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Enhancement of osteogenic and chondrogenic differentiation of human embryonic stem cells by mesodermal lineage induction with BMP-4 and FGF2 treatment

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ABSTRACT

Recently, it was reported that bone morphogenetic protein 4 (BMP4) alone or BMP4 combined with fibroblast growth factor 2 (FGF2) treatment enhanced mesodermal differentiation of human embryonic stem cells (hESCs) that were cultured feeder-free on Matrigel. In this study, we show that mesodermal lineage-induced embryoid bodies (EBs) generate greater numbers of osteogenic and chondrogenic lineage cells. To induce the mesodermal lineage, hESCs were treated with BMP4 and FGF2 during the EB state. Quantitative real-time reverse transcription-polymerase chain reaction analysis showed that the treatment decreased endodermal and ectodermal lineage gene expression and increased mesodermal lineage gene expression. Importantly, the mesodermal lineage-induced EBs underwent enhanced osteogenic and chondrogenic differentiation after differentiation induction. This method could be useful to enhance the osteogenic or chondrogenic differentiation of hESCs.

1. Introduction

The embryoid bodies (EBs) of embryonic stem cells (ESCs) offer suitable conditions for human embryonic stem cell (hESC) differentiation into all three germ layers [1,2]. The molecular and cellular morphogene signals of EB are similar to the signals of epiblast-stage embryos in vivo [2]. Following EB formation, EBs are plated onto tissue culture dishes, which leads to differentiation into many different cell lineages [3]. However, for tissue-engineering cell sources, differentiation into a homogeneous population of a desired cell type is needed [4].

The mesodermal cells develop into muscles, bones, and cartilage (from the paraxial mesoderm); kidneys, gonads, and their respective duct systems (from the intermediate mesoderm); and heart, blood vessels, and blood cells (from the lateral mesoderm) [5]. One of the well-known signals involved in the ESC differentiation to mesoderm is bone morphogenetic protein-4 (BMP4), which is a transforming growth factor-β superfamily member [6]. Fibroblast growth factor (FGF) also stimulates mesodermal differentiation [7,8]. Recently, treatment with BMP4 only [5] or co-treatment with BMP4 and FGF2 [9] promotes mesodermal induction of feeder-free cultured hESCs on Matrigel. hESCs were treated with MP4 or with BMP4/FGF2 directly without the EB formation step. In this study, we hypothesized that BMP4 and FGF2 treatment at the EB state, which is the initial step of hESC differentiation, stimulates mesodermal lineage induction and suppresses endodermal and ectodermal lineage induction. Additionally, we hypothesized that BMP4/FGF2-treated EBs generate increased numbers of osteogenic and chondrogenic lineage cells upon differentiation induction, which can be utilized as a useful cell source for regenerating bone and cartilage tissues.

2. Materials and methods

2.1. Cell culture

SNUhES31 (Institute of Reproductive Medicine and Population, Medical Research Center, Seoul National University, Seoul, Korea), a hESC line, was maintained as undifferentiated ESCs by culture on feeder layers of mitomycin-C (Sigma–Aldrich, St. Louis, MO,

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USA) treated STO cells (mouse embryonic fibroblast cell line) (American Type Culture Collection; ATCC, Manassas, VA, USA) in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Gibco BRL, Gaithersburg, MD, USA.) supplemented with 20% (v/v) knockout serum replacement (Invitrogen, Carlsbad, CA, USA), 4 ng/ml FGF2 (R&D Systems, Minneapolis, MN, USA), 1% nonessential amino acid (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), and 0.2% Primocin (InvivoGen, San Diego, CA, USA).The culture medium was changed daily, and hESCs were passaged onto fresh STO cells every week. The hESC colonies were fragmented into uniform sizes using the STEMPRO® EZPassage (Invitrogen). The culture medium for expansion of the STO cells consisted of DMEM (Gibco, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen).

2.2. Formation of EBs and mesodermal induction

To form the EBs, hESC colonies were detached from feeders using collagenase type IV (Worthington, Lakewood, NJ, USA). The detached hESC colonies were subsequently transferred to a low-attachment culture dish. EBs were formed by incubation in EB medium, which was FGF2-depleted ESC culture medium. To induce mesodermal induction, BMP4 (10 ng/ml Peprotech, Rocky Hill, NJ, USA) and FGF2 (20 ng/ml, R&D) were added to the EB medium for 4 days (B/F EB group). The medium was changed every other day. The EBs without BMP4 and FGF2 treatment served as the control (EB group). Four days later, the morphology of the EBs was observed using a light microscope (Model IX71, Olympus, Tokyo, Japan).

