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REGULAR ARTICLE

TSG-6 secreted by mesenchymal stem cells suppresses immune reactions influenced by BMP-2 through p38 and MEK mitogen-activated protein kinase pathway

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Abstract Bone morphogenetic protein 2 (BMP-2) has a critical function in bone and cartilage development and in repairing damaged organs and tissue. However, clinical use of BMP-2 at doses of 0.5–1 mg/ml for orthopedics has been associated with severe postoperative swelling requiring emergency surgical intervention. We determined whether a high concentration of BMP-2 induces inflammatory responses in macrophages and the suppression of osteogenesis in hMSCs. We obtained human periodontal ligament stem cells and bone marrow stem cells from the maxilla, i.e., human mesenchymal stem cells (hMSCs), from the periodontal ligament of extracted third molar teeth and from the bone marrow of the maxilla, respectively. Osteogenic differentiation was measured by alkaline phosphatase activity and alizarin red S staining. Proteins were assessed by flow cytometry, enzyme-linked immunosorbent assay, Western blot and immunocytochemistry. Changes of gene expression were measured by reverse transcription plus the polymerase chain reaction (RT-PCR) and real-time PCR. A high BMP-2 concentration inhibited the early stages of osteogenesis in hMSCs. Co-culturing THP-1 cells (human monocytic cells) with hMSCs reduced the late stages of osteogenesis compared with those seen in hMSCs alone. In addition, high-dose BMP-2 induced the expression of inflammatory cytokines in THP-1 cells and the expression of the anti-inflammatory cytokine tumor-necrosis-factor-α-inducible gene 6 protein (TSG-6) in hMSCs. Consistent with the anti-inflammatory effects of hMSCs when co-cultured with THP-1 cells, interleukin-1β expression was downregulated by TSG-6 treatment of THP-1 cells. Our findings suggest that a high BMP-2 concentration triggers inflammation that causes inflammatory cytokine release from THP-1 cells, leading to the suppression of osteogenesis, whereas TSG-6 secreted by hMSCs suppresses inflammatory reactions through p38 and ERK in the mitogen-activated protein kinase pathway.

Keywords Periodontal ligament (PDL) · Stem cells · Regeneration · Osteogenesis · Inflammation

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Introduction

Tissue regeneration with stem cells is a promising field in regenerative medicine. Among candidate stem cells for tissue regeneration, mesenchymal stem cells (MSCs) have potential clinical applications. Tissue defects in the oral cavity are challenging because of the complex composition and multiple cell types in this area. After a diseased tooth or jawbone is removed, primarily the jawbone must be regenerated (G.T. Huang et al. 2009). Among MSCs of dental origin,

periodontal ligament stem cells (PDLSCs), which are obtained from extracted third molars, have been identified and characterized as a multipotent stem cell that can be used for hard tissue regeneration (Seo et al. 2004; Vasandan et al. 2014). Another candidate for bone regeneration is represented by bone marrow stem cells (BMSCs) from marrow stromal cells (Akintoye et al. 2006; G.T. Huang et al. 2009).

Human bone morphogenetic protein-2 (BMP-2), which has been approved by the United States Food and Drug Administration (FDA) for use in bone regeneration and repair, is used at 0.5-1 mg/ml in autogenous bone grafts, spinal fusion, segmental defects, open tibia fractures and oral maxillofacial reconstruction (Boyne et al. 2005; Fiorellini et al. 2005; Swiontkowski et al. 2006; Tumialan et al. 2008; Wei et al. 2012). Unlike an in vitro study of treatment with 50-200 ng/ml BMP-2 (Guan et al. 2015), the clinical doses of BMP-2 are extremely high. In the first clinical reports of 1 mg/ml BMP-2 use, several adverse effects were reported (K.B. Lee et al. 2011a; Perri et al. 2007; Shields et al. 2006; Smucker et al. 2006; Zara et al. 2011). High BMP-2 concentrations increase osteoclast activation with transient bone resorption (Toth et al. 2009). A frequently reported side-effect of BMP-2 is inflammatory swelling. Patients undergoing anterior cervical spinal fusion generally exhibit severe soft tissue swelling and require surgery to explore and drain a swollen anterior neck with transplantation of rhBMP-2 sponges (Vaidya et al. 2007). Previous research has shown that BMP-2 triggers the chemotaxis of monocytes, macrophages and lymphocytes (G.T. Lee et al. 2010; Simoes Sato et al. 2014; Talati et al. 2014). During inflammation and bone formation, inflammatory cytokines triggered by immune cells not only perpetuate inflammation but also activate bone resorption in osteoporosis, rheumatoid arthritis and other bone diseases (Mundy 2007; Redlich and Smolen 2012). The inflammatory mechanisms acting between immune cells and hMSCs in bone formation have been investigated in vivo. With high doses of BMP-2, infiltrated immune cells, inducing inflammatory cytokines, have been found to be increased near the transplanted area that formed aberrant bone (Shen et al. 2013; Zara et al. 2011). To reduce life-threatening episodes and increase bone formation, the optimum BMP-2 concentration needs to be determined and the inflammatory environment induced by BMP-2 needs to be controlled. Moreover, the establishment of these conditions will elucidate the interactions between immune cells and hMSCs.

Recent reports have demonstrated that hMSCs regulate inflammation (Castro-Manrreza et al. 2014; Kim and Hematti 2009; Liu et al. 2016; Nazarov et al. 2013; Nemeth, et al. 2009). In addition to their immunomodulatory characteristics, hMSCs also have the capacity to differentiate into cell types such as adipocytes, osteoblasts and neurons (Kim et al. 2012). hMSCs secrete the anti-inflammatory protein, tumor necrosis factor-α (TNF-α)-inducible gene 6 protein (TSG-6), which attenuates inflammatory reactions. TSG-6 suppresses macrophages from expressing inflammatory cytokines in response to mitogen-activated protein kinase (MAPK) signaling (R.H. Lee et al. 2009a, 2014). However, the role of hMSCs on BMP-2-induced inflammation is not known.

