Biphasic effects of TGFβ1 on BMP9-induced osteogenic differentiation of mesenchymal stem cells

Short Title: Effect of TGF-β1 on BMP9-induced osteogenesis

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Abstract

We have found that the previously uncharacterized bone morphogenetic protein-9 (BMP9) is one of the most osteogenic factors. However, it is unclear if BMP9 cross-talks with TGFβ1 during osteogenic differentiation. Using the recombinant BMP9 adenovirus, we find that low concentration of rhTGFβ1 synergistically induces alkaline phosphatase activity in BMP9-transduced C3H10T1/2 cells and produces more pronounced matrix mineralization. However, higher concentrations of TGFβ1 inhibit BMP9-induced osteogenic activity. Real-time PCR and Western blotting indicate that BMP9 in combination with low dose of TGFβ1 potentiates the expression of later osteogenic markers osteopontin, osteocalcin and collagen type 1 (COL1a2), while higher concentrations of TGFβ1 decrease the expression of osteopontin and osteocalcin but not COL1a2. Cell cycle analysis reveals that TGFβ1 inhibits C3H10T1/2 proliferation in BMP9-induced osteogenesis and restricts the cells in G0/G1 phase. Our findings strongly suggest that TGFβ1 may exert a biphasic effect on BMP9-induced osteogenic differentiation of mesenchymal stem cells.

Keywords: BMP9; TGFβ1; Mesenchymal stem cells; osteogenic differentiation, osteoprogenitors.
INTRODUCTION

Mesenchymal stem cells (MSCs) can self-renew and differentiate into osteoprogenitors under external stimulation[1]. Osteogenic factors can induce and/or promote MSCs differentiation into osteoprogenitors and osteocytes. Bone Morphogenetic Proteins (BMPs) are the members of transforming growth factor (TGF) β superfamily that play important roles in regulating the differentiation of MSCs into bone, cartilage or tendon. As effective inducers MSCs undergoing terminal osteogenic differentiation, BMPs are known to exhibit high capability of osteogenic induction and ectopic ossification [2,3]. We previously found that BMP9 is one of the most potent BMPs in inducing osteogenic differentiation of MSCs both in vitro and in vivo [4].

Effective ossification and bone regeneration is required for many defects such as bony callus with long healing time and limited strength, which are probably related to the deficiency of collagen production and finite osteogenic ability [5,6]. TGFβ1 is one of the most abundant TGF members and important growth regulators in bone [7]. Unlike BMPs, TGFβ1 is unable to induce osteogenesis in mesenchymal multipotent cells [8]. However, TGFβ1 can regulate committed osteoprogenitors towards osteogenic differentiation and bone remodeling [9,10]. Thus, TGFβ1 is involved in bone formation, osteoblast proliferation and mineralization by increasing the strength and flexibility of bone [11]. It’s known that TGFβ1 interferes with late phases of differentiation and mineralization [12,13], which are also regulated by other growth factors such as BMPs [14]. Thus, it is conceivable that TGFβ1 may crosstalk with BMPs in a coordinated fashion during osteogenic differentiation.

Here, we investigate the effect of TGFβ1 on BMP9-induced osteogenic differentiation in a well-characterized MSC line C3H10T1/2 [15]. Our results indicate that the effects of TGFβ1 on ALP activity, matrix mineralization, osteogenic marker expression, cell proliferation and cell cycle in BMP9-transduced C3H10T1/2 cells are biphasic in a dose-dependent fashion. At lower concentrations, TGFβ1 acts synergistically with BMP9 whereas TGFβ1 inhibits BMP9-induced osteogenic differentiation at higher concentrations, suggesting that a balanced regulatory circuit between TGFβ1 and BMPs may play an important role in regulating osteogenic differentiation of MSCs.
RESULTS AND DISCUSSION

ALP activity in BMP9-transduced C3H10T1/2 cells stimulated with TGFβ1 at different concentrations

