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REGULAR ARTICLE

Characterization and evaluation of mesenchymal stem cells derived from human embryonic stem cells and bone marrow

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Abstract Embryonic stem cells (ESCs) and mesenchymal stem cells (MSCs) have been studied for years as primary cell sources for regenerative biology and medicine. MSCs have been derived from cell and tissue sources, such as bone marrow (BM), and more recently from ESCs. This study investigated MSCs derived from BM, H1- and H9-ESC lines in terms of morphology, surface marker and growth factor receptor expression, proliferative capability, modulation of immune cell growth and multipotency, in order to evaluate ESC-MSCs as a cell source for potential regenerative applications. The results showed that ESC-MSCs exhibited spindle-shaped morphology similar to BM-MSCs but of various sizes, and flow cytometric immunophenotyping revealed expression of characteristic MSC surface markers on all tested cell lines except H9-derived MSCs. Differences in growth factor receptor expression were also shown between cell lines. In addition, ESC-MSCs showed greater capabilities for cell proliferation, and suppression of leukocyte growth compared to BM-MSCs. Using standard protocols, induction of ESC-MSC differentiation along the adipogenic, osteogenic, or chondrogenic lineages was less effective compared to that of BM-MSCs. By adding bone morphogenetic protein 7 (BMP7) into transforming growth factor beta 1 (TGFβ1)-supplemented induction medium, chondrogenesis of ESC-MSCs was significantly enhanced. Our findings suggest that ESC-MSCs and BM-MSCs show differences in their surface marker profiles and the capacities of proliferation, immunomodulation, and most importantly multi-lineage differentiation. Using modified chondrogenic medium with BMP7 and TGFβ1, H1-MSCs can be effectively induced as BM-MSCs for chondrogenesis.

Keywords Embryonic stem cell · Mesenchymal stem cell · Differentiation · Transforming growth factor beta · Bone morphogenetic protein

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Introduction

For decades, adult tissue-derived mesenchymal stem cells (MSCs) have been studied as a cell source for tissue engineering and regenerative medicine, with the ultimate goal of treating numerous diseases (Brooke et al. 2007; Rivera and Aigner 2012; Toubai et al. 2009). These cells can be derived from various adult tissue sources including bone marrow (BM), adipose tissue, or synovial tissue, and their potential for various biomedical applications has been demonstrated (Giordano et al. 2007). More recently, MSCs have been derived from pluripotent embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) (Boyd, et al. 2009; Brown et al. 2009; de Peppo et al. 2013; Vodyanik et al. 2010). The multipotent characteristics of MSCs allow for the preparation of abundant connective tissue cells for cell-based therapies to treat diseases such as osteogenesis imperfecta (Grove et al. 2004; Horwitz et al. 2002; Jiang et al. 2002; Wang et al. 2005). Additionally, MSCs have been shown to play a critical role in regulating biological activities during injury and inflammation by recruiting tissue-specific progenitor cells to an area of injury and/or by regulating immune cell proliferation and differentiation for tissue regeneration (Alvarez-Dolado et al. 2003; Di Nicola et al. 2002; Hatzistergos et al. 2010; Jiang et al. 2005; Kinnaird et al. 2004; Seebach et al. 2012; Tolar et al. 2010).

Despite the aforementioned advantages of using MSCs for cell therapy, several issues associated with intrinsic properties of these cells limit their applications for clinical use in regenerative medicine. For example, while MSCs can be expanded during in vitro culture after isolation, inevitably cellular senescence occurs, thus limiting the ability of cells for robust proliferation and lineage-specific differentiation in subculture (Wagner et al. 2009, 2008). Isolated MSCs are often composed of heterogeneous cell populations and each population may have different proliferative and differentiation capabilities, thus increasing the difficulty for effective differentiation induction. In addition, these varying biological capabilities are also found in MSCs harvested from donors of different ages: cells of younger donors tend to proliferate and differentiate better than those of older donors (Alt et al. 2012). More importantly, there are unsolved challenges related to the control of lineage-specific differentiation in adult tissue-derived MSCs, especially with chondrogenesis. For example, in vitro differentiation of BM-derived MSCs toward chondrocytes often leads to production of collagen type 10 and alkaline phosphatase (ALP), the markers associated with hypertrophy. Hypertrophic chondrocytes can later undergo apoptosis towards endochondral bone formation (Coleman et al. 2013; Dickhut et al. 2009; Mueller and Tuan 2008; Pelttari et al. 2006). Researchers have therefore investigated the potential of other types of stem cells, such as ESCs and their derivatives, for musculoskeletal tissue engineering or regeneration (Brown et al. 2009; de Peppo et al. 2010; Hwang et al. 2008a; Karlsson et al. 2009; Oldershaw et al. 2010).

Several groups, including our collaborators, have reported successful derivation of MSCs from several ESC lines (ESC-MSCs) (Brown et al. 2009; de Peppo et al. 2010; Hwang et al. 2008b; Karlsson et al. 2009; Trivedi and Hematti 2008; Vodyanik et al. 2010). It is believed that these cells are capable of maintaining some of the unique properties of ESCs, such as their high proliferative capabilities, while gaining advantageous properties of MSCs, such as the inability to form teratomas. ESC-MSCs, like BM-MSCs, are capable of regulating proliferation of immune cells as demonstrated by Trivedi and others. In this study, they demonstrate that H1-, H7-, and H9-derived MSCs express HLA class I molecules but not HLA class II molecules. When induced with interferon-gamma (IFNγ), the cells express a low level of HLA class II but fail to elicit T-cell proliferation, demonstrating the ability to inhibit the proliferation of immune cells (Trivedi and Hematti 2008). Other studies have further shown that ESC-MSCs proliferate extensively in vitro while maintaining varying capacities of multipotent differentiation (de Peppo et al. 2010; Karlsson et al 2009).

In this study, we investigated biological properties of multiple ESC-MSC lines and BM-MSCs from different donors by directly comparing their phenotypic characteristics, proliferative ability, immunomodulatory property, and mulitpotency. Specifically, these ESC-MSC lines were derived from the same ESC line by different methods, or derived from different ESC lines using the same method to provide insight into whether properties of ESC-MSCs are dependent on derivation method and/or ESC line quality. Furthermore, we studied chondrogenic differentiation of the 3 ESC-MSC lines and established a viable induction approach to enhance the differentiation.

Materials and methods

Stem cell culture

Human BM, approved for use by the Institutional Review Board of the University of Wisconsin-Madison, was harvested from the femoral head of 3 patients between the ages of 24 and 58 who underwent hip arthroplasty. After further purification by a density gradient method using the Ficoll-Paque Plus medium (GE Healthcare, Little Chalfont, UK), mononuclear cells were cultured in low-glucose Dulbecco's Modified Eagle Medium (Life Technologies, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, USA) for further expansion. The medium was changed every 3 days. BM harvested from 3 donors was used in this study and each donor's cells were

cultured individually. Cells were maintained at 37 °C in a 5 % CO_2 and 95 % humidified incubator, and passaged using 0.05 % trypsin (Cellgro, Manassas, VA, USA) when reaching 70–80 % confluence.

Three ESC-MSC lines were derived from H1- and H9-ESCs (WiCell, Madison, WI, USA) as described previously (Trivedi and Hematti 2008; Vodyanik et al. 2010). Briefly, H1- and H9-ESCs were first cultured on OP9 feeder layer and then cultured in semisolid medium supplemented with fibroblast growth factor 2 (FGF2) and platelet-derived growth factor BB for 2 weeks. Selected mesodermal colonies were further cultured in serum-free medium with FGF2 to generate and expand MSCs. These cells are henceforth referred to as H1-MSCs and H9-MSCs. The third ESC-MSC line was derived from H1-ESCs (WA01, WiCell) using a different approach. Briefly, WA01 ESCs were originally maintained on mouse embryonic fibroblast (MEF) cells, and then passaged weekly on matrigel-coated plates and cultured with MEF conditioned medium and FGF2. The culture was continually passaged to generate MSCs with fibroblast-like morphology. These cells are henceforth referred to as WA1-MSCs. H1-MSCs and H9-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, USA) and 50 % Endothelial Serum Free Medium (Life Technologies), supplemented with 10 ng/ml FGF2 (Peprotech, Rocky Hill, NJ, USA), 100 μM monothioglutamate (Sigma-Aldrich), 0.05 % Ex-Cyte (StemCell Technologies, Vancouver, BC, Canada), 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). Culture medium was changed every 3 days. Cells were maintained at 37 °C in a 5 % CO_2 and 95 % humidified incubator, and passaged using StemPro Accutase (Life Technologies) when reaching 70–80 % confluence.