Therefore, we constructed a local inflammatory environment induced by a high BMP-2 concentration by co-culturing macrophages and hMSCs in order to study the role of hMSCs in an inflammatory environment. We hypothesized that a high concentration BMP-2 causes inflammatory reactions of THP-1 cells, leading to the suppression of the osteogenesis of hMSCs. Moreover, TSG-6 secreted by hMSCs suppresses BMP-2-induced inflammation. Overall, we identified the role of hMSCs on high BMP-2 concentrations, inducing TSG-6 to downregulate inflammatory reactions through the p38 and MEK/MAPK pathway.

Materials and methods

Cell culture conditions

hPDLSCs from impacted third molars of humans (n = 8, age 17–29) and BMSCs from human maxilla (n = 4, age 20-26) were collected at the Department of Oral and Maxillofacial Surgery, Seoul National University Dental Hospital. This protocol was approved by the Institutional Review Board of the Seoul National University School of Dentistry (IRB no. S-D20080009). Collected PDLSCs and BMSCs were digested separately in 3 mg/ml type 1 collagenase (BioBasic, Toronto, ON, Canada) and 4 mg/ml dispase II (Gibco BRL, Long Island, N.Y., USA) for 1 h with shaking at 37 °C in a 5 % CO_2 incubator as previously described (Seo et al. 2004). Cultures were in grown in alpha minimum essential medium (α-MEM) supplemented with 100 μM L-ascorbic acid, 2 mM L-glutamine, 100 U/ml antibiotics-antimycotics (all from Gibco) and 15 % fetal bovine serum (FBS; Equitech-Bio, Kerrville, Tex., USA). PDLSCs and BMSCs from the second to sixth passages were used for experiments. A human monocyte cell line, THP-1, was provided by the Korean Cell Line Bank (KCLB) and cultured in Roswell Park Memorial Institute medium (RPMI 1640 medium) supplemented with 100 U/ml antibiotics-antimycotics (both from Gibco) and 10 % FBS (Equitech-Bio).

Co-culture of macrophages and MSCs

THP-1 cells were activated with 50 nM phorbol 12-myristate 13-acetate (PMA) for 3 days, followed with 1000 or 5000 ng/ml recombinant human BMP-2 (rhBMP-2; Daewoong Pharmaceutical, Seongnam, Korea) for 24 h. THP-1 cells were stimulated for 3 days in RPMI 1640

medium with PMA and equal numbers of MSCs were seeded for 1 day and co-cultured in growth medium. MSCs and THP-1 cells were cultured alone as controls. Recombinant human TSG-6 protein (R&D Systems, Minneapolis, Minn., USA) was added to THP-1 cells at a concentration of 10 or 100 ng/ml.

Osteogenic differentiation

To induce osteogenic differentiation, MSCs with or without THP-1 cells were cultured in MSC growth medium until 80-90 % confluent. Co-cultured cells were changed to differentiation medium consisting of α-MEM supplemented with 10 % FBS, 10 nM dexamethasone (Sigma-Aldrich, St. Louis, Mo., USA), 5 mM glycerol phosphate (Sigma-Aldrich), 100 U/ml antibiotics-antimycotics (Gibco) and 100 μM L-ascorbic acid. After 5 days of incubation in the osteogenic medium with BMP-2, a QuantiChrom Alkaline Phosphatase (ALP) Assay Kit (BioAssay Systems, Hayward, Calif., USA) was used to detect ALP activity following the manufacturer's directions. Absorbance was measured at 405 nm with a microplate reader (Bio-Rad). An ALP staining kit (Sigma-Aldrich) was also used to detect ALP in MSCs after 5 days in osteogenic medium. After incubation in differentiation medium for 10 days, BMSCs and PDLSCs were stained with 40 mM alizarin red S solution (pH 4.2) to detect calcium deposits. Stained alizarin red particles were quantified by means of a solution of 20 % methanol and 10 % acetic acid and detected with a spectrophotometer (Fluostar Optima; BMG LABTECH, Ortenberg, Germany) at 450 nm.

Gene expression analysis

Total RNA was isolated from cells with an RNA Mini Kit (Ambion, Carlsbad, Calif., USA) and reverse-transcribed with a SuperScript III First-Strand Synthesis System kit (Invitrogen, Carlsbad, Calif., USA). The reverse transcription plus polymerase chain reaction (RT-PCR) was performed with the primers listed in Table S1 (Hong et al. 2009). Real-time RT-PCR was performed on a Real-time PCR System 7500 (Applied Biosystems, Foster City, Calif., USA) to quantify mRNA expression. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs99999905_m1) was used as an endogenous control. The following human-specific primers and probes (all from Applied Biosystems) were used: TNF-α (Hs01113624_g1), interleukin (IL)-1β (Hs00174097_m1) and TSG-6 (TNFAIP6; Hs01113602_m1). Expression was quantified by the ΔΔCT method.

Flow cytometry analysis

Activated THP-1 cells were subjected to flow cytometry to detect macrophage phenotypes showing CD11b and CD14 expression. Approximately 5×10^5 cells were activated with PMA for 3 days, followed with BMP-2 for 24 h. Activated cells were double-stained with allophycocyanin7 (APC-7)-conjugated mouse anti-human CD11b (BD Bioscience, Franklin Lakes, N.J., USA) and fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD14 (BD Bioscience). Stained cells were detected and analyzed with a FACS Aria IIII (BD Bioscience).

Enzyme-linked immunosorbent assay

To identify cytokines released from THP-1 cells with or without MSCs, the cultured cells were starved in serum-free medium overnight. After overnight starvation, cells were activated with 1000 and 5000 ng/ml BMP-2 for 24 h, supernatants containing inflammatory-related products induced by BMP-2 without FBS were collected and enzyme-linked immunosorbent assay (ELISA) was performed to determine TNF-α and IL-1β levels. A human TSG-6 ELISA kit was used for the quantitative measurement of human TSG-6 in supernatants (RayBiotech, Norcross, Ga., USA). The absorbance of each sample was measured with a microplate reader (Fluostar Optima, BMG LABTECH) at 450 nm.