2.3. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) of EBs

After 4 days, the EBs were harvested and analyzed using qRT-PCR. The total RNA was extracted from the B/F EB and the EB. The RNA was reverse-transcribed into cDNA. The expression of ZIC, PAX6, SOX1, FOXQ1, and Wnt3 mRNA was measured using qRT-PCR. qRT-PCR was performed using the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA) with FAST SYBR Green PCR master mix (Applied Biosystems). Each cycle consisted of the following times and temperatures: 94 °C for 3 s and 60 °C for 30 s. Primers specific for human GAPDH had the following sequences: 5′-GTC GGA GTC AAC GGA TTT GG-3′ (forward) and 5′-GGG TGG AAT CAA TTG GAA CAT-3′ (reverse). Primers specific for human ZIC1 had the following sequences: 5′- GCG CTC CGA GAA TTT AAA GA -3′ (forward) and 5′- CGT GGA CCT TCA TGT GTT TG -3′ (reverse). Primers specific for human PAX6 had the following sequences: 5′-GTG TCC AAC GGA TGT GTG AG-3′ (forward) and 5′-CTA GCC AGG TTG CGA AGA AC-3′ (reverse). Primers specific for human SOX1 had the following sequences: 5′-TAC AGC CCC ATC TCC AAC TC-3′ (forward) and 5′-GCT CCG ACT TCA CCA GAG AG-3′ (reverse). Primers specific for human FOXQ1 had the following sequences: 5′-GCG CGG ACT TGC ACT TT-3′ (forward) and 5′-GCA CGT TTG ATG GAG ATT TTA AAA-3′ (reverse). Primers specific for human WNT3 had the following sequences: 5′-CTG CCA GGA GTG TAT TCG CAT C-3′ (forward) and 5′-GAG AGC CTC CCC GTC CAC AG-3′ (reverse).

2.4. Osteogenic and chondrogenic differentiation of EBs

For osteogenic differentiation, the EBs and the B/F EBs were cultured on 0.1% (w/v) gelatin-coated dishes in osteogenic differentiation medium for 14 days. The osteogenic differentiation medium was DMEM (Gibco) supplemented with 10% FBS (Gibco), 0.1 μM dexamethasone (Sigma), 50 μM ascorbate-2-phosphate (Sigma), and 10 mM β-glycerophosphate (Sigma). The medium was changed every 2 days. For chondrogenic differentiation, the B/F EBs and the EBs were cultured in suspension in chondrogenic differentiation medium, which consisted of DMEM supplemented with 1% (v/v) ITS+1 (Gibco), 0.1 μM dexamethasone (Sigma), 50 μg/ml ascorbate-2-phosphate (Sigma) and 10 ng/ml transforming growth factor-β3 (R&D) for 14 days. The chondrogenic differentiation medium was changed every 2 days. Osteogenic and chondrogenic differentiation was analyzed using qRT-PCR and immunohistochemistry staining.

2.5. qRT-PCR of ostegenic and chondrogenic lineage cells

After 14 days, the cells were harvested and analyzed for osteogenic and chondrogenic differentiation using qRT-PCR. For the osteogenic differentiation analysis, alkaline phosphatase (ALP), collagen type I (Col I), runt-related transcription factor 2 (Runx2), and osteocalcin (OC) gene expression levels were analyzed. For chondrogenic differentiation analysis, collagen type II (Col II) and Sox9 gene expression levels were analyzed. qRT-PCR was performed using the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA) with the FAST SYBR Green PCR master mix (Applied Biosystems). Each cycle consisted of the following times and temperatures: 94 °C for 3 s and 60 °C for 30 s. Primers specific for human GAPDH had the following sequences: 5′-GTC GGA GTC AAC GGA TTT GG-3′ (forward) and 5′-GGG TGG AAT CAA TTG GAA CAT-3′ (reverse). Primers specific for human ALP had the following sequences: 5′-CCC TTG ACC CCC ACA ATG T-3′ (forward) and 5′-GTT GTT CCT GTT CAG CTC GTA-3′ (reverse). Primers specific for human Col I had the following sequences: 5′-CAG CCG CTT CAC CTA CAG C-3′ (forward) and 5′-TTT TGT ATT CAA TCA CTG TCT T-3′ (reverse). Primers specific for human Runx2 had the following sequences: 5′-GCA GCA CGC TAT TAA ATC CAA-3′ (forward) and 5′-ACA GAT TCA TCC ATT CTG CCA-3′ (reverse). Primers specific for human OC had the following sequences: 5′-CCT CAC ACT CCT CGC CCT ATT-3′ (forward) and 5′-CCC TCC TGC TTG GAC ACA AA-3′ (reverse). Primers specific for human Col II had the following sequences: 5′-ATA AGG ATG TGT GGA AGC CG-3′ (forward) and 5′-TTT CTG TCC CTT TGG TCC TG-3′ (reverse). Primers specific for human Sox9 had the following sequences: 5′-GTA CCC GCA CTT GCA CAA C-3′ (forward) and 5′-TCG CTC TCG TTC AGA AGT CTC-3′ (reverse).

