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Chondrogenesis of Embryonic Stem Cell-derived Mesenchymal Stem Cells Induced by TGF β 1 and BMP7 Through Increased TGF β Receptor Expression and Endogenous TGF β 1 Production

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Abstract

For decades stem cells have proven to be invaluable to the study of tissue development. More recently, mesenchymal stem cells (MSCs) derived from embryonic stem cells (ESCs) (ESC-MSCs) have emerged as a cell source with great potential for the future of biomedical research due to their enhanced proliferative capability compared to adult tissue-derived MSCs and effectiveness of musculoskeletal lineage-specific cell differentiation compared to ESCs. We have previously compared the properties and differentiation potential of ESC-MSCs to bone marrow-derived MSCs. In this study, we evaluated the potential of TGF β 1 and BMP7 to induce chondrogenic differentiation of ESC-MSCs compared to that of TGF β 1 alone and further investigated the cellular phenotype and intracellular signaling in response to these induction conditions. Our results showed that the expression of cartilage-associated markers in ESC-MSCs induced by the TGF β 1 and BMP7 combination was increased compared to induction with TGF β 1 alone. The TGF β 1 and BMP7 combination upregulated the expression of TGF β receptor and the production of endogenous TGF β s compared to TGF β 1 induction. The growth factor combination also increasingly activated both of the TGF and BMP signaling pathways, and inhibition of the signaling pathways led to reduced chondrogenesis of ESC-MSCs. Our findings suggest that by adding BMP7 to TGF β 1-supplemented induction medium, ESC-MSC chondrogenesis is upregulated through increased production of endogenous TGF β and activities of TGF β and BMP signaling.

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Keywords

embryonic stem cell; mesenchymal stem cell; chondrogenesis; transforming growth factor-beta; bone morphogenetic protein

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Introduction

Mesenchymal stem cells (MSCs) derived from embryonic stem cells (ESCs) represent an emerging cell source for tissue engineering and regenerative medicine and possess great potential toward understanding tissue development and cellular differentiation. MSCs derived from ESCs (ESC-MSCs) have essentially provided researchers with a tool needed to study the process of tissue development from pluripotent stem cells to MSC intermediates to terminally differentiated cells [Boyd et al., 2009; Oldershaw et al., 2010]. Multiple ESC-MSC lines have been established using different derivative methods and in general, ESC-MSCs have been shown to portray the characteristic phenotype of MSCs while maintaining the ability to differentiate into multiple cell lineages. Enhanced proliferative and immunosuppressive capabilities have also been demonstrated within ESC-MSCs compared to bone marrow MSCs (BM-MSCs) and other adult tissue-derived MSCs [Brown et al., 2009; de Peppo et al., 2010; Trivedi and Hematti, 2008; Vodyanik et al., 2010]. However, previous studies have shown that variation in the ability to differentiate into chondrocytes, osteoblasts, and adipocytes exists between different ESC-MSCs lines and also between ESC-MSCs and BM-MSCs [Karlsson and al, 2009; Marolt et al., 2012; Varga et al., 2011]. Chondrogenic induction of ESC-MSCs, for example, appears to be challenging as the cells produce less cartilage-associated extracellular matrix (ECM) in culture compared to BM-MSCs in response to transforming growth factor-beta (TGF β) induction [Karlsson and al, 2009; Marolt et al., 2012; Varga et al., 2011]. This presents a need to develop approaches capable of effectively inducing ESC-MSC chondrogenesis.

TGF β is a potent induction factor for chondrogenic differentiation of BM-MSCs and other adult tissue-derived MSCs [Johnstone et al., 1998]. Treatment with TGF β 1 or TGF β 3 induces the expression of SOX9 and other cartilage-associated ECM markers in high-density cell pellets. In fact, few growth factors other than TGF β have been demonstrated to be capable of effectively inducing chondrogenesis in human BM-MSC or other adult tissue-derived MSC [Longobardi et al., 2006; Neumann et al., 2007; Shintani and Hunziker, 2011]. We have previously shown that when induced with TGF β 1 alone for chondrogenesis, ESC-MSCs produces less cartilage-associated ECM markers than BM-MSCs, however, by adding BMP7 to TGF β 1-supplemented chondrogenic medium, ESC-MSCs produce significantly increased amounts of ECM markers [Brown et al., 2014].