Flow cytometry analysis

To analyze cell surface markers and receptors, BM-MSCs and ESC-MSCs between passages 4 and 6 were harvested from expansion culture, washed in a buffer solution [1× PBS, 1 % bovine serum albumin (BSA), and 0.09 % sodium azide], and incubated with phycoerythrin-conjugated antibodies detecting cell surface markers, CD29, CD34, CD44, CD45, CD73, CD90, CD106 (BD Biosciences, San Jose, CA, USA) and CD105 (AbD Serotec, Kidlington, UK). The expression of growth factor receptors, TGFβ receptor 2 (TGFβR2), Activin receptors 2A and B (ACVR2A, ACVR2B), BMP receptors 1B and 2A (BMPR1B, BMPR2A), Frizzled (FZD) receptors 3, 4, 5, and 7, IGF1 receptor (IGF1R) (R&D Systems, Minneapolis, MN, USA), Patched receptor, and PTH/PTHrP receptor (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were also analyzed. For the analysis of cell surface markers during chondrogenesis, ESC-MSC pellets after 6 days of chondrogenic induction were collected and washed with ice-cold PBS and fixed with 2 % formaldehyde (Polysciences, Warrington, PA, USA) before being dissociated using collagenase A (Roche, Mannheim, Germany). Cells were then passed through a 40-μm nylon strainer (BD Biosciences), washed with a buffer solution, incubated with antibodies for the detection of ACVR2B, BMPR1B, and IGF1R and analyzed by the FACSCalibur flow cytometer (BD Biosciences).

Cell proliferation analysis

For the analysis of cell growth in long-term culture, BM-MSCs, H1-MSCs and H9-MSCs were maintained in expansion culture for 85 days. During this period, cells reaching 70–80 % of density confluence were continually passaged using our standard protocol, and cell numbers were determined by counting using a hemocytometer. Population doublings and doubling time between cell passages were calculated following the method previously described (Cristofalo et al. 1998; Wagner et al. 2008) and cumulative doublings were determined. For the analysis of short-term cell proliferation, H1-MSCs, H9-MSCs, WA1-MSCs, and BM-MSCs were plated in triplicate in 6-well plates at 10,000 cells/well, collected after 3, 6, and 9 days of culture, and quantified using the Quant-iT PicoGreen dsDNA assay (Life Technologies). Cellular senescence at cell passages 2, 7, and 13 was detected by the β-galactosidase staining assay. The staining was performed using the Senescence β-Galactosidase Staining kit (Cell Signaling Technologies, Danvers, MA, USA) at pH 6 following the manufacturer's instructions.

Immunoregulation assay

To determine the capability of immunoregulation of ESC-MSCs and BM-MSCs, human peripheral blood mononuclear cells (PBMCs) were co-cultured with each of the MSC lines at the ratio of 1:1 in separate compartments of a transwell plate (Corning, Corning, NY, USA). PBMCs, acquired from Dr. Peiman Hematti at the University of Wisconsin-Madison through collaboration, were originally from the Interstate Blood Bank (Madison, WI, USA). PBMCs were activated by 100 U/ml IL2 (Peprotech) (Bocelli-Tyndall et al. 2009) in culture for 6 days and harvested at days 3 and 6. Collected cells were digested using Proteinase K (Sigma), and the DNA content was analyzed using the PicoGreen dsDNA assay.

Evaluation of multi-lineage differentiation

For adipogenic differentiation, ESC-MSCs or BM-MSCs at the density of 2×10^5 cells/well were plated in gelatin (Sigma)

coated 6-well plates and cultured with SFM supplemented with 5 ng/ml BMP4 (Peprotech) and 0.4 % Ex-Cyte for 6 days. Adipogenesis was induced by adipogenic medium composed of Iscove's Modified Dulbecco's Medium (Life Technologies), 5 % horse serum (Life Technologies), 0.5 mM isobutylmethylxanthine (Sigma), 1 μM dexamethasone (Sigma), and 1 μg/ml insulin. After 24 days, lipid droplets were visualized using Oil red O (Sigma). The stain was subsequently extracted and measured at the absorbance wavelength of 490 nm.

For osteogenic differentiation, ESC-MSCs or BM-MSCs at the density of 5×10^4 cells/well were plated in fibronectin (Life Technologies)-coated 6-well plates and induced by osteogenic medium composed of low-glucose DMEM, 10 % FBS, 50 μg/ml ascorbic acid (Sigma), 10 mM β-glycerolphosphate (Sigma), 0.1 μM dexamethasone, and 0.01 μM vitamin D (Enzo Life Sciences, Farmingdale, NY, USA) for 24 days. To measure mineralization, osteogenic culture plates were washed with PBS, fixed with formalin, and stained with Alizarin red S (Rowley Biochemical Institutes, Danvers, MA, USA).

For induction of chondrogenic differentiation, ESC-MSCs or BM-MSCs were centrifuged in 96-well plates at 600*g* for 5 min to create high-density cell pellets with 2.5×10^5 cells per pellet. Cell pellets were then induced in serum-free chondrogenic medium supplemented with 10 ng/ml TGFβ1 (Peprotech) or the combination of 10 ng/ml TGFβ1 and 150 ng/ml BMP7 (Peprotech). The chondrogenic medium was composed of high-glucose DMEM (Life Technologies), 1 % ITS+Premix (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenious acid, 1.25 mg/ml bovine serum albumin, and 5.35 μg/ml linoleic acid) (BD Biosciences), 1 % Penicillin/Streptomycin antibiotics, 1 mM sodium pyruvate, 50 μg/ml ascorbic acid, 40 μg/ml L-proline, and 0.1 μM dexamethasone was replaced every 3 days throughout the culture period. To quantify the production of glycosaminoglycan (GAG), chondrogenic cell pellets were harvested at days 7, 14, and 21, digested with 20 μg/ml papain solution at 60°C for 18 h, and analyzed using the dimethylmethylene blue (DMMB) assay (Biocolor, Carrickfergus, UK) following the manufacturer's instructions. Briefly, 400 μl of papain-digested solution was mixed with 1000 μl of DMMB solution to form insoluble sGAGdye complex. One hundred microliters of extracted solution was measured at the absorbance wavelength of 656 nm after the sGAG-dye complex was dissociated with 500 μl of dissociation buffer. GAG content was normalized with DNA content that was determined by measuring the same papain-digested sample using the PicoGreen dsDNA assay.

Histology and immunofluorescence analysis

Chondrogenic cell pellets were collected at day 21, fixed with 10 % formalin, and embedded in paraffin for histology sectioning. Embedded specimens were sectioned at a thickness of 7 μm. For hematoxylin and eosin (H&E) and Alcian blue staining, slides were prepared following our laboratory standard protocol before being stained with Gill's Hematoxylin #3 and 0.5 % Eosin Y or Alcian blue 8GX (Polysciences, Warrington, PA, USA). 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, Billerica, MA, USA) diluted in PBS at 1:200 for 1 h. Samples were then washed with PBS and incubated with FITC-conjugated secondary antibody (eBioscience, San Diego, CA, USA) diluted 1:100 in PBS for 1 h. Coverslips were affixed to slides using Prolong antifade reagent with DAPI (Life Technologies) prior to imaging.

Analysis of mRNA expression by real-time PCR

To analyze the expression of mRNA transcripts of lineage-specific markers, cells or cell pellets were harvested, washed with PBS, and lysed to isolate total RNA using the NucleoSpin RNA II kit (Macherey-Nagel, Germany) following the manufacturer's protocol. Synthesis of cDNA was carried out using the High Capacity cDNA Reverse Transcriptase kit (Life Technologies). For real-time PCR analysis, iQ SYBR Green Supermix (BioRad, Hercules, CA, USA) was used with cDNA samples and primers to detect target mRNA transcripts. The real-time PCR reaction was set for 40 cycles to amplify DNA products. The sequences of the primers 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 *ubiquitin C* (*UBC*) using the $2^{-\Delta CT}$ method.