2.6. Immunohistochemistry of osteogenic and chondrogenic lineage cells

After 14 days of osteogenic differentiation, the cells were fixed using 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature and were stained with anti-human OC antibodies (Abcam, Cambridge, UK). The chondrogenic differentiated cell pellets were frozen in Optimal Cutting Temperature compound (Tissue-Tek, Sakura Finetk, Torrance, CA, USA) at −20 °C and sectioned (a section thickness of 10 μm) using a Cryostat Cryocut Microtome (Leica, CM3050S, Nussloch, Germany). The sections were subsequently fixed using 4% paraformaldehyde in PBS for 10 min at room temperature and were stained with anti-human Col II antibodies (Abcam). The staining for OC was visualized using rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The staining for Col II was visualized using fluorescein-isothiocyanate-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). The cells were counter-stained with 4,6 diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA) and examined using a fluorescence microscope (Model IX71, Olympus, Tokyo, Japan).

2.7. Statistical analysis

All quantitative data are expressed as the mean ± standard deviation. Statistical analysis was performed using Student's *t*-test (SAS software, SAS Institute, Cary, NC, USA). A p value <0.05 was considered to be statistically significant.

3. Results

To induce mesodermal differentiation of EBs, the EBs were treated with BMP4 and FGF2 for 4 days. After induction, the morphology of the B/F EBs (EBs treated with BMP4 and FGF2) and the nontreated EBs were observed using an optical microscope. Both the B/F EBs and the EBs exhibited round and spherical shapes and showed no difference in morphology (Fig. 1A).

To determine whether BMP4 and FGF2 treatment induced mesodermal differentiation of EBs, the expression of the three germ layer lineage-specific genes was evaluated using qRT-PCR. Mesodermal gene (WNT3) expression in the B/F EBs increased compared to that of the nontreated EBs (Fig. 1B). In contrast, the expression of ectodermal genes (ZIC, PAX6, and SOX1) and the endodermal gene (FOXQ1) in the B/F EB decreased.

After 4 days of EB culture with or without BMP/4 FGF2 treatment, the B/F EBs and the EBs were cultured for 14 days in osteogenic or chondrogenic medium to investigate the mesodermal induction of EB-enhanced osteogenic and chondrogenic differentiation. qRT-PCR analysis showed that the B/F EBs had a greater mRNA expression of ALP, Col I, Runx2 and OC compared with the EBs that were cultured in osteogenic medium (Fig. 2A). In the chondrogenic medium, the B/F EBs expressed greater levels of Col II, and Sox9 compared with the nontreated EBs (Fig. 2B). The immunocytochemical analysis also showed that the B/F EBs expressed greater OC (red) and Col II (green) compared with the nontreated EBs (Fig. 3).

4. Discussion

In this study, we treated EBs with BMP4 and FGF2 to enhance mesodermal differentiation at the EB stage. A previous study reported that BMP4/FGF2 treatment of hESCs that were cultured feeder-free on Matrigel without EB formation induced mesodermal differentiation [9]. We hypothesized that BMP4/FGF2 treatment at the EB stage, which is the initial step of ESC differentiation, would also induce mesodermal differentiation. Due to the BMP4/FGF2 treatment, the mesodermal induction of EBs was promoted compared with untreated EBs. Furthermore, the osteogenic and chondrogenic differentiation of BMP4/FGF2-treated EBs was enhanced compared with that of untreated EBs.