Immunocytochemistry

A total of 10^5 THP-1 cells were plated and cultured in 2-well chamber slides with 50 nM PMA in RPMI 1640 medium for 3 days. Stimulated THP-1 cells were incubated without FBS overnight. Starved THP-1 cells were treated with 5000 ng/ml BMP-2 and 10-100 ng/ml TSG-6 for 3 h. After fixation, blocked cells were incubated with anti-nuclear factor kappa-B (NF-kB) p65 antibody (Abcam, Cambridge, UK) in blocking solution at 4 °C overnight. The cells were then incubated in anti-rabbit IgG secondary antibody (Abcam) for 1 h. Mounting medium containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, Calif., USA) was used to stain nuclei. Fluorescent imaging was performed on a confocal laser scanning microscope (FV300, Olympus America, Center Valley, Pa., USA).

Western blotting analysis

PMA-stimulated THP-1 cells were cultured and treated with 5000 ng/ml BMP-2 and 10 ng/ml TSG-6 for 10, 30, or 60 min. Proteins from THP-1 cells were collected in cell extraction buffer (Invitrogen) with protease

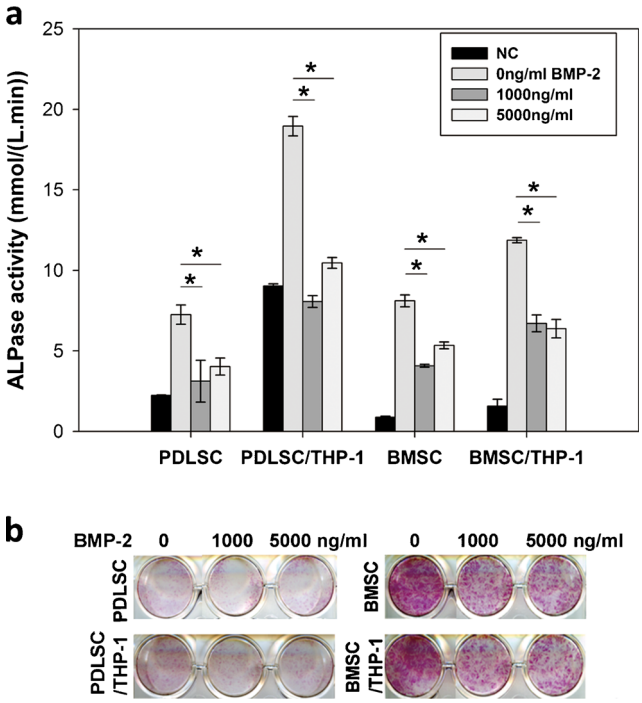


Fig. 1 Osteoblastic differentiation of mesenchymal stem cells (MSCs) is inhibited by a high bone morphogenetic protein 2 (BMP-2) concentration. **a** Alkaline phosphatase (ALP) activity from dental MSCs cultured alone or with human monocytic cells (THP-1). THP-1 cells co-cultured with bone marrow stem cells (BMSC)/periodontal ligament stem cells (PDLSC) or monocultured THP-1 cells were incubated in osteogenic medium with 1000 ng/ml or 5000 ng/ml BMP-2 for 5 days (*NC* negative control with no osteogenic differentiation). Each *bar* represents the mean ± SE of three independent experiments. **P* < 0.05 compared with the control group, *n* = 3. **b** ALP protein was detected on day 5 by an ALP staining kit

inhibitors and 1 mM phenylmethane sulfonyl fluoride. Proteins on membranes were detected with NF-κB, phosphorylated (p)-NF-κB, Erk1/2, p-Erk1/2, JNK (c-Jun N-terminal kinase), p-JNK, p38, and p-p38 primary antibodies (1:1000, Cell Signaling Technologies, Boston, Mass., USA) followed by horseradish peroxidase (HRP)-linked secondary antibody. As a control, we used β-actin (1:1000) antibody. The immunoblots were visualized with an HRP chemiluminescent detection kit (SurModics, Eden Prairie, Mnn., USA) and measured with a MicroChemi analyzer (DNR Bio-image Analyzer).

Statistical analysis

Statistical analysis was performed with a one-way analysis of variance followed by Tukey's HSD test with SPSS 22 (SPSS, Chicago, Ill., USA). ELISA data are presented as the means ± SE from quadruple replicates and others from triplicate replicates. A statistically significant difference between data was assigned if $P < 0.05$.

Results

High concentrations of BMP-2 inhibit early osteogenic differentiation of MSCs

In the early stages of osteogenic differentiation, hPDLSCs and hBMSCs had low levels of ALP activity in the presence of 1000 or 5000 ng/ml BMP-2 on day 5 (Fig. 1a). This pattern was identical to that of ALP staining (Fig. 1b). Both BMP-2 concentrations suppressed early osteogenic differentiation in hPDLSCs and hBMSCs. However, hMSCs co-cultured with THP-1 cells had higher levels of ALP activity in the early stages than did hMSCs alone.

