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The role of COX-2 in mediating the effect of PTEN on BMP9 induced osteogenic differentiation in mouse embryonic fibroblasts

Jun Huang [a, b], Shuang-Xue Yuan [a, b], Dong-Xu Wang [a, b], Qiu-Xiang Wu [a, b], Xing Wang [b, c], Chang-Jun Pi [b, c], Xiang Zou [b, c], Liang Chen [b, c], Liang-Jun Ying [b, c], Ke Wu [a, b], Jun-Qing Yang [a, b], Wen-Juan Sun [a, b], Zhong-Liang Deng [b, c], Bai-Cheng He [a, b, *]

[a] Department of Pharmacology, School of Pharmacy, Chongqing Medical University, Chongqing, People's Republic of China
[b] Key Laboratory for Biochemistry and Molecular Pharmacology of Chongqing, Chongqing, People's Republic of China
[c] Department of Orthopedic Surgery, Second Affiliated Hospital, Chongqing Medical University, Chongqing, People's Republic of China

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ABSTRACT

Mouse embryonic fibroblasts (MEFs) are multi-potent progenitor cells (MPCs), can differentiate into different lineages, such as osteogenic, and adipogenic. PTEN, a tumor suppressor, may be involved in regulating bone development through interacting with COX-2. BMP9, the most potent osteogenic BMPs, can up-regulate COX-2 in MPCs. Whether PTEN is involved in BMP9 induced osteogenic differentiation in MPCs remains unknown. The goal of this investigation is to identify the effect of PTEN on BMP9-induced osteogenic differentiation in MPCs and dissect the possible mechanism underlay this. We found that BMP9 down-regulates PTEN, and PTEN inhibitor (VO) effectively increases different osteogenic markers induced by BMP9 in MEFs. Exogenous expression of PTEN inhibits BMP9 induced ectopic bone formation apparently. Mechanistically, we found that VO can enhance BMP9 induced BMPs/Smads signaling prominently without no substantial effects on cell cycle. Further analysis indicates that VO can promote BMP9-induced expression of COX-2 in MEFs, which can be eliminated by PI3K inhibitor. Additionally, COX-2 knockdown abolishes the effect of VO on BMP9-induced ALP activities in MEFs. Our findings suggest that PTEN plays an important role in regulating BMP9 induced osteogenic differentiation in MPCs, which may be mediated by PTEN/PI3K/Akt signaling to modulate the expression of COX-2.

1. Introduction

Mouse embryonic fibroblasts (MEFs) are multi-potent progenitor cells (MPCs), which can differentiate into osteogenic, chondrogenic, adipogenic and myogenic lineages [1–4]. MEFs can be easily extracted from many type of tissues, including bone marrow stroma, periosteum, brain, liver, bone marrow, adipose, and so on [4,5]. For tissue engineering, MEFs can be used as promising seed cells to treat bone defect related diseases for their potential role in stem cell biology and regenerative medicine [5,6].

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor beta (TGF-β) super-family and consist of about 20 members [7]. BMPs are important for cell differentiation and proliferation during development through the classic BMPs/Smads pathway [7,8]. Bone morphogenetic protein 9 (BMP9) has been reported as one of the most potent BMPs to induce osteogenic differentiation in MPCs [9,10], and can be used for bone tissue engineering when combine with some special biomaterials. Although a few essential down-stream targets of BMP9 has been identified, such as Creld2 [11], Hey1 [12], and COX-2 [13], the detail mechanism for BMP9 to induce osteogenic differentiation in MPCs remains unclear yet.

Non-sterol anti-Inflammatory drugs (NSAIDs), as cyclooxygenase (COX) inhibitor, are used widely as pain killer for bone fracture or post-traumatic operation. However, it has been reported that NSAIDs have potential deleterious effects on bone fracture healing and ossification processes [14–16]. Several studies indicated that COX-2 plays critical role in bone fracture healing and BMP2 induced osteogenesis in MPCs through PGE2/EPs/cAMP/PKA signaling [16–18]. Our previous studies also indicated that COX-2

* Corresponding author. Department of Pharmacology, School of Pharmacy, Chongqing Medical University, No.1 of Yixueyuan Road, Yuzhong District, Chongqing 400016, China. Fax: +86 2368485161.
E-mail address: 894704897@qq.com (B.-C. He).