Similarly, other studies have shown that adipose-derived MSCs require additional BMP stimulation for chondrogenic differentiation [Afizah et al., 2007; Kim and Im, 2009]. In adult tissue-derived MSCs or other progenitor cells, combinations of TGF β 1 or TGF β 3 and various bone morphogenetic proteins (BMPs), including BMP2, 4, 6, and 7, have been shown to increase the production of collagen type 2 and other cartilage ECM proteins compared to TGF β alone [Brady et al., 2014; Hennig et al., 2007; Hildner et al., 2010; Kim

and Im, 2009; Koay et al., 2007; Xu et al., 2006]. The upregulation of cartilage-associated marker expression by these growth factor combinations is in part through increased activation of intracellular signaling pathways and downstream SOX9 [Montero et al., 2008]. While TGF β s and BMPs can act synergistically to upregulate chondrogenic induction of ESC-MSCs, the underlying regulatory mechanism is yet identified.

During cartilage development, multiple growth factors regulate the expression and endogenous production of TGFs and BMPs to drive differentiation of mesenchymal cells into chondrocytes. Similarly, exogenous administration of growth factors can induce MSCs and other cells to produce growth factors through autocrine regulation to modulate cellular behavior [Li et al., 2014; Li et al., 2013; Li et al., 2011]. Additionally, growth factors induce changes in expression of growth factor receptors, thus altering the cell phenotype as differentiation progresses. Activation of TGF β and BMP signaling through specific Type 1 and Type 2 serine/threonine kinase receptor complexes to regulate downstream molecules such as SOX9 [Raftery and Sutherland, 1999; Wrana and Attisano, 2000]. It is likely that increased production of endogenous growth factors and upregulation of specific growth factor receptors may synergistically increase chondrogenesis of MSCs. Specifically, the combination of TGF β 1 and BMP7 may regulate ESC-MSCs to increase production of TGF β and TGF β receptor to enhance chondrogenic differentiation compared to TGF β 1 alone.

The objective of this study was to investigate the effects of TGF β 1 and BMP7 combination on ESC-MSC chondrogenesis and further investigate the underlying regulatory mechanism. We hypothesized that TGF β 1 and BMP7 in combination upregulates the expression of TGF β receptor and the production of endogenous TGF to increase the activation of TGF β signaling, which collectively results in enhancement of ESC-MSC chondrogenesis.

Materials and Methods

Stem cell culture

Ethical approval of using human embryonic stem cells for this study was granted by the Institutional Biosafety Committee at the University of Wisconsin-Madison. Two ESC-MSC lines, H1-MSC and WA1-MSC, derived from H1-ESCs (WiCell, Madison, WI) as described previously were provided by Drs. Slukvin and Hematti and cultured following recommended protocols [Trivedi and Hematti, 2008; Vodyanik et al., 2010]. Briefly, one ESC-MSC line was derived by culturing H1-ESCs on OP9 feeder layer and then in semisolid medium supplemented with fibroblast growth factor-2 (FGF2) and platelet-derived growth factor BB for two weeks. Selected mesodermal colonies were further cultured in serum-free medium with FGF2 to expand ESC-MSCs. These cells are henceforth referred to as H1-MSCs. The other ESC-MSC line was derived from H1-ESCs by maintaining the cells on mouse embryonic fibroblast (MEF) cells and passaging them weekly on Matrigel-coated plates with MEF conditioned medium and FGF2. The culture was continually passaged to generate MSCs with fibroblast-like morphology that are henceforth referred to as WA1-MSCs. H1-MSCs were maintained in serum free, chemically defined medium (SFM) composed of 50% Stemline II Hematopoietic Stem Cell Expansion Medium (Sigma-Aldrich, St. Louis, MO) and 50% Endothelial Serum Free Medium (Life Technologies), supplemented with 10 ng/ml FGF2 (Peprotech, Rocky Hill, NJ), 100 μ M monothioglutamate (Sigma-Aldrich), 0.05%

Excyte (StemCell Technologies, Vancouver, BC), and 2 mM Glutamax (Life Technologies). WA1-MSCs were maintained in α -MEM (Life Technologies) supplemented with 5% FBS (Atlanta Biologicals), 2 mM Glutamax (Life Technologies), and 10 ng/ml FGF2 (Peprotech). The two culture media were previously optimized for achieving the best result of growth of each ESC-MSC line. Culture medium was changed every three days. Cells were maintained at 37°C in a 5% CO₂ and 95% humidified incubator and passaged using StemPro Accutase (Life Technologies) when reaching 70–80% confluence.