Statistical analysis

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

Results

ESC-MSCs and BM-MSCs express different morphologies and surface receptors

All ESC-MSC and BM-MSC lines exhibited the characteristic, spindle-shaped morphology of MSCs while BM-MSCs were markedly larger and more elongated than H1-MSC and H9-MSCs (Fig. 1a–d). To further analyze the phenotype of the

Table 1 List of primer sequences for quantitative PCR

Gene	Accession no.	Sequence (5′ to 3′)
Osteocalcin	NM_199173.3	(F) GACTGTGACGAGTTGGCTGA
		(R) CGTGGGAAGAAAGGAGAAGG
Alkaline phosphatase	NM_00478.3	(F) CAAAGGCTTCTTCTTGCTGG
		(R) GGAGCCTTCTGTGAGACTGG
CBFA1/RUNX2	NM_004348.3	(F) GGTTCCAGCAGGTAGCTGAG
		(R) CCGACACCTCAAACCACAGA
Collagen type 1	NM_000088.3	(F) GGCTCCTGCTCCTCTTAGCG
		(R) CTTGCCGGAGTCCATGGTAC
Collagen type 2	NM_001844.4	(F) GGAAACTTTGCTGCCCAGATG
		(R) CGTTAGGACCACTTGGACCACT
Collagen type 9	NM_001851.4	(F) ATATCCCAGTGGACTGCCTG
		(R) CCTTCAGAGGTTTGTCGTCG
Collagen type 10	NM_000493.3	(F) CCCAGGAAAACCAGGTCTCG
		(R) GCCTGTGTTACCTCTCCGAC
Aggrecan	NM_013227.2	(F)CACGATGCCTTTCACCACGAC
		(R) CTATCCGTGACAACTGGGCGT
SOX9	NM_000346.3	(F) TAAAGGCAACTCGTACCCAA
		(R) GCACCTCCTACTACCTCTTA
UBC	NM_021009.4	(F) TGAAGACACTCACTGGCAAGACCA
		(R) AACTAGAAACGGCCTTTCGTCGAC

cells, cell surface markers CD29, CD34, CD44, CD45, CD73, CD90, CD105, and CD106 were analyzed using flow cytometry. H1-MSCs, WA1-MSCs and BM-MSCs were positive for CD29, CD 44, CD73, CD90, and CD105 but negative for the hematopoietic markers CD34 and CD45 (Fig. 1e). Interestingly, CD106 was expressed on BM-MSCs but not on H1-MSCs and showed low expression on WA1-MSCs. On the other hand, H9-MSCs expressed a different cell surface marker profile with no or low expression of all the cell surface markers. The growth factor receptors likely involved in the regulation of MSC differentiation were also analyzed by flow cytometry. WA1-MSCs showed a profile of receptor expression similar to what was expressed by BM-MSC1 and 2 while H1-MSCs and BM-MSC3 exhibited similar profiles except for the expression of ACVR2B, BMPR1B and IGF1R. Overall, H1-MSCs and WA1-MSCs were more similar to BM-MSCs than H9-MSCs (Fig. 1f). When comparing the 3 BM-MSC lines, BM-MSC1 and 2 shared a similar profile of receptor expression, distinct from that of BM-MSC3. Specifically, expression levels of the growth factor receptors associated with TGFβ family signaling and WNT signaling were found to be different between BM-MSC1 or 2 and BM-MSC3.

ESC-MSCs proliferate more rapidly and undergo more population doublings than BM-MSCs

We then evaluated the growth of H1-MSCs, H9-MSCs, and BM-MSCs in long-term culture by measuring cell number to calculate population doublings. H1-MSCs and H9-MSCs were able to undergo 34 population doublings during the 85-day culture while BM-MSCs could only cumulate total numbers of population doublings between 11 and 18 (Fig. 2a). The replicative senescence of H1-MSCs, H9-MSCs, and BM-MSCs during long-term culture was analyzed using the staining of β-galactosidase activity at the early- (passage 2) (Fig. 2b–f), mid (passage 7) (Fig. 2g–k), and late- (passage 13) (Fig. 2l–p) stages of the culture period. At passage 2, minimal staining was seen within any of these cell lines whereas more cells began to show β-galactosidase staining and morphology changes at passage 7. With the increase of cumulated cell passage, all of the cell lines showed a more spread morphology deviating away from their phenotypic spindle shape and increased β-galactosidase staining. At passage 13, the majority of H1-MSCs, H9-MSCs, and BM-MSCs in culture showed positive staining of β-galactosidase activity. At this stage, when cultured for additional time, none of the cell lines were able to reach confluence.

We corroborated the proliferation finding by evaluating short-term proliferation of ESC-MSCs and BM-MSCs. The results showed that both ESC-MSCs and BM-MSCs proliferated during the 9-day culture but ESC-MSCs increased more rapidly than BM-MSCs, resulting in significantly more H1-MSCs, H9-MSCs, and WA1-MSCs than BM-MSCs at day 9 (Fig. 2q). Specifically, the H1-MSC culture showed the greatest cell numbers followed by the

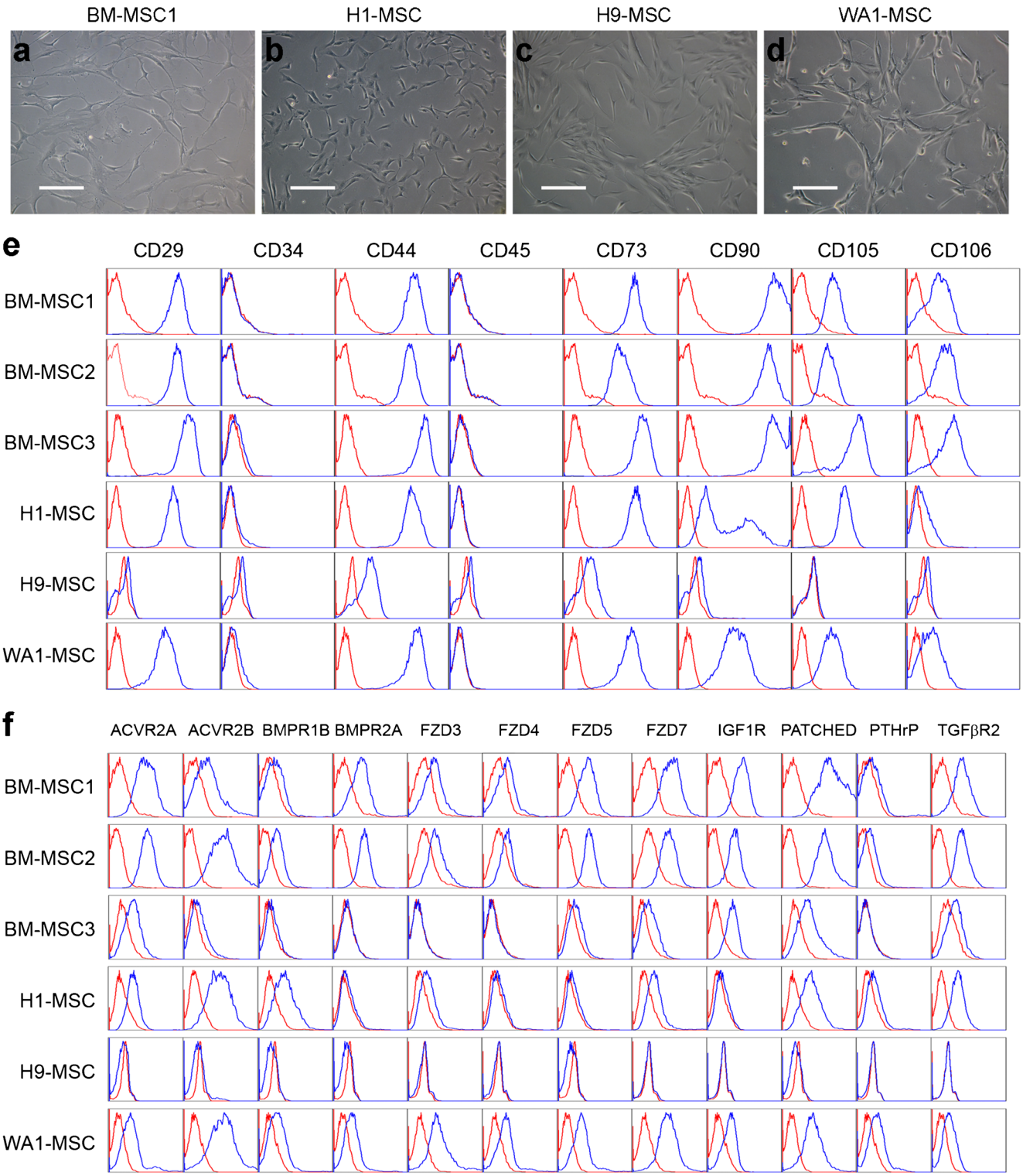


Fig. 1 Characterization of morphology and immunophenotype of human ESC-MSCs and BM-MSCs in expansion culture. Morphology of MSCs was analyzed using bright field microscopy (**a**–**d**). **e** Flow cytometry of ESC-MSCs and BM-MSCs was performed with PE-conjugated antibodies to detect cell surface markers. The expression of isotype controls are shown as *red histograms*. **f** The expression of growth factor receptors of ESC-MSCs and BM-MSCs was analyzed by flow cytometry using PE-conjugated antibodies. *Scale bars* 400 μm

H9-MSC, WA1-MSC and then BM-MSC culture. The results of both long- and short-term cell cultures concordantly suggest that ESC-MSCs have greater proliferation capability than BM-MSCs.