ALP is an early marker of osteogenic differentiation \[^{16,17,18}\] which peaks at around 7-9 days after BMP9 stimulation. We tested the effects of TGFβ1 on ALP activity. TGFβ1 alone exhibited a low level of ALP activity at days 3, 5, and 9 and had no significant difference over AdGFP control (\(p>0.05\)) (Fig. 1A), suggesting that TGFβ1 alone exhibits negligible osteogenic activity in C3H10T1/2 cells. AdBMP9-infected cells induced a remarked increase in ALP activity. AdBMP9 combined with 5ng/ml TGFβ1 had the ability stimulating earlier increase in and significantly higher ALP activity (\(p<0.01\)) (Fig. 1A), indicating that TGFβ1 may potentiate BMP9-induced osteogenic differentiation. ALP staining results were consistent with the quantitative ALP assays (Fig. 1B). These results indicate that under BMP9 stimulation TGFβ1 can promote osteogenic differentiation in a dose-dependent fashion. However, the increased ALP activity was diminished when the concentrations of TGFβ1 increased; and at 20ng/ml TGFβ1 inhibited BMP9-induced ALP activity, suggesting that high concentrations of TGFβ1 may inhibit BMP9-induced osteogenic differentiation.

Effect of TGFβ1 on BMP9-induced matrix mineralization in C3H10T1/2 cells

We next determined the effects of TGFβ1 on matrix mineralization in BMP9-stimulated C3H10T1/2 cells. Matrix mineralization nodules usually emerge at 10 days and reach peaks at 17-20 days after induction \[^{19}\]. TGFβ1 alone had no difference with AdGFP control that corresponded with ALP activity, indicating that TGFβ1 itself is unable to induce osteogenesis in MSCs \[^{20}\], while small mineralized nodules were visible in the group treated with AdBMP9 in combination with 5ng/ml or 10ng/ml TGFβ1 at day 5 whereas AdBMP9 alone exhibited apparent nodules at day 13 (Fig. 2). At the later stage (day 17), 5ng/ml TGFβ1 combination group, 10ng/ml TGFβ1 combination group exhibited no significant difference with AdBMP9 alone group, suggesting that the effects of TGF-β1 may primarily accelerate the early process of
matrix mineralization (Fig. 2). When the concentrations of TGFβ1 increased, the effects of TGFβ1 on potentiating mineralization decreased. At 20ng/ml TGFβ1 inhibited mineralization, and the matrix mineralization of 20ng/ml TGF-β1 was lower than that of the AdBMP9 alone group’s (Fig. 2). These results indicate that higher concentrations of TGF-β1 inhibit BMP9-induced matrix mineralization.

**Effect of TGFβ1 on the expression of osteogenic markers in BMP9-induced osteogenic differentiation of C3H10T1/2 cells**

Bone formation and mineralization require the production of type 1 collagen (COL1) and other bone matrix non-collagen proteins, such as osteopontin (OPN) and osteocalcin (OCN). COL1 plays an important functional role during osteogenesis as it augments the cell adhesion and lays a foundation for OPN, OCN and bone matrix mineralization [21,22]. OPN and OCN have high affinity of hydroxyapatite and can specifically integrate with the adhesion and connection of cells and tissues [23]. We previously demonstrated that BMP9 alone effectively induces the expression of these markers during osteogenic differentiation [3].

Here, we analyzed the expression of COL1a2, OPN and OCN at mRNA level by qPCR at different time points (Fig. 3A). As expected, BMP9 alone group effectively induced the expression of OPN and OCN except COL1a2. BMP9 plus 5ng/ml TGFβ1 group exhibited a significant higher expression than that of BMP9 only (p<0.01), and COL1a2 expression was readily detected. So were the later markers OPN and OCN. At 20ng/ml TGF-β1, COL1a2, but not OPN and OCN, remained at a high level (Fig. 3A). Thus, TGF-β1 may exert its effect on BMP9-induced osteogenesis by augmenting COL1 synthesis.