Chondrogenic differentiation

ESC-MSCs were collected upon reaching 80% confluence and centrifuged in 96-well round bottom plates at $600 \times g$ for 5 minutes to create high-density cell pellets with 2.5×10^5 cells per pellet. Cell pellets were then induced in serum-free chondrogenic medium composed of high-glucose DMEM (Life Technologies), 1% ITS+ Premix (6.25 μ g/ml insulin, 6.25 μ g/ml transferrin, 6.25 μ g/ml selenious acid, 1.25 mg/ml bovine serum albumin, and 5.35 μ g/ml linoleic acid) (BD Biosciences), 1% penicillin/streptomycin antibiotics, 1 mM sodium pyruvate, 50 μ g/ml ascorbic acid, 40 μ g/ml L-proline, and 0.1 μ M dexamethasone. Depending on the study, chondrogenic differentiation was induced with 10 ng/ml TGF β 1 (Peprotech) and with or without 100 ng/ml BMP7 (Peprotech) or BMP7 alone. Cell pellets were maintained at 37°C in a 5% CO₂ and 95% humidified incubator and the medium was replaced every 3 days during the culture period.

Inhibition of TGF β and BMP signaling

Inhibition of TGF β and/or BMP signaling was performed using the small molecule inhibitors, SB431542 (Torcris Biosciences, Bristol, United Kingdom) and Dorsomorphin (Abcam, Cambridge, United Kingdom), respectively. Inhibitors were added in culture with a final concentration of 5 μ M during medium change.

RNA isolation and quantitative PCR

Total RNA was isolated as previously described using the NucleoSpin RNA II kit (Macherey-Nagel). Synthesis of cDNA was carried out using the High Capacity cDNA Reverse Transcriptase kit (Life Technologies) and real-time PCR analysis was performed to determine the expression level of *collagen type 2*, *aggrecan*, *SOX9*, *TGF β 1*, and *TGF β 3* using iQ SYBR Green Supermix (BioRad). The sequences of the primers for these genes are listed in Table 1. The relative expression level of each target mRNA transcript was determined in reference to the expression level of the internal control *UBC* using the 2^{-CT} formula.

Flow cytometry analysis

After 1, 3, or 7 days of chondrogenic induction with TGF β 1 or the combination of TGF β 1 and BMP7, pellets were collected and washed with ice-cold PBS and fixed with 2% formaldehyde (Polysciences, Warrington, PA). Pellets were then dissociated using collagenase A and mechanical disruption (Roche Mannheim, Germany). Cells were passed through a 40- μ m nylon strainer (BD Biosciences), washed with a buffer solution, and incubated with antibodies detecting TGF β R2, BMPR1B and BMPR2. The data is presented

graphically as the fluorescence signal to noise ratio against the geometric mean of the isotype control.

Protein isolation and western blot analysis

Protein was isolated from chondrogenic pellets using radioimmunoprecipitation (RIPA) solubilization buffer (50 mM Tris-HCl, pH 7.5, 1% NP-40, 0.25% sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA) and complete protease inhibitor cocktail (Roche, Madison, WI). Pellets were physically dissociated with a pestle, vortexed for 1 minute, and sonicated for 5 minutes using a water bath sonicator. After centrifugation at 14,000 rpm for 10 minutes at 4°C, supernatant was collected and protein concentrations were determined using the BCA Protein Assay kit (Pierce, Rockford, IL). Protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) members for blotting. Membranes were incubated overnight at 4°C with primary antibodies in 5% BSA solution. The primary antibodies were used to detect pSMAD3 (Ser423/425), SMAD3, pSMAD1/5, SMAD1, and TGFβ. GAPDH was used as a loading control. All antibodies were purchased from Cell Signaling (Danvers, MA). A goat-anti-rabbit HRP-conjugated antibody was used as the secondary antibody. Signals were developed using Clarity™ Western ECL Substrate (BioRad) and imaged using Kodak Image Station 4000R Pro.

Histology and immunofluorescence analysis

Chondrogenic cell pellets were collected at day 21, fixed with 10% formalin, embedded in paraffin, and sectioned into 7 μm-thick slices. Slides were prepared following our laboratory standard protocol prior to staining with Gill's Hematoxylin #3 and 0.5% Eosin Y or Alcian blue 8GX (Polysciences). Immunohistochemical analysis was performed by blocking rehydrated samples with 1% BSA in PBS and then incubating the samples with mouse anti-human collagen type 2 primary antibody (Millipore) diluted in PBS at 1:200 for 1 hour. Samples were then washed with PBS and incubated with FITC-conjugated secondary antibody (eBioscience) diluted 1:100 in PBS for 1 hour. Coverslips were affixed to slides using Prolong Antifade Reagent with DAPI (Life Technologies) prior to imaging.

Enzyme-linked immunosorbent assay (ELISA) for TGFβ1

H1-MSC pellets were induced for chondrogenic differentiation with TGFβ1 alone or the combination of TGFβ1 and BMP7. At each medium change, 200 μl of culture medium was collected and immediately frozen at 20°C for analysis at a later time. TGFβ1 concentrations in the collected culture medium were measured using ELISA (R&D Systems) following the manufacturer's protocol.