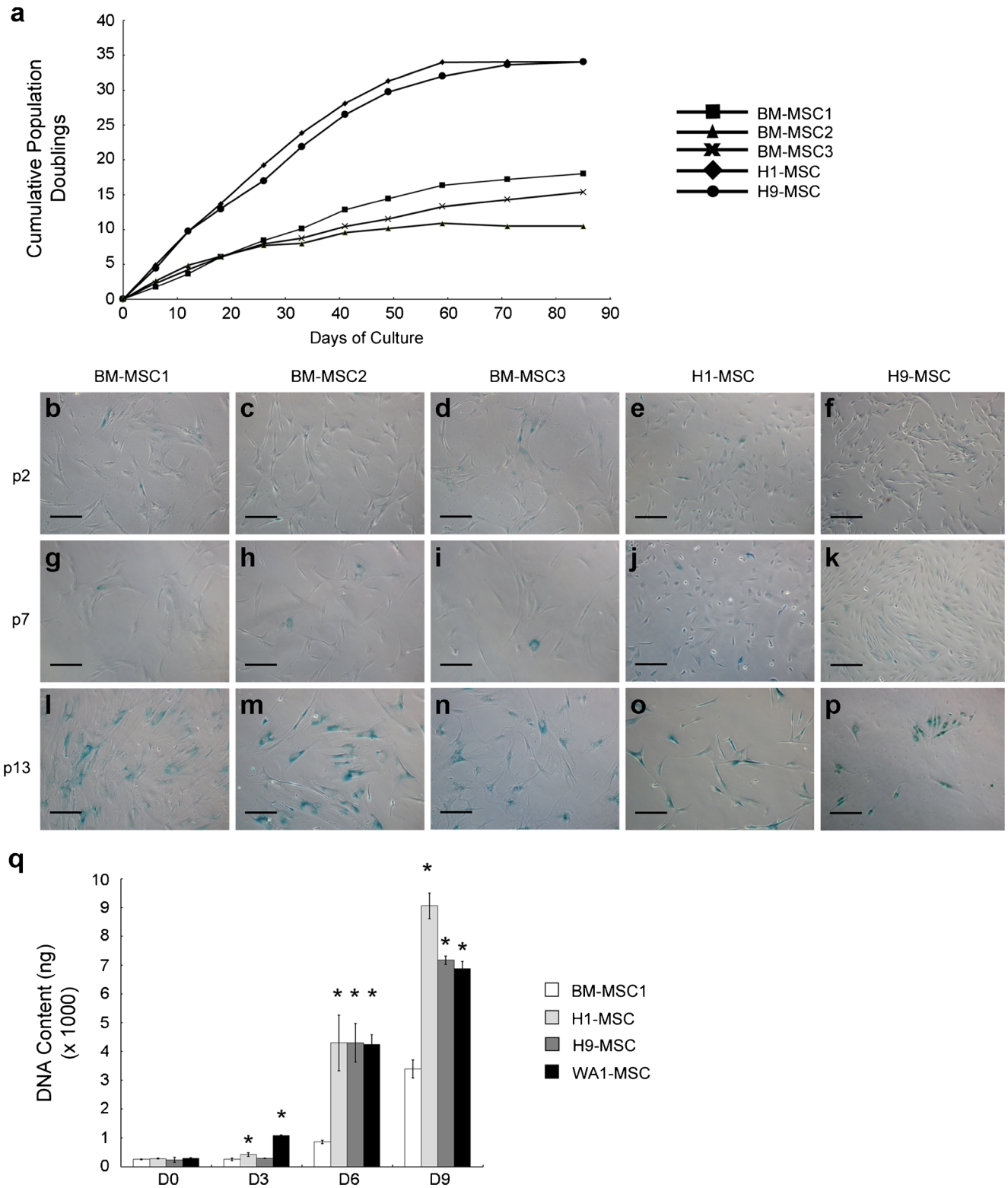


Fig. 2 Analysis of growth and senescence of ESC-MSCs and BM-MSCs in expansion culture. **a** Long-term cell growth of H1-MSC, H9-MSC and BM-MSC cultures was indicated by cumulative population doublings calculated based on cell numbers measured during the culture period. MSC lines were derived from H1 (*diamond*), H9 (*filled circle*) and BM (*square*, *triangle*, and *cross*). Analysis of cellular senescence in long-term culture was performed on H1-MSCs, H9-MSCs and BM-MSCs at passages 2 (**b**–**f**), 7 (**g**–**k**), and 13 (**l**–**p**) by staining β-galactosidase activity of the cells. **q** Short-term proliferation of H1-MSCs, H9-MSCs, WA1-MSCs and BM-MSC1 during a 9-day culture period was analyzed. Samples harvested on days 3, 6, and 9 were analyzed using PicoGreen. *$p<0.05$; $n=3$. *Scale bars* 400 μm

ESC-MSCs show greater immunoregulatory capability compared to BM-MSCs

We analyzed the proliferation of PBMCs co-cultured with ESC-MSCs or BM-MSCs to determine the immunoregulatory capability of different MSC lines (Fig. 3). With IL2 stimulation, PBMCs co-cultured with BM-MSCs and ESC-MSCs decreased during the 6-day culture. When comparing the effect of BM-MSCs and ESC-MSCs on PBMC proliferation, H1-MSCs and WA1-MSCs were able to more effectively suppress PBMC proliferation compared to BM-MSCs, suggesting that ESC-MSCs have greater capability to regulate immune cell activities than BM-MSCs.

Osteogenesis and adipogenesis are upregulated in BM-MSCs compared to ESC-MSCs

After 24 days of osteogenic induction, H1-MSC, WA1-MSC, and all BM-MSC cultures stained positive with Alizarin red S (Fig. 4a–f), suggesting that these cells are capable of differentiating into the osteogenic lineage to produce mineralized nodules. However, mineralization was not detected in H9-MSC culture. Interestingly, the mRNA expression levels of bone-related markers, *osteocalcin, ALP*, and *CBFA1/RUNX2*, of BM-MSCs were significantly higher than those of H1-MSCs and WA1-MSCs (Fig. 4g–i). The analysis of BM-MSC and ESC-MSC adipogenesis after 24 days of induction showed more lipid droplets detected by Oil red O staining in all of the BM-MSC cultures (Fig. 4j–l) compared to those in ESC-MSC cultures (Fig. 4m–-o). Of the ESC-MSCs, WA1-MSCs produced more lipid droplets than H1-MSCs or H9-MSCs (Fig. 4p). Taken together, the results of osteogenesis and adipogenesis suggest that when induced by the current differentiation protocols, ESC-MSC induction is less effective into osteoblast-like or adipocyte-like cells than BM-MSC.

BM-MSCs demonstrate greater chondrogenic potential than ESC-MSCs

We further investigated the chondrogenic capability of ESC-MSCs and BM-MSCs by analyzing mRNA expression of cartilage-related markers during chondrogenesis. Quantitative PCR showed that chondrogenic differentiation of BM-MSCs induced by the current differentiation protocol using TGFβ1 for 21 days greatly upregulated the expression levels of *collagen types 1, 2,* and *10, aggrecan*, and *SOX9* compared to those of ESC-MSCs (Fig. 5a, c–f). Specifically, the expression level of *collagen type 2* in BM-MSC pellets was significantly higher than that of ESC-MSC pellets during the 21-day culture (Fig. 5a). Other markers, *aggrecan* and *SOX9*, were also shown to be significantly upregulated in BM-MSC pellets compared to ESC-MSCs (Fig 5c, d). Of the ESC-MSCs, *collagen type 2* was expressed highest in WA1-MSCs. Interestingly, the expression level of *collagen type 9* was significantly higher in H9-MSCs compared to *collagen type 9* levels in the other cell lines (Fig. 5b). H1-MSCs also expressed more *aggrecan* compared to H9-MSCs and WA1-MSCs after 21 days. The expression of *collagen type 10*, a marker of hypertrophic chondrocytes, was increased in BM-MSC lines whereas it remained similar in ESC-MSC lines, suggesting that compared to ESC-MSCs, BM-MSCs induced for chondrogenesis tend to become hypertrophic chondrocytes (Fig. 5f). Lastly, the expression of *collagen type 1* was increased in both ESC-MSCs and BM-MSCs after 21-day chondrogenic induction with WA-MSC showing the greatest transient expression at day 7 (Fig. 5e). The results of mRNA expression of cartilage-related markers indicate that ESC-MSCs may be less inducible than BM-MSCs for chondrogenic differentiation using our current induction protocol.