Several studies have shown that BMP4 signaling is needed for mesoderm formation [10–12]. BMP4 gene- [10] or BMP

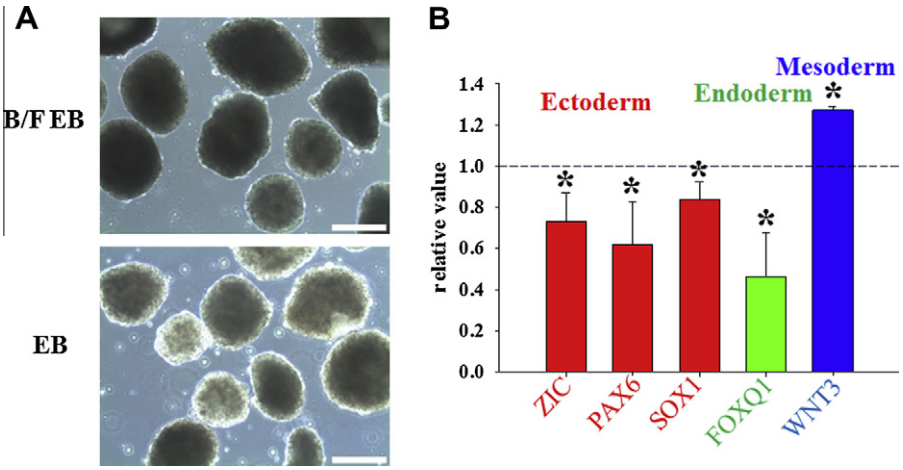

Fig. 1. Mesodermal lineage-induced EBs through the treatment of EBs with BMP4 and FGF2 (B/F EB). (A) Microscopic images of BMP4/FGF2-treated EBs (B/F EB) and nontreated EBs (EB). The scale bars are 200 μm. (B) Quantification of mRNA expression of the mesoderm gene (WNT3), endoderm gene (FOXQ1), and ectoderm genes (ZIC1, SOX1 and PAX6) in B/F EBs, as determined using qRT-PCR (n = 4). The expression levels were normalized to the levels of the nontreated EB group. $^{*}p < 0.05$ compared with EB.

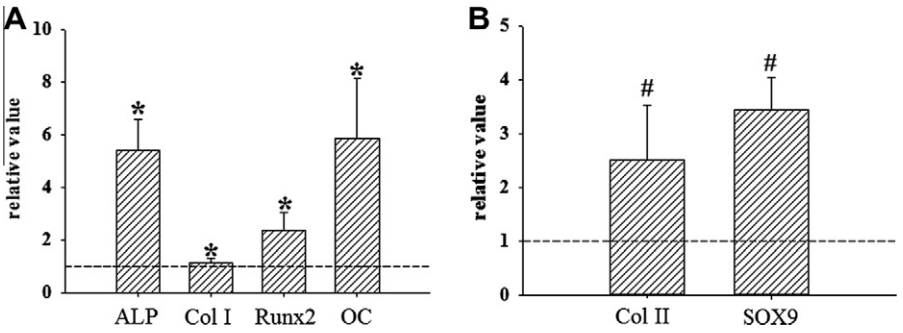

Fig. 2. Enhanced osteogenic and chondrogenic differentiation of mesodermal lineage-induced EBs (B/F EB). Quantification of the mRNA expression of (A) osteogenic differentiation marker genes in osteogenic differentiation-induced B/F EBs and (B) chondrogenic differentiation marker genes in chondrogenic differentiation-induced B/F EBs, as determined using qRT-PCR (n = 4). The expression levels were normalized to the levels of the nontreated EB group. $^{*}p < 0.05$ compared to the osteogenic differentiation-induced EBs, $^{\#}p < 0.05$ compared to the chondrogenic differentiation-induced EBs.

