crucial issue to optimize the use of mesenchymal stem cells (MSCs) and BMPs in bone tissue engineering. BMP9 is highly capable of promoting osteogenic differentiation of MSCs. Fibroblast growth factor 2 (FGF2) is abundantly secreted during the healing process of fractures or in surgery bone sites. Herein, we explore the detail effect of FGF2 on BMP9-induced osteogenic differentiation of MSCs. It was found that FGF2 inhibited BMP9-induced osteogenic differentiation by blocking BMP9-induced Smads signaling and subsequently reducing Smads dependent up-regulation of ALK1 and ALK2 in MSCs. This effect was rescued by exogenous expression of ALK1 and ALK2, which are proved to be receptors for BMP9. Our results discovered a clue to explain the mechanism involved in the inhibitory effect of FGF2 on BMP9-induced osteogenic differentiation of MSCs. This crosstalk between FGF2 and BMP9 should be emphasized in the future use of BMP9 in therapeutic purpose of fracture repair.

1. Introduction

Mesenchymal stem cells (MSCs) are non-hematopoietic multipotent cells present not only in the bone marrow, but in a number of other tissues, including dermis, umbilical cord blood, adult muscle, adipose tissue, synovium and deciduous teeth (Pittenger et al., 1999; Arthur et al., 2009; Estrela et al., 2011). These cells can self-renew and differentiate into osteoblasts, chondroytes, myblasts or adipocytes under various conditions or in response to different cytokines, including Wnt, FGFs, PDGF and BMPs (Deng et al., 2008). The extensive proliferation and differentiation potential of MSCs makes them best suited for tissue engineering application, especially in bone tissue engineering. In the area of bone tissue engineering, there are so many examples of applications involving in repair or regenerate bones with MSCs (Bruder et al., 1998; Kørbling and Estrov, 2003; Marcacci et al., 2007). The effectiveness of bone tissue engineering relies on the combination of three issues: cells, including MSCs and osteoprogenitors, a scaffold and osteogenic factors to promote cells differentiation and proliferation. However, one of the most crucial pillars in MSCs-based bone tissue engineering is to promote MSCs differentiation into osteoblasts by osteogenic factors such as BMPs to obtain a functional bone tissue.

BMPs are potent growth factors belonging to the transforming growth factors β (TGFβ) superfamily (Hogan, 1996). Several forms of recombinant BMPs, most notably BMP2 and BMP7, have been shown to promote osteogenic differentiation of MSCs and are currently used as adjunctive therapy to improve bone heal in the clinical setting (Rutherford et al., 2003; Boraiah et al., 2009). However, it remains unclear whether BMP2 and BMP7 are in fact the most potent BMPs in inducing osteogenic differentiation and bone formation. BMP9 (also known as growth differentiation factor 2, or GDF2) was originally isolated from fetal mouse liver cDNA libraries and is a potent stimulant of hepatocyte proliferation (Song et al., 1995). Other roles of BMP9 include inducing the cholinergic phenotype of embryonic basal forebrain cholinergic neurons (López-Coviella et al., 2000), regulating glucose and lipid metabolism in liver (Chen et al., 2003), and maintaining homeostasis of iron metabolism (Truksa et al., 2006). BMP9 is also a potent

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synergistic factor for murine hemopoietic progenitor cell generation and colony formation in serum-free cultures (Ploemacher et al., 1999). In previous studies, BMP9 has been proved to be most highly capable of inducing osteogenic differentiation of MSCs (Kang et al., 2004; Luu et al., 2007; Luo et al., 2010).

In addition to BMPs, a number of osteogenic factors, such as Wnt, Notch, and growth factors also contribute to the differentiation of MSCs into osteoblastic lineage (Deng et al., 2008). BMPs and these above mentioned osteogenic factors form an orchestrated signaling network to modulate the osteogenic differentiation process of MSCs. Therefore, understanding the crosstalk between signaling by BMPs and other osteogenic factors remains an urgent issue to optimize the use of BMPs and MSCs in therapeutic perspectives and bone tissue engineering.

Fibroblast growth factors (FGFs) are a family of growth factors involved in angiogenesis, wound healing, and embryonic development (Ornitz and Itoh, 2001). FGFs have a high affinity for heparan sulfate proteoglycans and require heparan sulfate to activate one of four cell-surface FGFs receptors (Ornitz and Itoh, 2001). FGFs are key players in the processes of proliferation and differentiation of wide variety of cells and tissues. In humans, 22 members of the FGFs family have been identified. FGFs are secreted during the healing process of fractures or in surgery bone sites, implying that FGFs are important factor in bone development and regeneration (Bolander, 1992).