Further analysis by histological staining demonstrated that the sizes of ESC-MSC and BM-MSC pellets were similar during the initial phase of chondrogenesis (data not shown) but BM-MSC pellets grew larger than ESC-MSC pellets after 21 days of culture (Fig. 5). Microscopically, H&E staining showed that BM-MSC pellets formed a dense structure

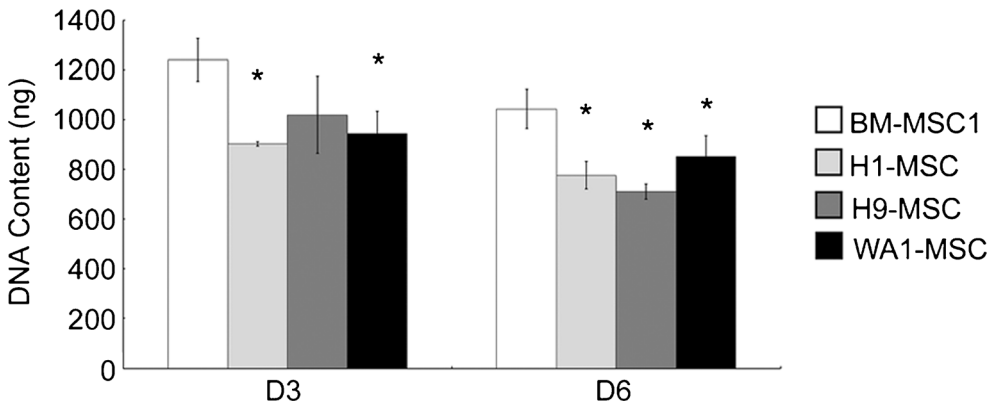


Fig. 3 Evaluation of immunomodulation of ESC-MSCs and BM-MSCs on PBMCs. PBMCs were co-cultured with BM-MSCs or ESC-MSCs in different compartments of a transwell plate and stimulated with IL2. PBMCs were collected on days 3 and 6 and quantified by PicoGreen. *$p<0.05$; $n=3$

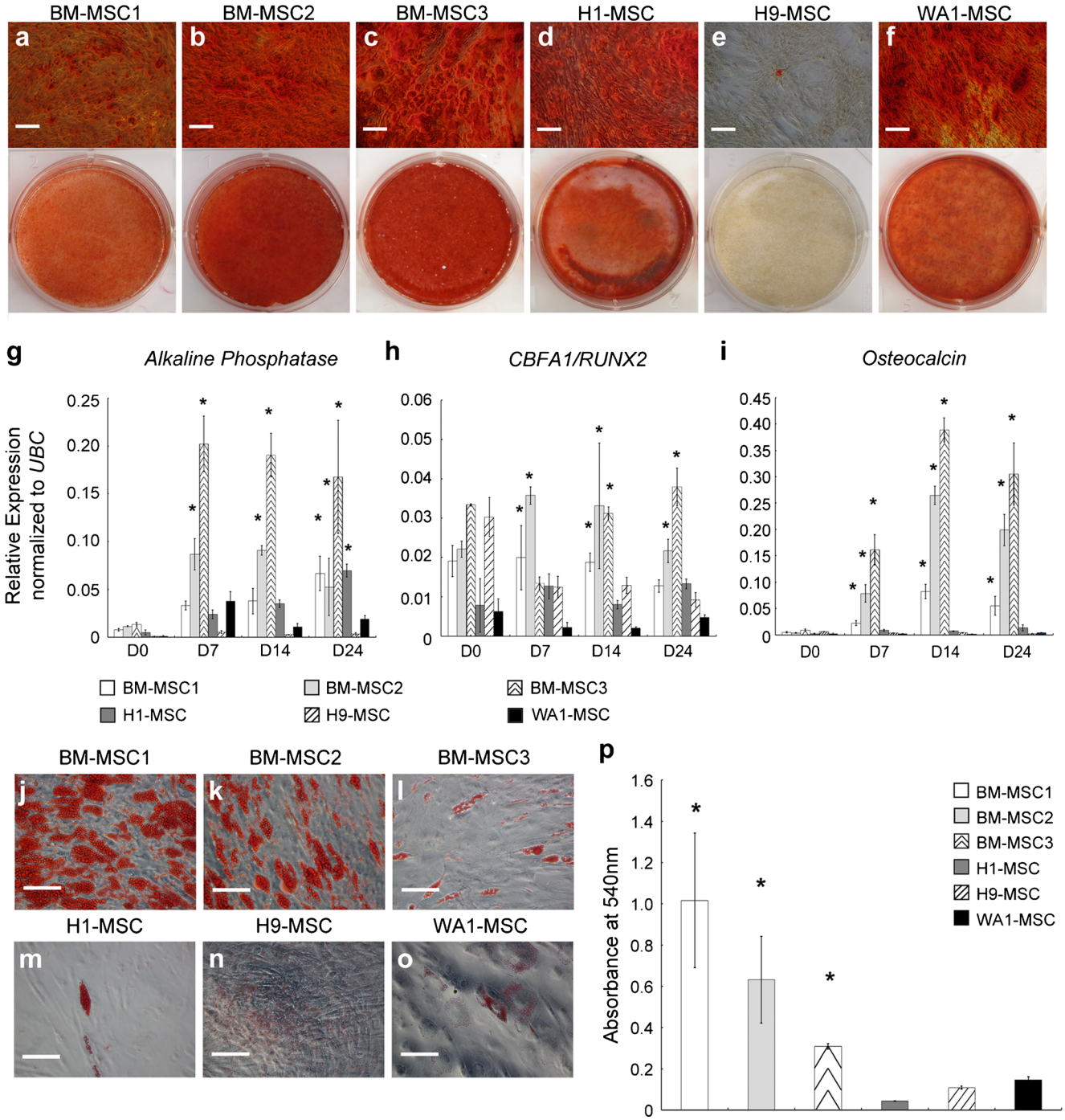
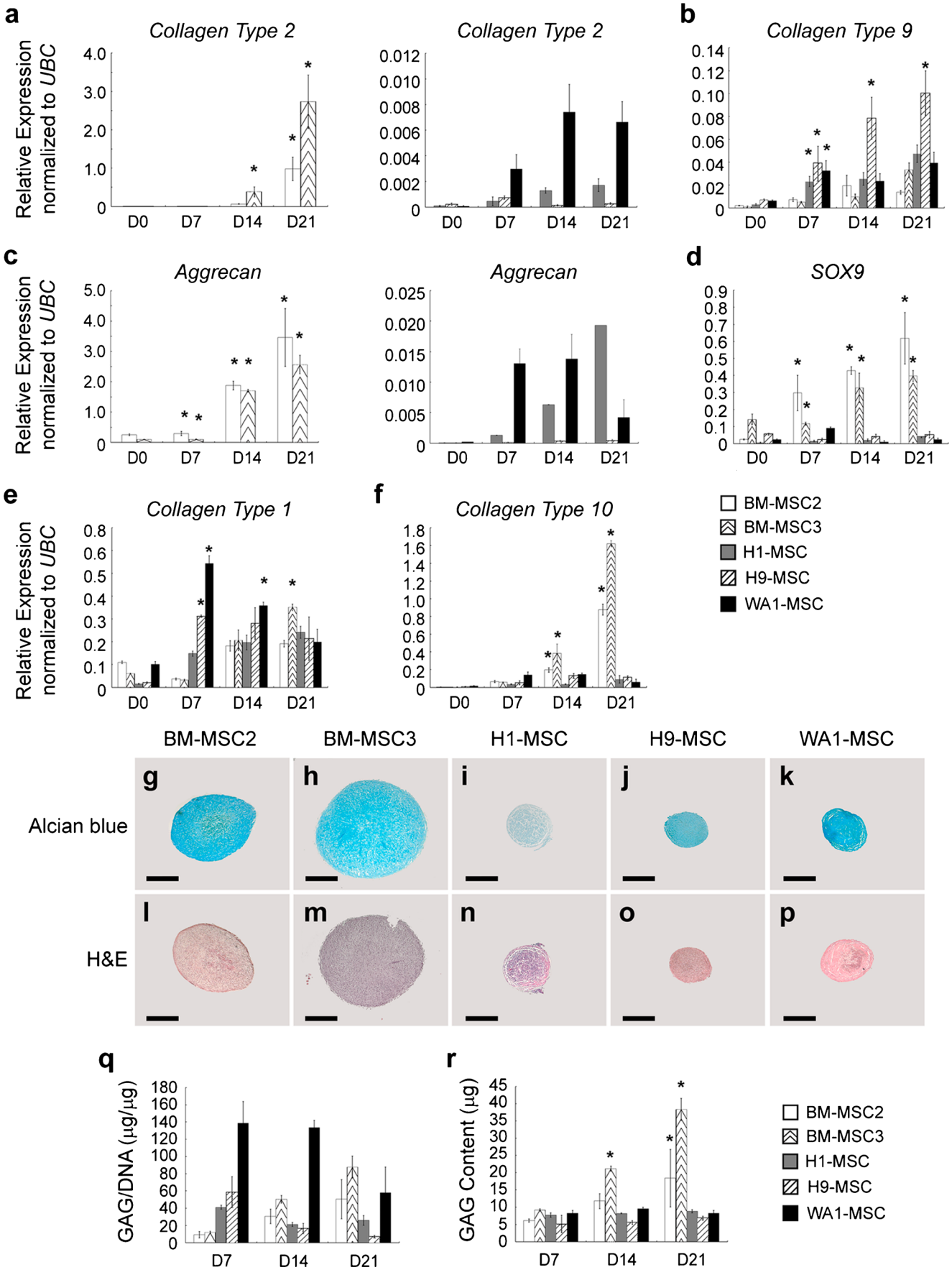