We further quantified the expression of COL1A1, OPN, and OCN at protein level. The protein levels of these markers were increased by 42.6%, 22.8% and 53.3% (p<0.01) in the 5ng/ml TGF-β1 plus BMP9 group when compared with BMP9 alone group (Fig. 3B). COL1A1 expression in the 20ng/ml TGF-β1+BMP9 group increased by 154.9%, whereas OPN and OCN decreased by 18.5% and 42.8% when compared to that from BMP9 alone group. The protein expression results are consistent with the qPCR results.
The effects of TGFβ1 on proliferation, cell cycle, and Smad activity of BMP9-induced C3H10T1/2 cells

TGFβ1 is shown to regulate the proliferation of osteoblasts [9,10,24,25-29,30]. We investigated whether TGFβ1 affected the proliferative activity of BMP9-stimulated C3H10T1/2 cells. The cell numbers of the BMP9 plus 5ng/ml TGFβ1 group were significantly different from that of the BMP9 alone group (Fig. 4A). However, when the cell cycle analysis was performed at 48h, we found that G0/G1 phase of the cells treated with BMP9 plus 5ng/ml TGFβ1 group was at 84.83% ± 1.29%, which was much higher than that of the BMP9 only group (71.46% ± 1.68%). Meanwhile, S phase and G2/M phase decreased to 6.58% ± 1.16% and 8.58% ± 0.62% comparing with that of the BMP9 alone group as 15.36% ± 0.85% and 13.18% ± 2.52% (Fig. 4B-4D). These results indicate that TGFβ1 may inhibit the proliferation of BMP9-induced C3H10T1/2 cells.

We further examined if TGFβ1 affected BMP9 function at the Smad signaling level. By transfecting C3H10T1/2 cells with the BMPR-Smad reporter p12xSBE-Luc or the TGFβR-Smad reporter, pBGLuc-Smad[31], we found that while BMP9 or TGFβ1 alone was able to activate its specific reporter activity, TGFβ1 combined with BMP9 exhibited significant lower activity of BMPR-Smad receptors comparing with BMP9 alone (p<0.01) (Fig. 4E). Interestingly, the combination group also showed a decrease in TGFR-Smad receptors activity comparing with single TGFβ1 group (p<0.01) (Fig. 4F). Nonetheless, these results suggest that the effect of TGFβ1 on BMP9-induced osteogenic differentiation of MSCs may be exerted at Smad signaling level, although the detailed molecular mechanisms underlying the crosstalk between TGFβ1 and BMP9 remain to be thoroughly elucidated.

MATERIALS AND METHODS

Cell culture and chemicals
HEK293, HCT116 and C3H10T1/2 lines were obtained from ATCC (Manassas, VA). HEK293 cells were maintained in complete Dulbecco's Modified Eagle's Medium (DMEM). HCT116 cells were maintained in complete Minimal Essential Medium (MEM). C3H10T1/2 cells were
maintained in complete Basal Medium Eagle (BME). Unless otherwise indicated, all chemicals were purchased from Sigma or Fisher Scientific.

**Construction and amplification of recombinant adenoviruses expressing RFP, GFP, BMP9 and TGFβ1**

Recombinant adenoviruses were generated previously using the AdEasy system \[^{16,17,31,32}\]. Briefly, the coding regions of human BMP9 and mouse TGFβ1 were PCR amplified and cloned into an adenoviral shuttle vector and subsequently used to generate recombinant adenoviruses in HEK293 cells. The resulting adenoviruses were designated as AdBMP9 (also expressing GFP) and AdTGFβ1 (also expressing RFP). Analogous adenoviruses expressing only GFP (AdGFP) and RFP (AdRFP) were used as mock virus controls \[^{17,31,32}\].

**Preparation of conditioned medium**

TGFβ1 conditioned medium were prepared as previously described \[^{18,33}\]. Briefly, subconfluent HCT116 cells seeded in 75cm^2 flasks were infected with an optimal titer of AdTGFβ1 or AdRFP control. At 15h after infection, the culture medium was changed to serum-free BME. Conditioned medium was collected at 48h after infection and assayed the concentration of TGFβ1 with the double antibody sandwich method by TGFβ1 ELISA kit.