Statistical analysis

All values of quantitative results are expressed as mean ± standard deviations. Statistical significance was determined by performing Two-factor ANOVA to test for significant differences between means of the experimental groups, followed by pairwise t-test as prescribed by the Fisher's Least Significant Difference (LSD) test to determine statistical significance between any paired groups. A *P*-value < 0.05 was considered significant.

Results

TGF β 1 and BMP7 combination enhances H1-MSC and WA1-MSC chondrogenesis

To demonstrate the effect of the TGF β 1 and BMP7 combination on chondrogenic differentiation of ESC-MSCs, we induced chondrogenesis in H1-MSCs and WA1-MSCs using TGF β 1 with or without BMP7 and analyzed the mRNA expression of cartilage-related markers. H1-MSCs or WA1-MSCs induced with the TGF β 1 and BMP7 combination expressed significantly higher levels of *collagen type 2*, *aggrecan*, and *SOX9* than those induced with TGF β 1 alone; H1-MSCs expressed greater levels of these mRNA transcripts than WA1-MSCs (Fig. 1A). Histological analysis showed that cell pellets induced with the TGF β 1 and BMP7 combination produced greater amounts of ECM compared to pellets induced with TGF β 1 alone (Fig. 1B). H&E and Alcian blue staining showed denser collections of ECM within the pellets induced by the TGF β 1 and BMP7 combination. Similarly, compared to TGF β 1 induction, the TGF β 1 and BMP7 combination resulted in increased collagen type 2 production throughout both H1-MSC and WA1-MSC pellets as shown by immunofluorescence staining (Fig. 1B).

BMP7 induces much less expression of cartilage-associated ECM markers than TGF β 1

After demonstrating that the addition of BMP7 to standard TGF β 1-supplemented chondrogenic medium enhanced chondrogenesis of ESC-MSCs, we sought to determine if BMP7 alone was able to induce chondrogenesis of the cell by analyzing the mRNA expression of cartilage-associated ECM markers, *collagen type 2* and *aggrecan* (Fig. 2). While comparable mRNA levels of *collagen type 2* were found between TGF β 1- and BMP7-induced chondrogenesis, BMP7 induced significantly lower mRNA levels of *aggrecan* than TGF β 1, suggesting that compared to TGF β 1, BMP7 is an ineffective inducer for chondrogenesis.

TGF β 1 and BMP7 in combination upregulates expression of TGF β 2 and production of endogenous TGF β

We analyzed the expression of TGF β 2, BMPR1B, and BMPR2 during chondrogenesis induced by TGF β 1 with or without BMP7 using flow cytometry (Fig. 3A). During the 7-day induction, the fluorescence intensity of TGF β 2 on H1-MSCs treated with TGF β 1 and BMP7 was greater than that on the cell treated with only TGF β 1. While the expression of BMPR1B induced by either treatment showed a dramatic decrease from day 1 to day 3 before increased again at day 7, the fluorescence intensity was greater in TGF β 1-treated cells than that in TGF β 1 and BMP7-treated cells. On the other hand, the expression of BMPR2 was decreased with culture time and showed no difference between TGF β 1- and TGF β 1 and BMP7-treated cells.

After determining that TGF β 1 and BMP7 upregulated chondrogenic differentiation of ESC-MSCs and increased the expression of TGF β 2, we next investigated the mRNA expression of TGF β s and BMPs that might be produced endogenously in cells during chondrogenic induction (Fig. 3B). Quantitative PCR analysis revealed no significant difference in the expression of *BMP2*, *BMP4*, *BMP6* or *BMP7* between H1-MSCs induced with TGF β 1 and the TGF β 1 and BMP7 combination. However, the cells induced by the combination of

growth factors upregulated expression of *TGFβ1* and *TGFβ3* with a significant increase in their expression levels at hour 48 compared to those induced by TGFβ1 alone. Specifically, in the TGFβ1-induced cells, the expression of *TGFβ1* began to decrease between hours 24 and 48 while TGFβ1 and BMP7 induction resulted in a consistent increase in the expression of *TGFβ1* from hour 6 to hour 48 with significant expression differences between the TGFβ1 alone and the TGFβ1 and BMP7 combination groups at hours 12 and 48. The expression of *TGFβ3* increased steadily between hours 6 and 48 in cells induced by both conditions, but induction with the TGFβ1 and BMP7 combination resulted in significantly greater expression of *TGFβ3* than that with TGFβ1 alone at hour 48.