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can regulate BMP9 induced osteogenic differentiation though the BMPs/Smads signaling in MPCs [13].

Usually, COX-2 has been thought as an inducible enzyme [19], as one downstream target of Wnt/β-catenin and PI3K/Akt signalings [20,21]. It is reported that PI3K/Akt signaling is involved in BMP9 induced osteogenic differentiation in MPCs(22, 23), although the mechanism underlaying this remains unclear. PTEN, as a tumor suppressor, has been found functional lose or mutation in many cancers [24,25]. In addition, it can also regulate the bone development through negatively regulating the PI3K/Akt signaling [26]. These imply that PTEN and COX-2 may be involved in BMP9 induced osteogenic differentiation.

In this study, we investigated the effect of PTEN on BMP9 induced osteogenesis in MEFs. We found that BMP9 can down-regulate the expression of PTEN in MEFs, and PTEN specific inhibitor effectively promotes BMP9 induced different osteogenic markers in MEFs. However, exogenous expression of PTEN inhibits BMP9 induced osteogenic differentiation greatly. For mechanism, we found that inhibition of PTEN can promote BMP9 induced BMPs/Smads signaling transduction. The effect of PTEN on BMP9 induced osteogenic differentiation may be initialized by modulating the expression of COX-2 through negatively regulating the PI3K/Akt signaling. Our investigation provides another possible mechanism through which to regulate BMP9 induced osteogenic differentiation in MPCs. This will benefit to promote BMP9 mediated bone tissue engineering greatly.

2. Materials and methods

2.1. Cell culture and chemicals

HEK293 cells were obtained from ATCC (Manassas, VA, USA). Cells were maintained in the Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in 5% CO_2. Antibodies were purchased from Santa Cruz Biotechnology (CA, USA). The PTEN inhibitor VO-OHpic trihydrate (VO) was bought from Sigma–Aldrich (St Louis, MO, USA).

2.2. Recombinant adenovirus construction

Recombinant adenoviruses were generated with AdEasy system, as described previously [27,28]. Briefly, the coding regions of BMP9, PTEN, GFP (green fluorescent protein) and RFP (red fluorescent protein) were amplified with PCR and cloned into adenoviral shuttle vectors, so did the COX-2 siRNA oligo fragments. These vectors were used to generate recombinant adenoviruses in HEK293 cells subsequently. The recombinant adenoviruses were designated as AdBMP9, AdGFP, AdPTEN and AdsimCOX-2. GFP or RFP was used as tag for tracking the viruses, and AdGFP was used as vector control.

2.3. Isolation of mouse embryonic fibroblast cells (MEFs)

MEFs were isolated from post-coitus day 12.5 NIH mice, as described [13]. Each embryo, voided of its internal organs, was dissected and sheared through an 18-gauge syringe in the presence of 1 ml of 0.25% trypsin. After 15 min of incubation with gentle shaking at 37 °C, 10 ml DMEM with 10% fetal bovine serum (FBS) was added to inactivate the trypsin. The cells were plated on 100 mm dishes and incubated for 24 h at 37 °C. The adherent cells were used as MEFs cells. All MEFs used in this study were within five passages.

2.4. Immunocytochemical staining

Cultured cells were infected with AdBMP9 or AdGFP. At the indicated time points, cells were fixed with 4% formalin and washed with PBS. Then, permeabilized with 0.25% Triton X-100 and blocked with 10% goat serum, followed by incubating with anti-PTEN anti-body and HRP labeled second anti-body sequentially. Finally, visualized with DAB staining and imaged under a microscope. Mouse IgG was run as negative control.

2.5. Flow cytometry cell cycle analysis

Sub-confluent cells were seeded in 6-well plates and treated with different reagents as experiment design for 48 h. Cells were harvested, washed with PBS, fixed with cold (4 °C) 70% ethanol, washed with 50% and 30% ethanol, and PBS finally. Then, cells were stained with 1 ml of 20 mg/ml PI containing RNase (1 mg/ml) in PBS for 30 min followed by fluorescence activated cell sorting for cell cycle analysis. Each assay was done in triplicate.