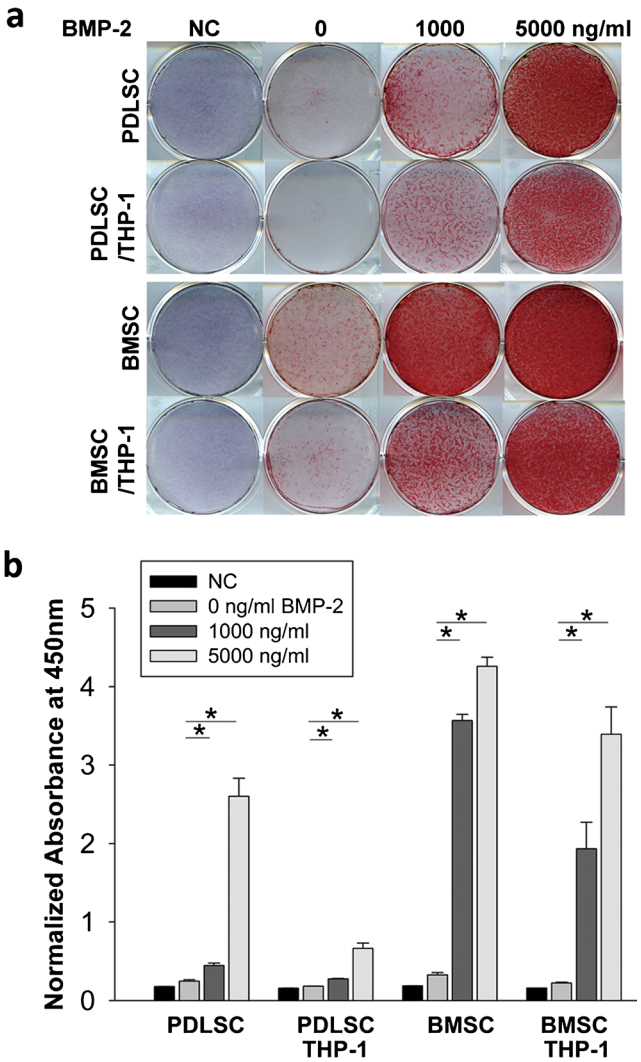


Fig. 2 Co-culturing THP-1 cells with MSCs inhibits late osteogenic differentiation of dental MSCs. **a** Calcium deposits from osteogenic differentiation of both BMSCs and PDLSCs with or without THP-1 cells were stained with alizarin red S solution at day 10 (*NC* negative control with no osteogenic differentiation). **b** Stained calcium deposits were destained and quantified with 20 % methanol and 10 % acetic acid. Each *bar* represents the mean ± SE of three independent experiments **P* < 0.05 compared with the control group, *n* = 3

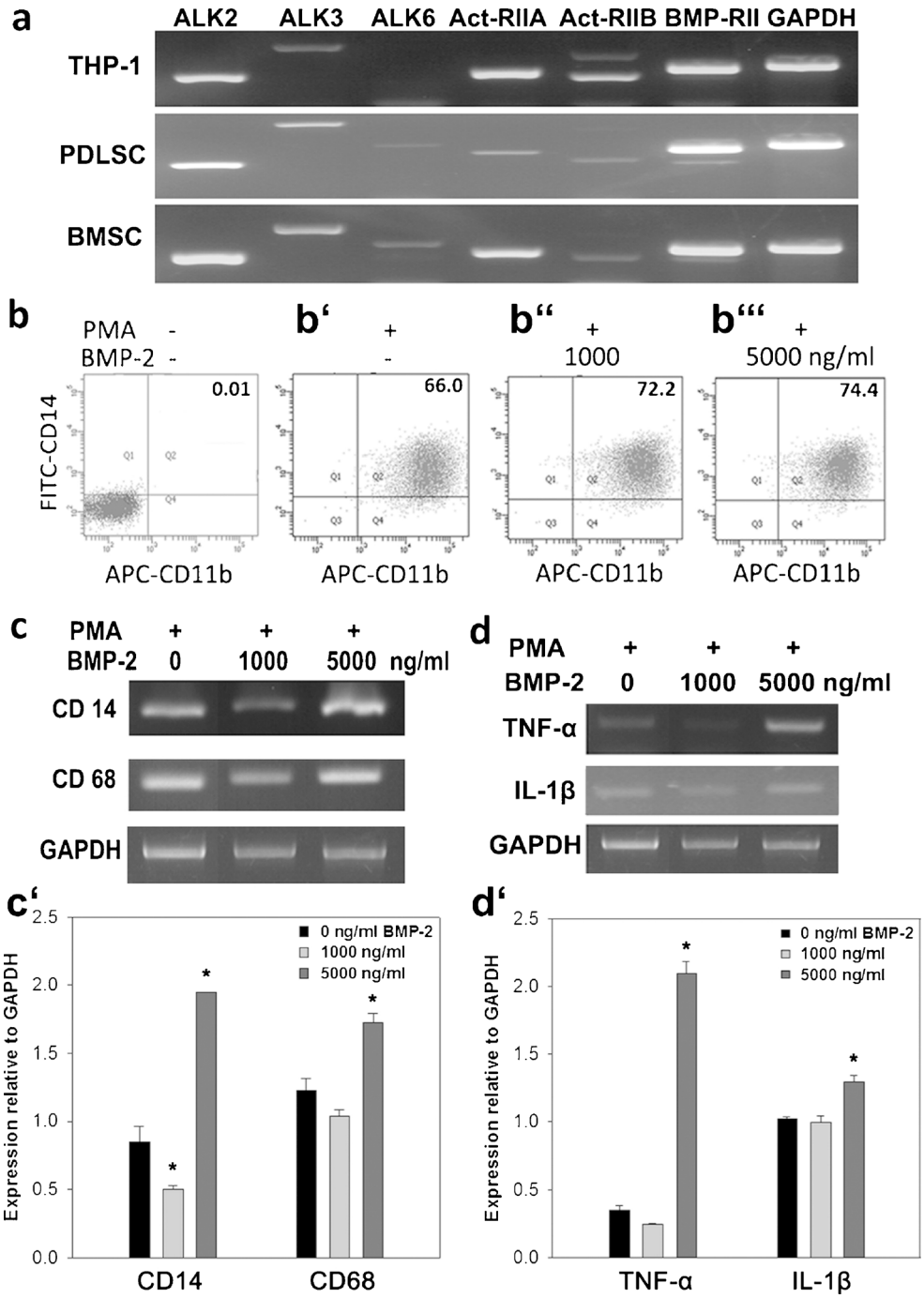


Fig. 3 Characteristics of THP-1 cells during BMP-2-induced inflammation. **a** Reverse transcription plus the polymerase chain reaction (RT-PCR) showed that MSCs and phorbol 12-myristate 13-acetate (PMA)-stimulated THP-1 cells had different levels of BMP receptors, although some similarities were apparent. **b–b'''** Flow cytometric analysis of CD14 and CD11b expression in THP-1 cells. Results for THP-1 activated with 50 nM PMA for 3 days followed by BMP-2 for 1 day were obtained by using fluorescein isothiocyanate (*FITC*)-conjugated anti-human CD14 and allophycocyanin (*APC*)-conjugated anti-human CD11b as surface markers of mature macrophages. The number of CD14 and CD11b double-positive cells was increased by BMP-2 treatment, regardless of dose. **c**, **c'** RT-PCR for macrophage markers (CD14 and CD68) on THP-1 cells after treatment with various BMP-2 concentrations. Gene expression was normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) per sample. **d**, **d'** Gene expression determined by RT-PCR. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) gene expression relative to GAPDH were measured after culture with various BMP-2 concentrations for 24 h ($n = 3$). Each *bar* represents the mean ± SE. *$P < 0.05$ compared with the control group

Co-culturing MSCs with THP-1 cells inhibits late osteogenic differentiation

The late stages of osteogenic differentiation were evaluated by alizarin red S staining (Fig. 2a, b). The inhibitory effect of THP-1 cells on osteogenic differentiation was overcome by BMP-2 in the late stages of differentiation. However, co-cultured hMSCs and THP-1 cells underwent less osteogenic differentiation in the late stages than did MSCs alone. Consequently, the initially inhibitory effect of BMP-2 concentrations changed to an inductive osteogenic signal in the late stages.