**Measurement of alkaline phosphatase (ALP) activity**

C3H10T1/2 cells were seeded in 24-well culture plates and infected with AdBMP9 or AdGFP. After 8h infection, cells were stimulated with TGFβ1-conditioned media of indicated concentrations. At indicated days after infection, ALP activity was assessed by a modified Great Escape SEAP Chemiluminescence Assay (BD Clontech, Mountain View, CA, USA) and histochemical staining assay (using a mixture of 0.1 mg/ml of napthol AS-MX phosphate and 0.6 mg/ml of Fast Blue BB salt), as described previously \[^{16,17,31}\]. For the chemilluminescence assays, each assay condition was performed in triplicate, and the results were repeated in at least three independent experiments.
Matrix mineralization assay (Alizarin Red S staining)
Cells were treated as ALP methods and were cultured in the presence of ascorbic acid (50μg/ml) and β-glycerophosphate (10mM). At indicated days after infection, mineralized matrix nodules were stained for calcium precipitation by means of alizarin red S staining, as described previously [16,17,31,34-38]. Cells were fixed with 0.05% (v/v) glutaraldehyde at room temperature for 10min. After being washed with distilled water, fixed cells were incubated with 0.4% Alizarin Red S for 5 min, followed by extensive washing with distilled water. The staining of calcium mineral deposits was recorded under bright field microscopy.

Total RNA isolation and quantitative real-time PCR (qPCR) analysis
Total RNA was isolated using TRIZOL Reagents (Invitrogen) according to the manufacturer’s instructions. qPCR were carried out as described [16-18,31,36]. Total RNA was subjected reverse transcription using hexamer and Superscript II reverse transcriptase (TOYOBO, JPN). The cDNA products were further diluted 10-fold and used as qPCR templates. The qPCR primers were designed by using the Primer3 program: COL1a2, 5’-AGC GAA GAA CTC ATA CAG CCG-3’, and 5’-TGC CCG TCT CCT CAT CCA-3’; OPN, 5’-CTT TCA ACT CCA ATC GTC CCT AC-3’ and 5’-CTG CCC TTT CCG TTG TTG TC-3’; OCN, 5’-GGA CCA TCT TTC TGC TCA CTC TG-3’ and 5’-ACC TTA TTG CCC TCC TGC TT-3’; β-actin, 5’-CTG AGA GGG AAA TCG TGC GT-3’ and 5’-CCA CAG GAT TCC ATA CCC AAG A-3’. SYBR Green-based qPCR (TOYOBO, Japan) analysis was carried out using Real-Time PCR Detection System (SLAN, HONGSHI, CHN). The specificity of each qPCR reaction was verified by melting curve analysis. Duplicate reactions were carried out for each sample. All samples were normalized by the expression level of β-actin.

Western blotting analysis
Western blotting was performed as previously described [16-18,31,34-36]. 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. After electrophoretic separation, proteins were transferred to an Immobilon-P membrane. Membrane was blocked with Super-Block Blocking
Buffer, and probed with primary antibodies for COL1, OPN, OCN, or β-actin (Santa Cruz Biotechnology, Santa Cruz, USA), followed by an incubation with a secondary antibody conjugated with horseradish peroxidase. The proteins of interest were detected by using ECL Western Blotting Detection Substrate (Beyotime Institute of Biotechnology, CHN).

**Viable cell counting**
Cells were seed in 25 cm$^2$ flasks as $1.2 \times 10^5$ and treated as indicated. After 24h, 48h and 72h culture, cells were harvested, suspended in PBS mixed with 0.4% Trypan blue and counted on bright-line counting chamber under a microscope. Each assay was performed in triplicate.

**Flow cytometry**
Cells were seeded in 25cm$^2$ flasks and were collected, washed with PBS and fixed with 70% iced-ethanol in 4°C after 48h treatment. The fixed cells were washed with PBS including 100ug/ml Ribonuclease H and incubated at 37°C for 30min. Staining with PBS including 20ug/ml propidium iodide away from light for 30min, the cells were assayed the fluorescence intensity by BD.LSR flow cytometry (w/l 488nm). The MODFIT2.0 software analyzed the DNA content of the cells.