2.6. RNA extraction, cDNA preparation and polymerase chain reaction analysis

Total RNA were isolated with TRIZOL reagents (Invitrogen) and used to generate cDNA templates by reverse transcription (RT) reaction with hexamer and Superscript II RT. The first strand cDNA products were further diluted 5–10 fold and used as templates for polymerase chain reaction (PCR). All samples were normalized with the expression level of GAPDH. The primers used in this investigation are shown in Supplemental Tab. 1.

2.7. Western blot analysis

Cells were plated in 6-well plates and treated as the schedule. At the indicated time point, cells were lysed and the lysate were denatured by boiling for 10 min. Total protein were separated by SDS-PAGE, transfered with polyvinylidene difluoride (PVDF) membrane, blocked in 10% skimmed milk and probed with anti-body against the target proteins. Finally, the images of target bands were developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, IL, USA).

2.8. Alkaline phosphatase (ALP) assay

ALP activities were assessed with a modified Great Escape SEAP Chemiluminescence Assay (BD Clontech) and/or histochemical staining as described previously [13,22,29]. MEFs were treated with AdGFP, different concentrations of VO, AdBMP9, AdBMP9 combine with different concentrations of VO, AdPTEN, and AdBMP9 combine with AdPTEN. For the chemilluminescence assay, each assay was performed in triplicate, the results were repeated in at least three independent experiments. The ALP activities were normalized with the total cellular protein level. ALP activities were expressed as mean ± SD.

2.9. Transfection and luciferase reporter assay

Cells were seeded in T25 cell culture flasks and transfected 2 μg BMPR Smads binding elements luciferase reporter (p12xSBE-Luc) per flask with Lipofectamine (Invitrogen) [30]. 16 h after transfection, cells were seeded to 24-well plates and infected with AdBMP9, AdGFP, and AdBMP9 combine with different concentrations of VO at 4 h after replating. 24 h after infection, cells were lysed and subjected to luciferase assay using Promega's Luciferase Assay Kit. Each assay condition was performed in triplicate. The results were repeated in at least three independent experiments. Luciferase activities were normalized with the total cellular protein concentrations among the samples. Reporter activities were expressed as mean ± SD.

2.10. Matrix mineralization assay (Alizarin Red S staining)

Cells were seeded in 24-well culture plates and treated with AdGFP, AdBMP9, different concentrations of VO, and AdBMP9 combine with different concentrations of VO. On d14 and d20 after treatment, mineralized matrix nodules were stained for calcium precipitation by means of Alizarin Red S staining assay, as described [13,22,29]. Cells were fixed with 0.05% (v/v) glutaraldehyde at room temperature for 10 min. After being washed with distilled water, cells were incubated with 0.4% Alizarin Red S (Sigma–Aldrich) for 5 min, followed by extensive washing with distilled water. The results were repeated in at least three independent experiments.

2.11. Ectopic bone formation assay

Cells were infected with AdGFP, AdBMP9, AdPTEN, and AdBMP9 combine with AdPTEN. 16 h after infection, cells were harvested and resuspended in PBS for subcutaneous injection (5×10^6 per injection) into the flanks of athymic nude mice (five animals per group, four to six week old females). The nude mice were ordered from the Animal Centre of Chongqing Medical University (Chongqing, China). All animal experiments were approved by Institutional Animal Care and Use Committee (IACUC) of Chongqing Medical University (Chongqing, China). Four weeks after implantation, animals were euthanized, and the bone masses from implantation sites were retrieved for μCT scan and histological evaluation. The results were repeated in at least three independent experiments.

2.12. Micro–computed tomographic (μCT) imaging analysis

All retrieved specimens were scanned with μCT (VivaCT 40, SCANCO Medical AG, Switzerland). The image data analysis and 3D reconstruction were performed following the software with the scanner (μCT 516.1).

2.13. Histologic evaluation and staining

The retrieved bone masses were fixed with 10% formalin (decalcified, if necessary) and embedded in paraffin. Serial sections of the embedded specimens were stained with hematoxylin and eosin (H&E), Masson's trichrome, or alcian blue after been deparaffinized and rehydrated.

2.14. Statistical analysis

Microsoft Excel was used to calculate standard deviations and statistically significant differences between samples using the two-tailed Student's *t* test. For all