Similarly, the expression of endogenous TGFβ in both cultures analyzed by western blotting was increased with induction time, and the addition of BMP7 to TGFβ1-supplemented chondrogenic medium induced more endogenous TGFβ production than TGFβ alone (Fig. 3C). Western blots detected by a pan-specific antibody showed two bands of TGFβ isoforms with the molecular weight around 60 kDa. The production of endogenous TGFβ by ESC-MSCs induced with TGFβ alone or with the TGFβ1 and BMP7 combination increased with time during the 48 hours and similar to the results of mRNA expression, induction with the TGFβ1 and BMP7 combination resulted in the upregulated production of endogenous TGFβ compared to induction by TGFβ1.

We further used ELISA to determine the concentration of soluble TGFβ1 and TGFβ3 released into the media after growth factor induction. Culture medium was collected before each cell feeding for 9 days, and again at the end of the culture period (Fig. 3D). The results showed that the TGFβ1 content in medium was increased with induction time in response to both treatments while more TGFβ1 was detected in the culture induced with TGFβ1 and BMP7 than that in the culture with TGFβ1 alone. Interestingly, the concentration of TGFβ3 in medium was undetectable in either of the cultures (data not shown).

TGFβ1 and BMP7 combination increases activation of SMADs during H1-MSC chondrogenesis

We analyzed SMAD signaling activated by TGFβ1 or the TGFβ1 and BMP7 combination to determine whether the growth factor treatments induce differential activation of the intracellular signaling in an ESC-MSC line. After growth factor induction, the TGFβ1 and BMP7 combination increased phosphorylation of SMAD3 at both hours 2 and 6 compared to TGFβ1 alone (Fig. 4A). Similarly, TGFβ1 alone was able to induce activation of SMAD1/5 but the level of phosphorylation was less than that in hMSCs induced by the TGFβ1 and BMP7 combination.

As our results showed that TGFβ1 and BMP7 in combination induced the production of endogenous TGFβ1 in culture medium, we were then interested in determining if SMAD signaling could be activated using the conditioned chondrogenic medium. The conditioned medium was collected from chondrogenic culture of H1-MSCs induced with TGFβ1 or the TGFβ1 and BMP7 combination for 3 days. H1-MSC pellets were then treated with the conditioned medium for 6 hours and analyzed for SMAD signaling molecules. While the conditioned medium of TGFβ1-induced culture weakly activated SMAD3 and SMAD1/5,

the medium from the TGF β 1 and BMP7-induced culture more robustly activated both of the SMAD signaling molecules (Fig. 4B).

Attenuating TGF β or BMP signaling inhibits H1-MSC chondrogenesis

With our earlier results showing that the TGF β 1 and BMP7 combination enhanced chondrogenesis of ESC-MSCs compared to TGF β 1 alone, we then sought to determine the effect of blocking TGF β and/or BMP receptors on chondrogenic differentiation of H1-MSCs. We used small molecule inhibitors SB431542 (SB) to block ALK4 (Activin receptor 1B), ALK5 (TGF β receptor 1) and ALK7 (Activin receptor 1C) to attenuate TGF β 1-induced signaling and dorsomorphin (DM) to block ALK2 (Activin receptor 1), ALK3 (BMP receptor 1A) and ALK6 (BMP receptor 1B) to attenuate BMP7-induced signaling during chondrogenic induction. Quantitative PCR analysis showed that at both days 10 and 20 the TGF β 1 and BMP7 combination induced the expression of *collagen type 2*, *aggrecan*, and *SOX9*, as expected (Fig. 5A). With the addition of SB, DM or both, the induced expression of mRNA transcripts of these markers was significantly reduced. Particularly, the expression of *collagen type 2* and *aggrecan* was reduced to near undetectable levels.

Furthermore, the histological results of H1-MSC pellets corroborated the quantitative PCR results. In the TGF β 1 and BMP7-treated pellets, Alcian blue and H&E staining showed a dense cellular structure rich in glycosaminoglycan and immunofluorescent staining revealed a large deposition of collagen type 2 (Fig. 5B). When treated with the inhibitors DM and/or SB, chondrogenic cell pellets showed a less dense cellular structure with a decrease in the glycosaminoglycan content compared to the control pellets without inhibitor treatment. Additionally, in terms of the evaluation of pellet size, DM-treated pellets were similar in size to TGF β 1 and BMP7-treated pellets while SB-treated pellets were clearly the smallest among all pellets cultured in different conditions. The production of collagen type 2 was also reduced in inhibitor-treated pellets compared to that in the pellets induced by the TGF β 1 and BMP7 combination.