**Transfection and luciferase reporter assay**
Exponentially growing cells were seeded in 25cm$^2$ cell culture flasks and transfected with 2µg per flask of BMP-R Smad-responsive luciferase reporter p12xSBE-Luc or TGFβ-R Smad –responsive luciferase reporter pBGLuc-Smad using LipofectAmine (Invitrogen). At 16h after transfection, cells were replated to 24-well plates and infected with AdBMP9, AdTGF-β1, AdGFP and AdRFP. At 48h after infection, cells were lysed and subjected to luciferase assays using Promega’ Luciferase Assay Kit or New England Biolabs’ Gaussia Luciferase Assay Kit. Each assay condition was performed in triplicate. The results were repeated in at least three independent experiments. Luciferase activity was normalized by total cellular protein concentrations among the samples.
Statistical analysis

Microsoft Excel was used to calculate standard deviations (SDs) and statistically significant differences between samples using the one-factor analysis of variance and the two-tailed Student’s t-test. For all quantitative assays, each assay condition was performed in triplicate. A \( p \)-value<0.05 was defined as statistically significance.
ACKNOWLEDGEMENTS

We thank Dr. Huayu Deng of Chongqing Medical University for her advice and technical supports. This work was supported in part by research grants from the Natural Science Foundation of China (Grant# 31070875 to WH, Grant# 31000434 to LC).
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FIGURE LEGENDS

Fig. 1 Effect of TGFβ1 on ALP activity in BMP9-induced C3H10T1/2 cells. Subconfluent C3H10T1/2 cells were infected with a comparable titer (MOI = 30pfu/cell) of AdBMP9 (BMP) or AdGFP (GFP). After 8h post infection, cells were stimulated with 5ng/ml (BT5), 10ng/ml (BT10) or 20ng/ml (BT20) TGFβ1 respectively. Control cells were infected with AdGFP and stimulated with 5ng/ml TGFβ1 alone (TGF). (A) Relative ALP activity was assessed using a chemiluminescent assay at the indicated time points. The dotted lines indicate the same level of relative ALP activity. Data are present as mean ± S.D. All ALP assays were carried out in triplicate. (B) ALP activity was histochemically stained at days 5 and 9. Representative results are shown (magnification, 100×). Each assay condition was done in triplicate.

Fig. 2 Effect of TGFβ1 on BMP9-induced matrix mineralization in C3H10T1/2 cells. Cells were treated as previously described. Alizarin Red S staining was conducted at the indicated time points. Representative results are shown (magnification, 100×).

Fig. 3 Effect of TGFβ1 on BMP9-induced mRNA and protein expression of osteogenic markers in C3H10T1/2 cells. Cells were treated as described in the text. (A) Total RNA was isolated at the indicated time points and was subjected to reverse transcription and qPCR analysis. All samples were normalized for β-actin expression. Data are present as mean ± S.D. and reactions were done in triplicate. (B) Cells were lysed and subjected to Western blotting analysis using COL1A1, OPN (at day 5), or OCN antibody (at day 9). β-actin antibody was used to assure equal loading of all samples. Right panels are shown the quantitative analysis of density ratio corresponding with proteins blots. Data are presented as mean ± S.D. Experiments were carried out in triplicate. Representative results are shown.

Fig. 4. TGFβ1 inhibits proliferation and Smad activity of BMP9-induced C3H10T1/2 cells
(A) TGFβ1 inhibits the proliferation of BMP9-induced C3H10T1/2. Cells were seeded in 25cm² flasks as 1.2×10⁵ per flask and treated. At the indicated time points, cells were collected
and suspended in Trypan blue-containing PBS. Viable cells were counted under a microscope and calibrated with the dilution ratios. (B) (C) (D) TGF-β1 blocked G_{0}/G_{1} phase of cell cycle of BMP9-transduced C3H10T1/2 cells. Cells were treated for 48h, harvested, suspended and fixed with 70% iced-ethanol. After being washed with Ribonuclease H and staining with propidium iodide, the cells were assayed for fluorescence intensity and DNA content. The dotted lines indicate the same level of cell numbers. Each assay condition was done in triplicate. (E) and (F). TGFβ1 inhibited BMPR-Smad and TGFR-Smad reporter activity. Subconfluent C3H10T1/2 cells were transfected with BMPR-Smad or TGFβR-Smad luciferase reporters. After 16h, cells were replated to 24-well plates and infected with AdGFP, AdRFP, AdBMP9 and/or AdTGF-β1. At 48h after infection, cells were lysed for luciferase assays. Each assay condition was performed in triplicate.