Fig. 4 Evaluation of osteogenesis and adipogenesis of ESC-MSCs and BM-MSCs. Matrix mineralization during osteogenesis of BM-MSCs and ESC-MSCs was detected by Alizarin red S after 24 days of culture (**a**–**f**). The mRNA expression levels of bone-related markers *alkaline phosphatase* (**g**), *CBFA1/RUNX2* (**h**) and *osteocalcin* (**i**) of ESC-MSCs and BM-MSCs during osteogenic differentiation were determined by qPCR. *$p<0.05$; $n=3$. Lipid droplets produced in ESC-MSC and BM-MSC cultures after 24 days of adipogenesis were stained by Oil red O (**j**–**o**). **p** Resulting stain was extracted and measured at the absorbance wavelength of 480 nm. *Scale bars* 400 μm

composed of chondrocyte-like cells and evenly distributed extracellular matrix (ECM) (Fig. 5l, m). In contrast, H1-MSC pellets developed into a loose structure in which cells and ECM were localized unevenly in the pellet (Fig. 5i, n) while cells and ECM in H9-MSC pellets appeared to be evenly distributed throughout the pellet (Fig. 5j, o). WA1-MSC pellets appeared to have a dense cluster of cells in the center surrounded by ECM (Fig. 5k, p). The intensity of Alcian blue staining in BM-MSC pellets was similar to that in WA1-MSC pellets and stronger than that in H9-MSC and H1-MSC pellets. Quantitatively, the amount of total sGAG produced by BM-MSCs was significantly greater than that


a
Collagen Type 2
Relative Expression normalized to UBC
4.0
3.0
2.0
1.0
0
D0 D7 D14 D21
Collagen Type 2
0.012
0.010
0.008
0.006
0.004
0.002
0
D0 D7 D14 D21
b
Collagen Type 9
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
D0 D7 D14 D21
c
Aggrecan
Relative Expression normalized to UBC
5.0
4.0
3.0
2.0
1.0
0
D0 D7 D14 D21
Aggrecan
0.025
0.020
0.015
0.010
0.005
0
D0 D7 D14 D21
d
SOX9
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
D0 D7 D14 D21
e
Collagen Type 1
Relative Expression normalized to UBC
0.8
0.6
0.5
0.4
0.3
0.2
0.1
0
D0 D7 D14 D21
f
Collagen Type 10
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
D0 D7 D14 D21
BM-MSC2
BM-MSC3
H1-MSC
H9-MSC
WA1-MSC
BM-MSC2
BM-MSC3
H1-MSC
H9-MSC
WA1-MSC
Alcian blue
H&E
g
h
i
j
k
l
m
n
o
p
q
GAG/DNA (μg/μg)
180
160
140
120
100
80
60
40
20
0
D7 D14 D21
r
GAG Content (μg)
45
40
35
30
25
20
15
10
5
0
D7 D14 D21
BM-MSC2
BM-MSC3
H1-MSC
H9-MSC
WA1-MSC

◀ **Fig. 5** Evaluation of chondrogenesis of ESC-MSCs and BM-MSCs. The mRNA expression of cartilage-related markers, *collagen types 2* (**a**), and *9* (**b**), *aggrecan* (**c**), *SOX9* (**d**), and *collagen types 1* (**e**) and *10* (**f**) of ESC-MSC and BM-MSC pellets was analyzed by qPCR during 21 days of chondrogenic differentiation. *$p<0.05$; $n=3$. Histological sections of ESC-MSC and BM-MSC pellets induced for chondrogenesis for 21 days were stained with Alcian blue (**g**–**k**) and H&E (**l**–**p**). The production of sGAG in ESC-MSC and BM-MSC pellets during chondrogenesis determined by DMMB was normalized by the amount of DNA quantified by PicoGreen (**q**, **r**). *$p<0.05$; $n=3$. *Scale bars* 400 μm

produced by ESC-MSCs (Fig. 5q, r). Consistent with the results of Alcian blue staining, the amount of sGAG production per cell at day 21 in BM-MSCs was greater than that in ESC-MSC pellets.

Induction with TGFβ1 and BMP7 enhances chondrogenic differentiation of ESC-MSCs

The results of multi-lineage differentiation demonstrated that ESC-MSCs did not respond to our induction medium as robustly as BM-MSCs. We suspected that the composition of induction medium might favorably support BM-MSC differentiation and not ESC-MSC differentiation. Therefore, we aimed to enhance chondrogenesis of ESC-MSCs with modified induction medium. To determine which growth factors added to TGFβ1-supplemented induction medium are able to increase chondrogenesis, expression of growth factor receptors in chondrogenic pellets was analyzed to determine the choice of growth factor ligands. Our results showed that in reference to the IgG control, ACVR2B was highly expressed in H1-MSC, H9-MSC, and WA1-MSC pellets during the first 6 days of chondrogenesis (Fig. 6a). The expression of BMPR1B in H1-MSCs was increased at days 1 and 3 before returning to the near baseline level at day 6. H9-MSCs barely expressed BMPR1B while WA1-MSCs showed low expression of the receptor during the 6-day culture. IGF1R was not expressed in pellets of any of the three ESC-MSC lines during 6 days of chondrogenesis.

Based on the results of growth factor expression and previous published findings (Mrugala et al. 2009; Nakagawa et al. 2009), we then decided to investigate the effect of BMP7 on TGFβ1-induced chondrogenesis of ESC-MSCs. Quantitative PCR analysis showed that expression of *collagen type 2* and *aggrecan* in ESC-MSCs treated with TGFβ1 and BMP7 was significantly upregulated compared to that in the cells treated with TGFβ1 alone (Fig. 6b, c). When comparing different ESC-MSC lines, H1-MSC and WA1-MSC pellets were more responsive to chondrogenic induction with the growth factor combination than H9-MSC pellets. We further compared chondrogenesis of H1-MSCs to that of gold-standard BM-MSCs induced by TGFβ1 with or without BMP7, and found that when induced with TGFβ1 and BMP7, H1-MSC pellets expressed a comparable level of *collagen type 2* and significantly higher levels of *aggrecan* and *SOX9* than BM-MSC pellets at day 21 (Fig. 6e–g).

Histologically, ESC-MSC pellets were smaller than BM-MSC pellets after 21 days of chondrogenesis induced by TGFβ1 with or without BMP7 while BM-MSC, H1-MSC, and WA1-MSC pellets treated with TGFβ1 and BMP7 were larger than those treated with TGFβ1 alone (Fig. 6h). In H1-MSC pellets induced by TGFβ1 and BMP7, a dense cell and ECM mass was formed in the core of the pellets, creating an inhomogeneous structure, suggesting that H1-MSCs at the peripheral and core regions may undergo different extents of differentiation during chondrogenesis (column 4). Similar structural inhomogeneity was also found in WA1-MSC pellets (columns 7 and 8). H9-MSC pellets (columns 5 and 6) were structurally homogeneous but smaller than the other 2 ESC-MSC pellets. Immunofluorescence staining revealed that under TGFβ1 induction, collagen type 2 was detected throughout BM-MSC pellets (column 1, row 3) but barely in H1-MSC pellets (column 3, row 3). However, when induced by TGFβ1 and BMP7, both BM-MSC (column 2, row 3) and H1-MSC (column 4, row 3) pellets increased production of collagen type 2 in the peripheral region of pellets but with a greater amount in H1-MSC than in BM-MSC pellets. The similar effects of TGFβ1 and BMP7 was not found in H9-MSC (column 6, row 3) and WA1-MSC pellets (column 8, row 3). Our findings indicate that although forming inhomogeneous cell pellets, H1-MSCs induced by TGFβ1 and BMP7 are capable of differentiating into hyaline chondrocyte-like cells and producing more collagen type 2 than BM-MSCs induced by the same growth factors.