Discussion

Although the roles of TGF β s and BMPs in regulation of chondrogenesis of MSCs derived from adult tissues have been studied for years, the mechanism of how these growth factors induce chondrogenesis of ESC-MSCs still remains elusive. In this study we demonstrate that adding BMP7 to the gold standard TGF β 1-supplemented chondrogenic medium can greatly upregulate chondrogenic induction of ESC-MSCs compared to TGF β 1 alone, which is considered a gold-standard induction molecule for chondrogenesis. In addition, we further show that the upregulation of chondrogenesis by TGF β 1 and BMP7 is correlated to increased TGF β R2 expression and the production of endogenous TGF β . Concomitantly, TGF β 1 and BMP7 in combination induce greater activation of both TGF β - and BMP-related SMAD signaling compared to TGF β 1. Inhibition of the signaling pathways using small molecule inhibitors reduces the ability of the TGF β 1 and BMP7 combination to induce ESC-MSC chondrogenesis. Together, our data provide insight into how the TGF β 1 and BMP7 combination upregulates chondrogenesis of ESC-MSCs.

We investigate the effect of BMP7 in TGF β 1-supplemented culture on chondrogenesis because BMP7 has been shown to promote adipogenesis, osteogenesis, and chondrogenesis in several previous studies including ours [Brown et al., 2014; Knippenberg et al., 2006; Neumann et al., 2007; Shen et al., 2010]. In chondrocytes of developing cartilage, BMP7 along with BMP2, 3, 4, and 5 are expressed in the perichondrium but only BMP7 is expressed in proliferating chondrocytes and mature cartilage [Hidaka and Goldring, 2008]. This may suggest that BMP7 may be a potent growth factor capable of inducing chondrogenesis compared to other BMPs that are found mostly in hypertrophic chondrocytes. BMP7 can additionally function as a potent anabolic stimulus for adult chondrocytes *in vivo* and exert an anti-apoptotic effect to promote cell survival [Gokce et al., 2012; Hidaka and Goldring, 2008; Wei et al., 2008]. Our results show that TGF β 1 and BMP7 in combination increasingly induce ESC-MSC chondrogenesis.

Our results show that BMP7 generates synergistic effects in TGF β 1-supplemented culture on the upregulation of ESC-MSC chondrogenesis but BMP7 alone in culture is limited in driving ESC-MSCs to differentiate into chondrocytes. Shen et al. have demonstrated a similar finding in their study in which BMP7 is unable to effectively induce chondrogenesis of BM-MSCs with reduced mRNA expression of *collagen type 2* comparable to TGF β 3 [Shen et al., 2010]. Together, the results of both studies suggest that it is not preferable to use BMP7 alone in culture to induce chondrogenesis of MSCs and BMP7 should be used along with TGF β s to upregulate chondrogenic differentiation of the cell.

Previous studies using ESCs, MSCs, tissue-specific stem cells, and chondro-progenitor cells have demonstrated the synergistic effects of TGF β s and BMPs on chondrogenesis [Gong et al., 2010; Hildner et al., 2010; Nakagawa et al., 2009; Rui et al., 2010; Xu et al., 2006]. However, the chondrogenic effects of different TGF β and BMP combinations in these studies are dependent on the growth factor isoform and concentration [Mehlhorn et al., 2007; Shintani and Hunziker, 2011]. For example, Shen et al. have demonstrated that compared to TGF β 3 alone, chondrogenic induction by BMP7 and TGF β 3 upregulates the expression of *collagen types 2 and 9* and *aggrecan* but downregulate the expression of *collagen type 1* and *biglycan* suggesting a critical role of BMP7 in regulating hyaline- and fibro-chondrogenesis [Shen et al., 2010]. On the other hand, it has also been shown that TGF β s and BMPs can exert opposing effects on each other depending on cell type. For example, induction by both TGF β and BMP does not yield a synergetic effect on chondrogenesis of ESCs in embryoid bodies [Toh et al., 2009]. Furthermore, Keller et al. have demonstrated that when TGF β 1 and BMP2 in combination are used to induce chondrogenesis in the rat ATCD5 cell line, TGF β 1 was able to enhance BMP signaling while BMP2 significantly reduced levels of TGF β signaling [Keller et al., 2011]. These examples demonstrate that the effect of TGF β s and BMPs on chondrogenesis may also depend on target cell types.

Induction by TGF β 1 and BMP7 increases the expression of TGF β R2 in H1-MSCs during early chondrogenesis. TGF β R2 recruits and phosphorylates TGF β R1 after binding TGF β 1 to initiate its downstream signaling pathway [Ehrlich et al., 2012]. Studies have shown that depletion of TGF β R2 or abolishing its function interferes with TGF β -induced chondrogenesis and the development of cartilage, demonstrating the importance of this

receptor [Hiramatsu et al., 2011; Li et al., 2014]. Similar findings reported by Hennig et al. have shown that alteration of the expression of TGF β R1 in adipose-derived MSCs results in reduced chondrogenesis [Hennig et al., 2007]. When the cells are induced with BMP6 and TGF β 3, the expression of TGF β R1 is increased. On the other hand, studies have also reported that TGF β can upregulate BMP receptors further suggesting additional layers of regulation [Montero et al., 2008; Xu et al., 2006].