Characteristics of THP-1 cells in BMP-2-induced inflammation

To determine the response to BMP-2 of each cell, the gene expression of BMP-related receptors on THP-1

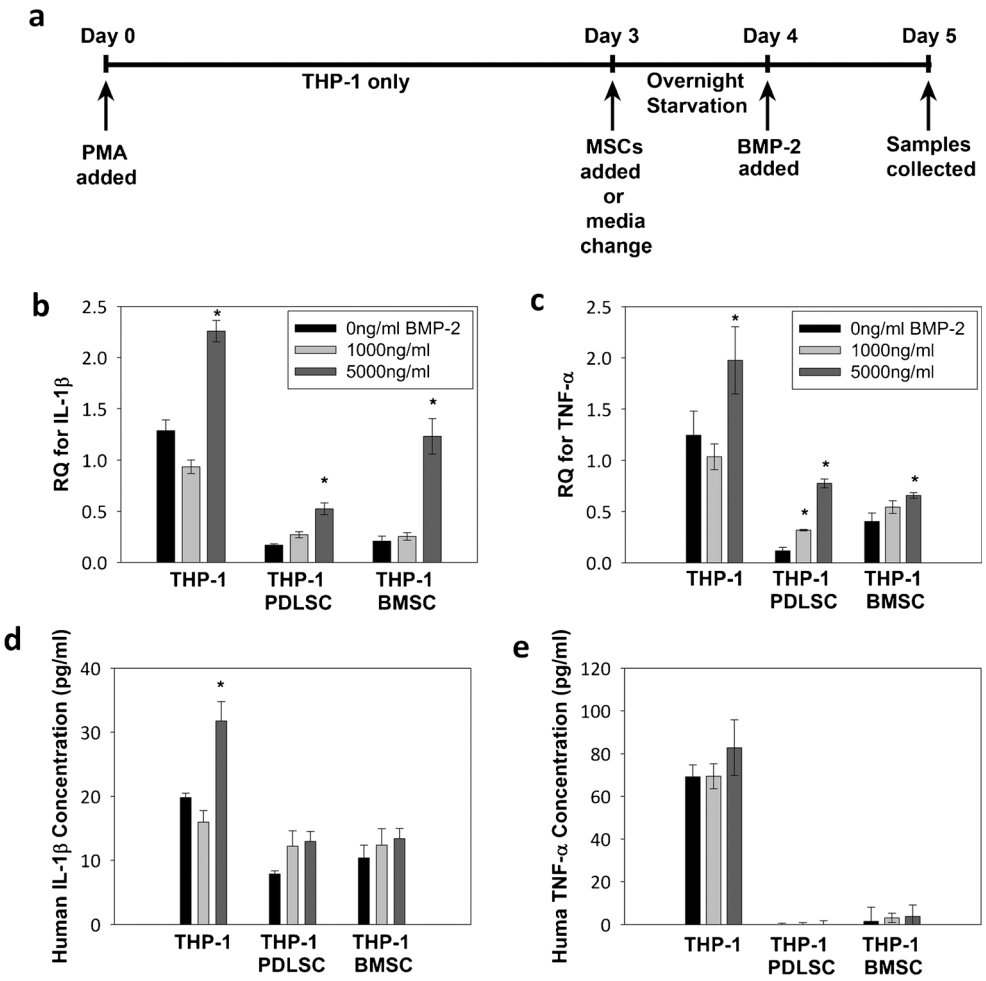


Fig. 4 MSCs suppress the expression of inflammatory cytokines in the presence of various BMP-2 concentrations. **a** Experimental scheme. Samples were collected for enzyme-linked immunosorbent assay (ELISA) and real-time PCR. **b**, **c** Real-time RT-PCR ($n = 3$) normalized to GAPDH expression showed that the expression of IL-1β and TNF-α was blocked by 24-h co-culture of MSCs with PMA-stimulated THP-1 cells (*RQ* relative quantification). **d**, **e** ELISA assay ($n = 4$) for IL-1β and TNF-α in supernatants following BMP-2 treatment for 24 h. Each *bar* represents the mean ± SE. *$P < 0.05$ compared with the control group

cells, PDLSCs and BMSCs was determined by RT-PCR. The BMP receptors related to signaling consist the type I receptors ALK2, ALK3 and ALK6 and the type II receptors Act-RIIA, Act-RIIB and BMP-RII. The gene expression of BMP-related receptors differed among THP-1 cells, PDLSCs and BMSCs (Fig. 3a). The activation of THP-1 cells was examined via flow cytometry analysis. CD11b and CD14 expressed on activated macrophages from monocytes were double-stained to examine the activation of THP-1 cells. In the absence of stimulation by PMA, THP-1 cells were not activated (Fig. 3b). Surface expression of activated macrophage markers, namely CD14 and CD68, on activated THP-1 cells changed in response to BMP-2 treatment. CD14 and CD68 were overexpressed at 5000 ng/ml BMP-2. BMP-2 at a concentration of 5000 ng/ml also activated the inflammatory stage of THP-1 cells (Fig. 3c). As shown in Fig. 3d, 5000 ng/ml BMP-2 induced an inflammatory response in THP-1 cells. THP-1 cells highly expressed TNF-α and IL-1β in the presence of 5000 ng/ml BMP-2.