FGF2 (also known as bFGF, Basic fibroblast growth factor) is one of the most abundant FGFs in adult tissues, and is an important regulator of bone and cartilage cells (Huang et al., 2007). Sustained FGF2 treatment inhibits bone formation (Canalis et al., 1988; McCarthy et al., 1989; Rodan et al., 1989), while intermittent FGF2 treatment stimulates bone formation *in vitro* and *in vivo* (Canalis et al., 1988; Mayahara et al., 1993). FGF2 treatment has both inhibitory (Biver et al., 2012) and stimulatory effects (Hanada et al., 1997; Kizhner et al., 2011) on osteogenic differentiation of MSCs. However, the preponderance of studies showed that FGF2 enhanced the osteogenic phenotype of MSCs (Hanada et al., 1997; Kizhner et al., 2011). The effects of FGF2 on BMPs-induced osteogenic differentiation and bone formation have been investigated previously. Synergic effects between BMPs (BMP2 and BMP6) and FGFs to promote osteogenesis have already been reported (Kaewsrichan et al., 2010; Visser et al., 2012). Most studies come to the conclusion that bFGF enhances bone formation only at low dose, while suppressing osteogenesis with higher amounts. However, the results are quite disputable when trying to determine the optimal dose of FGF2.

Here, we investigate the effect of FGF2 on BMP9-induced osteogenic differentiation of MSC. Our results showed that FGF2 significantly inhibits BMP9-induced osteogenic differentiation of MSCs. The regulatory effect of FGF2 on BMP9-osteogenic induction is mediated by blocking BMP9-activted Smads signaling and subsequently neutralizing Smads-dependent expression of BMP9 type I receptor ALK1 and ALK2.

2. Materials and methods

2.1. Reagents and antibodies

Anti-total Smad1/5/8, anti-phosphorylated Smad1/5/8, anti-Smad4, anti-total ERK1/2, anti-phosphorylated ERK1/2, anti-total p38, anti-phosphorylated p38 and anti-β-actin antibodies were purchased from Cell Signaling (Danvers, MA). Anti-OPN, anti-OCN, anti-Runx2, anti-ALK1, anti-ALK2 and the secondary goat and rabbit IgGs were obtained from Santa Cruz (Santa Cruz, MA). Anti-BMP9 and anti-FGF2 were obtained from Abcam (Cambridge, MA). All cell culture media and supplements were obtained from Hyclone (Thermo Fisher, Carlsbad, CA). Unless indicated otherwise, all chemicals were purchased from Sigma–Aldrich (Saint Louis, MO).

2.2. Construction of recombinant adenoviruses

Recombinant adenovirus expressing BMP9 (Ad-BMP9) and FGF2 (AdR-FGF2) were generated previously using the AdEasy system, as demonstrated (Kang et al., 2004). Adenoviruses expressing only GFP (Ad-GFP) and RFP (Ad-RFP) were used as negative controls.

2.3. Cell cultures

C3H10T1/2 and C2C12 cells were obtained from ATCC and maintained in complete DMEM (Dulbeccos Modified Eagle Medium) supplemented with 10% fetal bovine serum and 100 units/ml streptomycin/penicillin at 37 °C in a humidified atmosphere of 5% CO_2.

A single-step primary bone marrow stomal cells primary (BMSCs) purification method using adhesion to cell culture plastic was employed as described (Luo et al., 2010). Mouse Embryo Fibroblasts (MEFs) were isolated from post coitus day 13.5 mice, as previously described (Luo et al., 2010).

2.4. Crystal violet viability assay

Crystal violet assay was conducted as described previously (He et al., 2010). Cells were treated with BMP9 and/or FGF2. At 72 or 120 h after treatment, cells were carefully washed with PBS and stained with 0.5% crystal violet formalin solution at room temperature for 20–30 min. The stained cells were washed with tap water and air dried for taking images, and quantified by Image J.

2.5. Determination of ALP activity

ALP activity was assessed by a modified Great Escape SEAP chemiluminescence assay (BD Clontech, Mountain View, CA) and/or histochemical staining assay (using a mixture of 0.1 mg/ml napthol AS-MX phosphate and 0.6 mg/ml Fast Blue BB salt) as described (Luo et al., 2010). For the bioluminescence assays, each assay condition was performed in triplicate and the results were repeated in at least three independent experiments. ALP activity was normalized by total cellular protein concentrations among the samples.

2.6. Matrix mineralization assay (Alizarin Red S staining)

Mineralized matrix nodules were stained for calcium precipitation by means of Alizarin Red S staining, as described previously (Luo et al., 2010). Cells were fixed with 0.05% (v/v) glutaraldehyde at room temperature for 10 min. After being washed with distilled water, fixed cells were incubated with 0.4% Alizarin Red S (Sigma–Aldrich) for 5 min, followed by extensive washing with distilled water. The staining of calcium mineral deposits was recorded under bright field microscopy.

2.7. Western blotting

Western blotting was performed as previously described (Zhao et al., 2012). Briefly, cells were collected and lysed in laemmli buffer. Cleared total cell lysate was denatured by boiling and loaded onto a 4–20% gradient SDS–PAGE. The membrane protein was extracted by using Thermo Scientific Mem-PER Eukaryotic Membrane Protein Extraction Kit. After electrophoretic separation, proteins were transferred to an Immobilon-P membrane. Membrane was blocked with Super-Block Blocking Buffer, and probed with the primary