In addition to upregulated expression of the TGF β receptor, we demonstrated that the induction by TGF β 1 and BMP7 increased the production of endogenous TGF β compared to induction by TGF β 1 alone during chondrogenesis. Interestingly, the level of soluble TGF β 1 or TGF β 3 in culture measured by ELISA was either comparable between experimental groups or undetectable. Considering that our results did show a noticeable increase in the expression of both TGF β 1 transcript and protein in H1-MSCs induced by the TGF β 1 and BMP7 combination, it is possible that ELISA is limited to detecting a fraction of total TGF β s released from cells since some of the released TGF β s may have already bound to cells and some of them may have been sequestered by the ECM. In any way, our results suggest that the TGF β 1 and BMP7 combination upregulates production of endogenous TGF β in H1-MSCs. The production of endogenous TGF β s or BMPs during cartilage development *in vitro* in response to growth factors has been investigated by several groups and is suggested to improve differentiation into mesenchymal lineages [Choi et al., 2012; Leonard et al., 1991; Mizuta et al., 2002; Seib et al., 2009]. For example, TGF β s are known to regulate the production of endogenous TGF β s [Mizuta et al., 2002] and transient administration of TGF β can induce the production of endogenous TGF β s during the differentiation process [Kulyk et al., 1989]. Likewise, transient induction of TGF β 3 between 30 minutes and 6 hours is also sufficient to continuously induce cellular condensation and matrix production in chick limb mesenchymal cells for a few days following induction through the activity of endogenous TGF β [Leonard et al., 1991]. Taken together these studies demonstrate the importance of endogenous growth factors to the differentiation process as seen in this study.

In addition to differences in the expression of growth factor receptors, we also observe differential activation of downstream SMAD signaling after growth factor stimulation. TGF β 1 and BMP7 in combination induce greater activation of both SMAD3 and SMAD1/5 compared to TGF β 1 alone. Additionally, we also demonstrate that H1-MSC pellets treated with the TGF β 1 and BMP7-conditioned medium resulted in increased activation of SMAD signaling compared to those treated with the TGF β 1-conditioned medium. Together, these results suggest that the TGF β 1 and BMP7 combination may induce ESC-MSCs to release endogenously produced growth factors into the medium that continuously activate SMAD signaling during chondrogenesis. Notably, our results show that when TGF β 1 and BMP7 are administered together, both the SMAD3-mediated and SMAD1/5-mediated signaling pathways are activated whereas TGF β 1 alone only activated SMAD3. It has been reported that SMAD activation by TGF β leads to an increased SOX9 level which in turn upregulates the production of collagen type 2 and cartilage extracellular matrix proteins in MSCs or in the limb during chondrogenesis [Song et al., 2009; Zehentner et al., 1999]. We have confirmed that in this study the TGF β 1 and BMP7 combination increases activation of

SMAD signaling and expression of *SOX9* to upregulate production of collagen type 2 and aggrecan as enhanced chondrogenesis in ESC-MSCs.

We also show that inhibition of TGF β and BMP signaling reduces the effect of the TGF β 1 and BMP7 combination on chondrogenesis, suggesting that activation of both of the signaling pathways is critical for ESC-MSC chondrogenesis. Interestingly, the addition of both small molecule inhibitors results in cell pellets with a less dense structure than those treated with or without one of the inhibitors. Our results suggest that TGF β 1 and/or BMP7 may be involved in mediating cell-cell interaction or cell condensation in a pellet for chondrogenesis. The effect of TGF β s and BMPs on cell-cell interaction has been demonstrated in previous studies, in which BMPs and TGF β s increase cellular adhesion through regulation of N-cadherin to enhance chondrogenic differentiation [Haas and Tuan, 1999; Tuli et al., 2003]. In addition, it seems to be more detrimental to the formation of chondrogenic pellets to block TGF β signaling with SB than to block BMP signaling with DM. Pellets treated with DM retained a structure much similar to those with TGF β 1 and BMP7 than pellets treated with SB. This suggests that TGF β 1 may be a predominant growth factor that induces chondrogenesis of H1-MSCs while BMP7 may act synergistically with TGF β 1 to increase production of cartilaginous matrix proteins.

In conclusion, our results show that BMP7, when combined with TGF β 1, exerts a synergistic effect on the induction of ESC-MSC chondrogenesis through the upregulation of TGF β R2 expression and an increase in the production of endogenous TGF β s. Inhibition of TGF β 1 and/or BMP7 signaling with receptor inhibitors attenuates the synergistic effect of TGF β 1 and BMP7 on promoting ESC-MSC chondrogenesis. Our findings provide further insight into the mechanism of how the combination of TGF β 1 and BMP7 synergistically induces ESC-MSC chondrogenesis.