MSCs inhibit inflammatory cytokine expression in THP-1 cells

To examine the immunosuppressive effects of hMSCs on BMP-2-induced inflammation, pro-inflammatory gene expression and protein accumulation in response to BMP-2 treatment were determined by real-time RT-PCR (Fig. 4b, c) and ELISA (Fig. 4d, e) following the experimental scheme shown in Fig. 4a. With a 24-h BMP-2 treatment, 5000 ng/ml BMP-2 was required for PMA-stimulated THP-1 cells to release pro-inflammatory cytokines. Stimulated THP-1 cells increased expression of the TNF-α and IL-1β genes in the presence of 5000 ng/ml BMP-2. Co-culturing THP-1 cells in the presence of PDLSCs or BMSCs decreased levels of inflammatory cytokines IL-1β and TNF-α. PDLSCs significantly suppressed the gene expression of inflammatory cytokine IL-1β to a greater extent than did BMSCs. The suppression of TNF-α genes on co-culturing THP-1 cells with PDLSCs was similar to the suppression in BMSCs (Fig. 4b, c). The levels of TNF-α and IL-1β proteins followed a pattern similar to gene expression in response to BMP-2 treatment

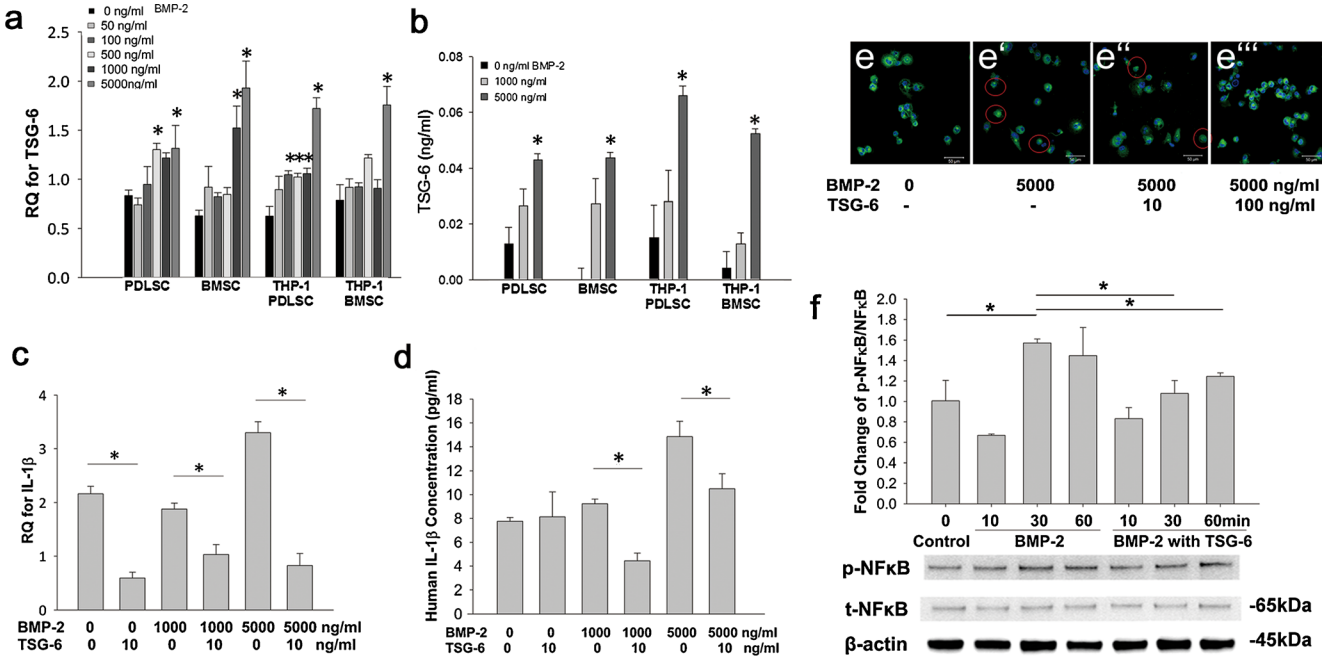


Fig. 5 Tumor-necrosis-factor-α-inducible gene 6 protein (TSG-6) secreted by MSCs in response to BMP-2 induction interferes with the inflammatory reactions of THP-1 cells through nuclear factor kappa-B (NF-κB) signaling. **a** Real-time RT-PCR revealed that PDLSCs and BMSCs in the presence or absence of THP-1 cells secreted TSG-6 in response to BMP-2 treatment for 24 h, as normalized to GAPDH. **b** Quantitative measurement of human TSG-6 was performed with an ELISA kit. Levels of TSG-6 were measured upon BMP-2 treatment for 24 h. **c**, **d** Real-time analysis and ELISA showed that TSG-6 inhibited IL-1β expression in THP-1 cells. THP-1 cells were exposed to 50 nM PMA for 3 days and with 1000 ng/ml or 5000 ng/ml BMP-2 and 10 ng/ml TSG-6 for 24 h. **e–e'''** Immunofluorescence analysis of NF-κB. THP-1 cells were pre-stimulated with 50 nM PMA and then exposed to BMP-2 for 3 h in the presence of 10 or 100 ng/ml TSG-6. NF-κB translocation into the nucleus (*red circle*) was decreased by TSG-6 treatment. Magnification ×200. *Bars* 50 μm. **f** Western blots were performed to measure NF-κB phosphorylation. THP-1 cells were stimulated with 50 nM PMA and treated with 5000 ng/ml BMP-2 or 10 ng/ml TSG-6 for 10, 30, or 60 min. Proteins were collected and the ratio of phospho-NF-κB to total NF-κB was determined by immunoblotting. The immunoblot data were normalized to β-actin. Each *bar* represents the mean ± SE. $^*P < 0.05$ compared with the control group, $n = 3$

(Fig. 4d, e). This result demonstrated that PMA-stimulated THP-1 cells secreted excessive levels of inflammatory cytokines in the presence of 5000 ng/ml BMP-2, thereby worsening the local inflammatory environment. In contrast, TNF-α and IL-1β expression induced by PMA was significantly suppressed by co-culture with MSCs.