Discussion

In this study, we have investigated and compared biological properties of MSCs derived from multiple BM and ESC lines, and further explored a strategy of using TGFβ1 and BMP7 to stimulate ESC-MSC chondrogenesis. Our results show that differences in expression of cell surface markers, morphology, and capabilities of cell proliferation and multi-lineage differentiation are found between BM-MSCs and ESC-MSCs and between ESC-MSC lines. ESC-MSCs are more proliferative and more capable of immunomodulation but less inducible for multi-lineage differentiation by current protocols than BM-MSCs. H1-MSCs and WA1-MSCs are more inducible for multi-lineage differentiation than H9-MSCs. We furthermore demonstrate that our modified induction medium composed of TGFβ1 and BMP7 is able to greatly improve chondrogenic differentiation of H1-MSCs compared to the gold standard TGFβ1. Our findings suggest that compared to the H9-MSC and WA1-MSC

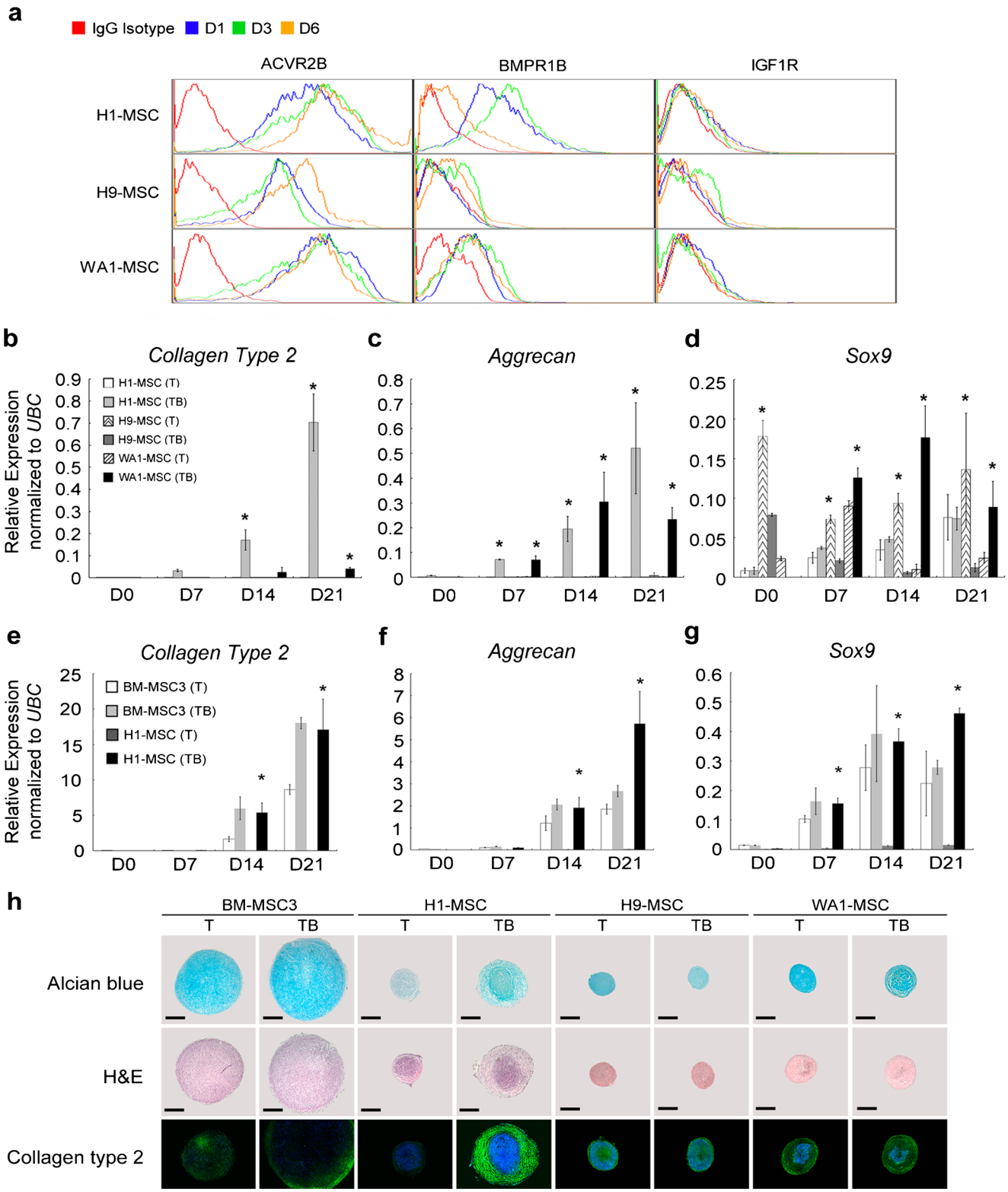


Fig. 6 Characterization of growth factor receptors and evaluation of chondrogenesis of ESC-MSC pellets. **a** Changes in the expression levels of ACVR2B, BMPR1B, and IGF1R during the first 6 days of ESC-MSC chondrogenesis were analyzed by flow cytometry. The mRNA expression of cartilage-related markers of ESC-MSC pellets induced by TGFβ1 and BMP7 (TB) for 21 days was compared to those of ESC-MSCs treated with TGFβ1 (T) (**b**–**d**). *$p<0.05$; $n=3$. H1-MSCs induced by TB for 21 days was compared to H1-MSCs induced by T, BM-MSCs treated with T, and BM-MSCs treated with TB (**e**–**g**). *$p<0.05$; $n=3$. **h** Histological sections of ESC-MSC and BM-MSC chondrogenic pellets induced by T or TB for 21 days were analyzed by Alcian blue (*top row*) and H&E (*middle row*) staining, and immunofluorescent staining detecting collagen type 2 (*bottom row*). Collagen type 2 was labeled *green*, and nuclei were stained *blue* with DAPI. *Scale bars* 400 μm

lines and BM-MSCs, the H1-MSC line may be a viable MSC source for induction of chondrogenic lineage differentiation.

Our results show that while sharing several phenotypic characteristics with BM-MSCs, ESC-MSCs are different from BM-MSCs in their expression of surface markers. H9-MSCs used in this study express low levels of CD44, CD73 and CD90, indicating that they are not phenotypic MSCs. In contrast, H1-MSCs, WA1-MSCs, and BM-MSCs express MSC surface markers yet H1-MSCs and WA1-MSCs express a lower level of CD106 compared to BM-MSCs. Similar findings demonstrating that ESC-MSCs express low levels of MSC markers, CD105 and CD106, have been reported (Hwang et al. 2008a, b; Kopher et al. 2010). Additionally, CD106 has been shown to be associated with the multi-lineage differentiation potential of adult tissue-derived MSCs, as the expression level of CD106 decreases with progression of differentiation (Liu et al. 2008). The low expression of CD106 on H1-MSCs and WA1-MSCs may partially explain why the cells are less potent for multi-lineage differentiation compared to BM-MSCs. We also found that H1-MSCs and WA1-MSCs express a lower level of CD90 than BM-MSCs. This has also been demonstrated in studies using different ESC-MSC lines by other groups (Hwang et al. 2008b; Li et al. 2013; Varga et al. 2011; Yen, et al. 2011), and these cell lines demonstrate varying potential of differentiation compared to BM-MSCs. In this study, lower expression of CD90 and CD106 in ESC-MSCs may be correlated to less potent multi-lineage differentiation.

We demonstrate that ESC-MSCs are capable of extensive proliferation with results showing that H1-MSCs and H9-MSCs accumulate over twice as many population doublings than BM-MSCs before reaching their growth plateau. Previous studies have similarly demonstrated that ESC-MSCs are more proliferative compared to BM-MSCs or other adult tissue-derived MSCs (de Peppo et al. 2010; Trivedi and Hematti 2008; Yen et al. 2011). The greater proliferative capacity of ESC-MSCs compared to adult tissue-derived MSCs is attributed to the activity of longer telomeres in ESC-MSCs (de Peppo et al. 2010; Kopher et al. 2010). Additionally, Yen et al. (2011) have shown that the genes related to control of DNA replication and repair are greatly upregulated in ESC-MSCs compared to those in BM-MSCs, suggesting that other mechanisms may be involved in the regulation of the extensive proliferation of ESC-MSCs. Though not investigated in our current study, these previously reported findings might possibly explain our results of potent proliferation of ESC-MSCs before reaching their growth plateau.