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Figure 1.

Analysis of cartilage-related markers during chondrogenic differentiation of ESC-MSCs. (A) The mRNA expression of cartilage-related markers in two ESC-MSC lines induced by TGF β 1 (T) with or without BMP7 (B) was analyzed by quantitative PCR. * $p < 0.05$; $n = 3$. (B) Histological sections of ESC-MSC pellets induced with T or TB for chondrogenesis were analyzed by Alcian blue (top row), H&E staining (middle row), and immunofluorescence staining of collagen type 2 (bottom row). Scale bar = 200 μ m.

Figure 2. Chondrogenic induction of H1-MSCs with BMP7 or TGFβ1. H1-MSCs induced by either BMP7 or TGFβ1 for 21 days were analyzed for the mRNA expression of cartilage-associated ECM markers. * $p < 0.05$; $n = 3$

Figure 3.

Expression of growth factor receptors and production of endogenous growth factors. (A) Chondrogenic pellets of H1-MSCs induced with T or TB for a week were analyzed by flow cytometry to detect the expression of TGFβR2, BMPR1B, and BMPR2. Values are represented as the fluorescence signal to noise ratio against the geometric mean of the isotype control. (B) The mRNA transcripts of *BMPs 2,4,6* and *7*, and *TGFβ1* and *β3* in H1-MSC pellets induced with T or TB for chondrogenesis were analyzed by quantitative PCR. (C) Protein extracts from H1-MSC pellets induced with T or TB for chondrogenesis were analyzed by Western blotting to detect the expression of TGFβ. (D) Soluble TGFβ1 released into culture medium during ESC-MSC chondrogenesis was determined by ELISA.

Figure 4.

Activation of SMADs during chondrogenesis of H1-MSC pellets. (A) H1-MSC pellets induced with T or TB for chondrogenesis were analyzed by western blotting to detect the expression of pSMAD3 and pSMAD1/5. GAPDH was used as a loading control. (B) H1-MSC pellets were treated with conditioned medium collected from separate 3-day chondrogenic culture of H1-MSCs for 6 hours prior to protein isolation and analysis of pSMAD3 and pSMAD1/5.

Figure 5.

Analysis of cartilage-related markers during chondrogenic differentiation of ESC-MSCs attenuated by TGF β and BMP signaling inhibitors. (A) H1-MSC pellets induced with TB are treated with dorsomorphin (DM), SB431542 (SB) or both before the analysis of expression of the cartilage-related markers. * $p < 0.05$; $n = 3$ (B) Histological sections of H1-MSC pellets induced with TB and treated with DM and/or SB were analyzed by Alcian blue (top row), H&E staining (middle row), and immunofluorescence staining of collagen type 2 (bottom row). Scale bar = 200 μ m.

Table 1

List of primer sequences for quantitative PCR

Gene	Accession number	Sequences (5' to 3')
<i>Aggrecan</i>	NM_013227.2	(F) CACGATGCCTTTCACCACGAC (R) CTATCCGTGACAACTGGGCGT
<i>BMP2</i>	NM_00478.3	(F) CGTCAAGCCAAACACAAACAG (R) GAAGTCCACGTACAAAGGGTG
<i>BMP4</i>	NM_004348.3	(F) TAGGAGCCATTCCGTAGTGCC (R) ACAGTCCATGATTCTTGACAGC
<i>BMP6</i>	NM_000088.3	(F) GTCTTACAGGAGCATCAGCAC (R) GGAGTCACAACCCACAGATTG
<i>BMP7</i>	NM_001844.4	(F) TCCTGATGCGTAAACAGAAGC (R) TCTTGGATTGGGCAAACCTC
<i>Collagen Type 2</i>	NM_001844.4	(F) GGAAACTTTGCTGCCCAGATG (R) CGTTAGGACCACTTGGACCACT
<i>SOX9</i>	NM_000346.3	(F) TAAAGGCAACTCGTACCCAA (R) GCACCTCCTACTACCTCTTA
<i>TGFβ1</i>	NM_013227.2	(F) CTATTGCTTCAGCTCCACGG (R) TCCAGGCTCCAAATGTAGGG
<i>TGFβ3</i>	NM_000346.3	(F) TGACTTCCGACAGGATCTGG (R) AGCCCTTAGGTTTCATGGACC
<i>UBC</i>	NM_021009.4	(F) TGAAGACACTCACTGGCAAGACCA (R) AACTAGAAACGGCCTTTCGTCGAC

Forward and reverse primers are indicated as "F" and "R", respectively.