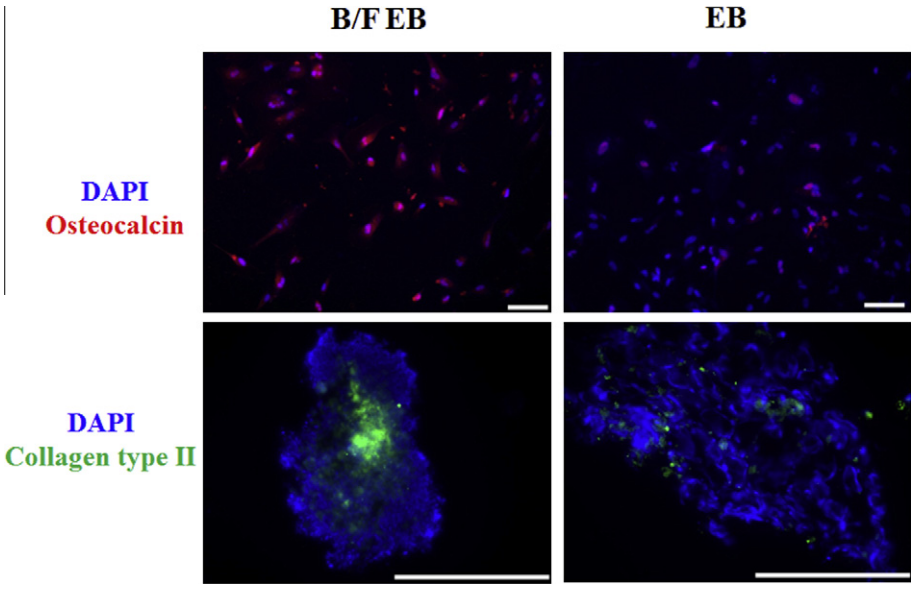

Fig. 3. Enhanced osteogenic and chondrogenic differentiation of the mesodermal lineage-induced EBs (B/F EB). Immunohistochemistry for human osteocalcin (red) in osteogenic differentiation-induced B/F EBs and nontreated EBs and for human collagen type II (green) in chondrogenic differentiation-induced B/F EBs and nontreated EBs at day 14. The nuclei were stained with DAPI (blue). The scale bars are 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

receptor- [11,12] deficient mouse embryos failed to develop a mesoderm. BMP4 induces mouse ESCs to differentiate into a mesoderm in vitro [13–15]. The expression of mesodermal markers T, WNT3, and MIXL1 was enhanced not only in mice but also in BMP4-treated hESCs that were cultured feeder-free [6]. In our results, the BMP4 and FGF2 treatment at the EB stage decreased ectodermal (ZIC, PAX6, and SOX1) and endodermal gene (FOXQ1) expression and enhanced mesodermal gene (WNT3) expression, which suggests that there is stimulation of mesodermal induction but not endodermal induction using BMP4/FGF2 treatment.

Mesodermally induced EB using BMP/4FGF2 treatment showed enhanced osteogenic and chondrogenic differentiation when cultured in osteogenic and chondrogenic medium. The B/F EBs showed increased mRNA expression of Runx2, which is an osteogenic transcription factor [16], and OC, which is a terminal osteoblastic differentiation marker [17]. The B/F EBs also showed increased chondrogenic gene expression. Sox9 is an essential transcription factor in chondrogenesis [18]. Sox9 is known to activate type II collagen [19]. In our results, the mRNA expression levels of both Sox9 and Col II increased.

Teratoma formation is one of the serious problems in using hESC therapy [20]. When hESCs are injected into immune-deficient mice, they form teratomas, which are tumor-like formation containing three germ layer tissues [21]. To prevent in vivo teratoma formation, many methods have been proposed. This teratoma formation could be prevented either by selectively removing, killing or inactivating pluripotent hESCs from the differentiated cell population or by ensuring the loss of pluripotent cells during the differentiation process [22]. Other methods include generating genetically engineered hESCs [23–25], selecting specific ectopic markers of expression [26,27], separating cells by density gradient centrifugation or by the differences in cell adherence [28,29], eliminating pluripotent cells by cytotoxic drugs [30], mitotically inactivating the cell therapy product [31], and inducing differentiation of the remaining undifferentiated cells [32,33]. In our study, direct treatment of EBs with BMP4 and FGF2 during the EB formation step enhanced mesodermal lineage differentiation and suppressed the differentiation of the ectodermal and endodermal lineage. Therefore, this method may help to suppress ESC teratoma formation.

In summary, BMP4 and FGF2 treatment of EBs promoted differentiation to a mesodermal-specific lineage. Mesodermal lineage-induced EBs enhanced osteogenic and chondorgenic differentiation when cultured in osteogenic and chondrogenic medium compared with control EBs. The modulation of hESCs at the EB formation stage with growth factors could be a useful method to enhance hESC differentiation into desirable cells.

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