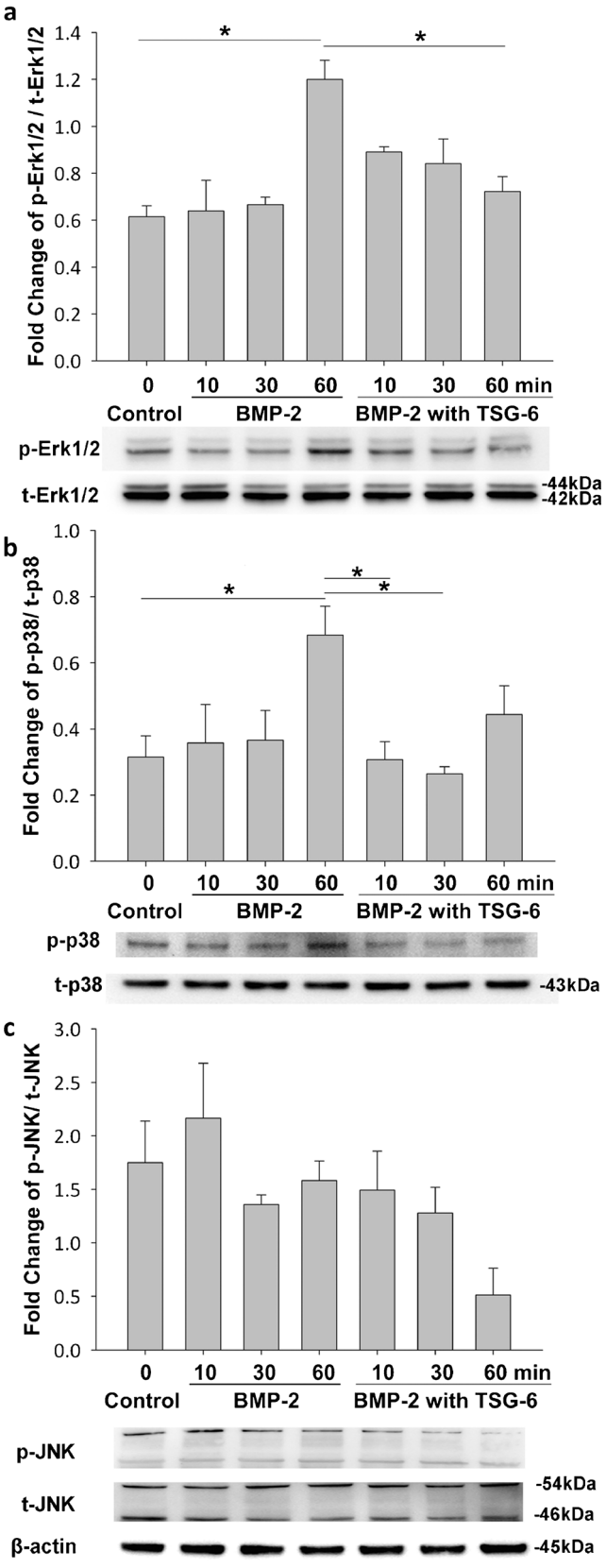
TSG-6 secreted by MSCs in response to BMP-2 interferes with inflammatory reactions of THP-1 cells through MAPK signaling

In the presence of 5000 ng/ml BMP-2, both PDLSCs and BMSCs secreted a significant amount of TSG-6 as shown by real-time RT-PCR (Fig. 5a). PDLSCs and BMSCs treated with 5000 ng/ml BMP-2 secreted significant protein levels of TSG-6 (Fig. 5b). Furthermore, PDLSCs and BMSCs co-cultured with THP-1 cells in 5000 ng/ml BMP-2 also showed increased protein expression of TSG-6. TSG-6 lowered IL-1β expression by BMP-2-treated THP-1 cells. Moreover, a BMP-2 concentration of 5000 ng/ml increased IL-1β expression, which was significantly inhibited by exogenous TSG-6 (Fig. 5c, d). TNF-α expression did not change in response to TSG-6 induced by 5000 ng/ml BMP-2 (data not shown). Treatment with 100 ng/ml TSG-6 for 3 h with BMP-2 induction blocked NF-κB translocation to the nucleus in THP-1 cells (Fig. 5e). Western blotting revealed that TSG-6 blocked NF-κB phosphorylation (Fig. 5f).

The effect of TSG-6 on downstream NF-κB signaling in THP-1 cells showed that extracellular signal-regulated kinase 1/2 (ERK1/2; Fig. 6a) and p38 signaling (Fig. 6b) were blocked at 60 min, whereas TSG-6 did not affect JNK signaling during BMP-2 treatment (Fig. 6c). Moreover, TSG-6 treatment for 60 min inhibited JNK signaling. This result indicates that TSG-6 secreted by MSCs blocks NF-κB translocation and IL-1β secretion through NF-κB/Erk1/2/p-38 signaling in THP-1 cells.

Discussion

This study was designed to investigate the immunosuppressive effects of MSCs on immune reactions triggered by macrophages at high BMP-2 concentrations. In current clinical and experimental applications of BMP-2, the inflammatory response induced by high concentrations of exogenous BMP-2 may decrease initial bone formation. Our study



◀ **Fig. 6** TSG-6 inhibits p38 and extracellular signal-regulated kinase 1/2 (*Erk1/2*) signaling in THP-1 cells activated by a high BMP-2 concentration. THP-1 cells were treated with 5000 ng/ml BMP-2 and/or 10 ng/ml TSG-6 for 10, 30, or 60 min. Immunoblotting data were analyzed by the ratio of phospho-ERK1/2 (p-Erk1/2), phospho-p38 (p-p38), and phospho-c-Jun N-terminal kinase (*p*-JNK) to total ERK1/2 (*t-Erk1/2*), total p38 (*t*-p38), and total JNK protein (*t*-JNK), respectively. **a** ERK1/2 signaling was activated by BMP-2 at 60 min, whereas TSG-6 treatment inhibited ERK1/2 signaling. **b** Moreover, p38 signaling was inhibited by TSG-6 treatment at high BMP-2 concentrations. **c** JNK signaling had no significant influence on protein levels. Each *bar* represents the mean ± SE. $^{*}P < 0.05$ compared with the control group, $n = 3$

suggests that high BMP-2 concentrations boost the initial local inflammatory reaction by releasing inflammatory cytokines, whereas MSCs reduce the inflammatory reaction through TSG-6.

Dense infiltration of inflammatory cells was observed around the grafted collagen sponge with 20 μg/ml of BMP-2 on the dorsal side of the mouse (Fig. S1). Bone remodeling/regeneration is a consequence of the balancing mechanism between osteoblasts and osteoclasts (Manolagas and Jilka 1995; Teitelbaum 2000). Monocytic macrophages were selected as candidate immune cells in this study because they are derived from a hematopoietic lineage related to osteoclasts. Monocytes from blood vessels differentiate and activate into macrophages after infiltrating blood vessels in inflammatory sites and can then differentiate into osteoclasts (Haynes et al. 2001; Hume et al. 2002). Similarly, we observed that high doses of BMP-2 triggered the infiltration and activation of macrophages. Our in vitro experimental scheme mimicked the in vivo clinical applications. In our research, a monocytic macrophage cell line, THP-1, was activated with PMA in order to convert them into activated inflammatory cells (for details of THP-1 cells, see Figs. S2, S3, S4). At the same time, osteogenic medium was applied to MSCs to drive differentiation into osteoblasts or THP-1 cells. A previous study had shown that BMP-2 secretion was increased in co-cultured MSCs and monocytes/macrophages in the early stages of osteogenic differentiation (Pirraco et al. 2013). ALP is an early osteogenic marker; however, some researchers have also found ALP to be an indicator of inflammation. ALP is induced during acute and chronic inflammation in rodent and human models (Krötzsch et al. 2005; Takabayashi et al. 2014). In our experiment, THP-1 cells were influenced by an inflammatory condition, namely PMA activation, which might affect early ALP activity. Our co-cultured MSCs and macrophages exhibited enhanced early osteogenic differentiation (ALPase activity) relative to MSCs alone. However, a high concentration of exogenous BMP-2 inhibited the early stages of osteogenesis in both MSCs alone and MSCs co-cultured with THP-1 cells. Previous studies have demonstrated that the inflammatory environment inhibits osteoblastic differentiation (R.L. Huang et al. 2014b). In the present study, alizarin red S staining indicated that the late stages of osteogenic differentiation were suppressed in

MSCs co-cultured with macrophages. In addition, osteogenic differentiation induced by high BMP-2 concentrations was reversed, resulting in higher calcium deposits in the late stages of osteogenesis. This reversal in the late stages of osteogenic induction can be explained by the discontinued PMA treatment of THP-1 cells. Therefore, the effects of inflammatory macrophages were reduced in late osteogenesis in our experiments. Reduced inflammatory effects increased the osteogenesis of hMSCs. This is a possible explanation of the contrasting effects of BMP-2 on the early and late stages of osteogenesis.

Clinical disorders of osteoporosis involving inflammatory conditions include rheumatoid arthritis, cystic fibrosis and periodontitis (Redlich and Smolen 2012; Shead et al. 2010). Our results indicate that an inappropriately high BMP-2 concentration stimulates macrophages to secrete inflammatory cytokines and delays osteogenic differentiation in MSCs. This implies that the reduced bone formation seen during the clinical use of high BMP-2 concentrations is caused by increased inflammatory cytokines. BMP-2 is thought to be responsible for inducing a pro-inflammatory environment. The inflammatory characteristics of rhBMP-2 have been observed in vitro and in vivo in the form of soft tissue swelling and inflammatory cytokine release in a rodent model (K.B. Lee et al. 2011b, 2012). We also found that monocytic macrophages express the genes for type I and type II BMP receptors. PDLSCs and BMSCs also express BMP receptors at differing levels, suggesting that BMP-2 affects downstream signaling heterogeneously in immune cells and MSCs. Our results show that BMP-2-induced macrophages cause the secretion of several inflammatory cytokines. In MSCs, however, BMP-2 participates in both Sma- and Mad-related family (SMAD) and MAPK signaling to increase the expression of osteogenic-related genes. In BMP-2-induced inflammation, MSCs differentiate into osteoblasts to a lesser degree, supporting previous studies showing that TNF-α and IL-1β can inhibit MAPK signaling to reduce osteogenic gene expression in murine MSCs (R.L. Huang et al. 2014a; G.T. Lee et al. 2010).

In addition to osteoblast differentiation, another MSC function is immunomodulation (Gibon et al. 2016; Ma et al. 2014; Prockop and Oh 2012). In disease models, MSCs suppress inflammatory responses through several mechanisms (R.H. Lee et al. 2009b, 2014; D.E. Lee et al. 2016; Manning et al. 2015; Wang et al. 2012). Our results suggest that MSCs secrete TSG-6 in response to BMP-2. This result indicates that the administeration of a high BMP-2 concentration to MSCs triggers an anti-inflammatory reaction that affects macrophages. In MSC and macrophage co-cultures, we observed the inhibited macrophage secretion of inflammatory cytokines, such as IL-1β and TNF-α. PDLSCs suppressed inflammation more effectively at a higher BMP-2 concentration than did BMSCs. Less IL-1β was produced upon treatment of the macrophages with TSG-6; this was consistent with results from the macrophage and MSC co-cultures. Previous studies have shown that TSG-6 from MSCs regulates macrophages through NF-κB signaling triggered by lipopolysaccharide (LPS) or zymosan-induction (Choi et al. 2011; R.L. Huang et al. 2014a; Sullivan et al. 2014). We found that, at a high BMP-2 concentration, macrophages induced NF-κB translocation into the nucleus, similar to that seen in LPS- or zymosan-induced inflammation. On TSG-6 treatment, NF-κB phosphorylation was inhibited in THP-1 cells at a high BMP-2 concentration. Although the inhibition of inflammation by TSG-6 has been tested in other studies, little agreement is evident regarding the manner in which inflammatory cytokines and osteogenic stimulation are differentially activated during NF-κB signaling in MSCs (R.L. Huang et al. 2014a; R.H. Lee et al. 2014; Mahoney et al. 2008).

In conclusion, the release of inflammatory cytokines by PMA-activated macrophages is increased by a high BMP-2 concentration. TSG-6 secreted by MSCs suppresses inflammatory reactions related to high BMP-2 concentrations through MAPK signaling in macrophages. The differing tendencies of PDLSCs and BMSCs in the presence of BMP-2 should be studied in the context of oral maxillofacial reconstruction therapy. By determining the anti-inflammatory effects of TSG-6 from dental-tissue-derived MSCs, we can expand the use of MSCs in clinical trials, especially in bone-related diseases, by establishing optimal therapeutic BMP-2 concentrations.

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