The results of immunomodulation between ESC-MSCs and BM-MSCs demonstrate that proliferation of PBMCs is suppressed more by ESC-MSCs than by BM-MSCs. This suggests that allogeneic ESC-MSCs may be advantageous in the regulation of host immune response compared to BM-MSCs in vivo. Similar findings are reported in previous studies using separate culture or direct-contact culture for ESC-MSCs and lymphocytes (Trivedi and Hematti 2008; Varga et al. 2011). In addition, it has also been shown that ESC-MSCs are able to effectively modulate the activity of other immune cells, such as natural killer (NK) cells. Studies have shown that ESC-MSCs co-cultured with activated NK cells are more resistant to cell lysis than BM-MSCs (Sotiropoulou et al. 2006; Spaggiari et al. 2006; Yen et al. 2009). Together, these studies demonstrate that ESC-MSCs may have greater capability for immunoregulation than BM-MSCs.

Our results show that BM-MSCs are more inducible for multi-lineage differentiation than ESC-MSCs. Interestingly, osteogenic differentiation of H1-MSCs and WA1-MSCs results in mineralization that is comparable to BM-MSC cultures, despite lower mRNA expression of osteogenic markers in these 2 ESC-MSC lines than in BM-MSCs. Other groups have demonstrated the similar finding that ESC-MSCs and BM-MSCs possess comparable osteogenic potential (de Peppo et al. 2013; Karlsson et al. 2009; Marolt et al. 2012). However, these previous studies have also reported that the chondrogenic potential of ESC-MSCs is often less than that of BM-MSCs. In this current study, we demonstrate that induced by chondrogenic medium supplemented with BMP7 and TGFβ1, H1-MSCs are able to vigorously undergo chondrogenesis and produce cartilage ECM. Our results suggest that with optimized induction protocols, multi-lineage differentiation of ESC-MSCs can be greatly improved.

Interestingly, we observed that with the induction of TGFβ1 and BMP7, H1-MSCs in a cell pellet underwent different extents of chondrogenic differentiation. This resulted in cells and matrix that were unevenly distributed in pellets: the core of the pellet was mainly composed of cells whereas collagen type 2 was abundantly localized to the peripheral region. A possible explanation for this occurrence is that the extent of chondrogenesis at different regions of a pellet may be dependent on the amount of endogenous growth factors locally exposed to the cells. Chondrogenic induction is not only regulated by administered growth factors but also by endogenously produced ones. As exogenous growth factors have a half-life of a few minutes (Kaminska et al. 2005), cell activities may be largely affected by continuously released endogenous growth factors. In this study, the unresponsiveness of H1-MSCs to chondrogenic induction in the core of a pellet may be in part due to a low amount of released endogenous growth factors. That being said, further studies are required to test this hypothesis.

The TGF superfamily, including TGFβs, activins and BMPs, has been shown to play a pivotal role in the differentiation of mesodermal cells into skeletal tissue cells (Montero et al. 2008; Oshimori and Fuchs 2012; Wan and Cao 2005). During limb development, TGFβs are critical for initiating and maintaining the process of cartilage maturation (Ferguson

et al. 2004; Schmierer and Hill 2007). Similarly, several BMPs, including BMP2, 4, 6, 7, and 9 have been shown to promote chondrogenesis and contribute to limb morphogenesis (Schmitt et al. 2003; Shen et al. 2010; Yoon and Lyons 2004). Moreover, previous studies have shown that combinations of BMPs and TGFβs are able to upregulate expression of cartilage-related markers in ESCs and adult tissue-derived MSCs (Nakagawa et al. 2009; Nakayama et al. 2003; Oldershaw et al. 2010; Rui et al. 2010; Shen et al. 2010), consistent with our results reported in this study. A study has also shown that knockdown of BMP signaling can inhibit TGFβ-induced chondrogenesis of ESC-MSCs (Nakayama et al. 2003). Altogether, these findings suggest that TGFβs and BMPs may synergistically regulate chondrogenesis and chondrocyte activities. Our results demonstrate that, during early chondrogenesis, the expression of ACVR2B and BMPR1B is increased in H1-MSCs and WA1-MSCs. As BMP7 binds to BMP receptors as well as activin receptors to activate BMP and activin signaling through SMAD1/5/8 and SMAD2/3, respectively (Xu et al. 2006), it is possible that chondrogenesis can be enhanced through synergistic activation of SMAD2/3 by BMP7 and TGFβ1.

While previous studies have reported the multipotency of ESC-MSCs (Boyd et al. 2009; Trivedi and Hematti 2008), there exist differences in the extent of multi-lineage differentiation capability between MSCs derived from different ESC lines (Marolt et al. 2012; Varga et al. 2011). In this study, we demonstrate that H1-MSCs and H9-MSCs undergo different extents of adipogenesis, chondrogenesis, and osteogenesis. A previous study has shown that MSCs derived from H13-ESCs are less inducible for chondrogenesis compared to MSCs derived from H9-ESCs while both ESC-MSC lines are capable of undergoing similar extents of osteogenesis and adipogenesis (Marolt et al. 2012). Varga et al. (2011) have also reported that only one of the ESC-MSC lines tested in their study is able to differentiate into all three mesenchymal lineages, suggesting inherent differences existing between different ESC lines. These previous and our current findings suggest that differentiation of a particular cell lineage may be dependent on the property of an ESC-MSC line. Moreover, our results show that H1-MSCs and WA1-MSCs derived from the H1-ESC line undergo different extents of multi-lineage differentiation, suggesting that the properties of ESC-MSCs may be affected by the method used to derive MSCs from ESCs. This is because MSCs derived by different methods may represent cells at different stages of the mesodermal lineage and possess distinct differentiation capabilities (de Peppo et al. 2010; Hwang et al. 2008a, b; Karlsson et al. 2009; Varghese et al. 2010). To obtain ESC-MSCs with stable phenotypes, further studies are needed to develop effective methods to derive ESCs into fully committed MSCs.

In conclusion, ESC-MSCs represent a cell source that holds promise for the future of tissue engineering and regenerative medicine. Although ESC-MSCs are less responsive to induction for mesenchymal differentiation by current protocols compared to BM-MSCs, we have shown that the modified differentiation protocols can enhance ESC-MSC differentiation. Our key finding demonstrates that chondrogenesis of H1-MSCs can be greatly increased by BMP7 and TGFβ1 compared to that by the gold standard TGFβ1. Thus, for tissue engineering applications or as a model to study the development of musculoskeletal tissues, H1-MSCs are considered promising as an alternative MSC source.

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References

Alt EU, Senst C, Murthy SN, Slakey DP, Dupin CL, Chaffin AE, Kadowitz PJ, Izadpanah R (2012) Aging alters tissue resident mesenchymal stem cell properties. Stem Cell Res 8:215–225

Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A (2003) Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature 425:968–973

Bocelli-Tyndall C, Bracci L, Schaeren S, Feder-Mengus C, Barbero A, Tyndall A, Spagnoli GC (2009) Human bone marrow mesenchymal stem cells and chondrocytes promote and/or suppress the in vitro proliferation of lymphocytes stimulated by interleukins 2, 7 and 15. Ann Rheum Dis 68:1352–1359

Boyd NL, Robbins KR, Dhara SK, West FD, Stice SL (2009) Human embryonic stem cell derived mesoderm-like epithelium transitions to mesenchymal progenitor cells. Tissue Eng A 15:1897–1907

Brooke G, Cook M, Blair C, Han R, Heazlewood C, Jones B, Kambouris M, Kollar K, McTaggart S, Pelekanos R, Rice A, Rossetti T, Atkinson K (2007) Therapeutic applications of mesenchymal stromal cells. Semin Cell Dev Biol 18:846–858

Brown SE, Tong W, Krebsbach PH (2009) The derivation of mesenchymal stem cells from human embryonic stem cells. Cells Tissues Organs 189:256–260

Coleman CM, Vaughan EE, Browe DC, Mooney E, Howard L, Barry F (2013) Growth differentiation factor-5 enhances in vitro mesenchymal stromal cell chondrogenesis and hypertrophy. Stem Cells Dev 22:1968–1976

Cristofalo VJ, Allen RG, Pignolo RJ, Martin BG, Beck JC (1998) Relationship between donor age and the replicative lifespan of human cells in culture: a reevaluation. Proc Natl Acad Sci U S A 95:10614–10619

de Peppo GM, Svensson S, Lenneras M, Synnergren J, Stenberg J, Strehl R, Hyllner J, Thomsen P, Karlsson C (2010) Human embryonic mesodermal progenitors highly resemble human mesenchymal stem cells and display high potential for tissue engineering applications. Tissue Eng A 16:2161–2182

de Peppo GM, Marcos-Campos I, Kahler DJ, Alsalman D, Shang L, Vunjak-Novakovic G, Marolt D (2013) Engineering bone tissue

substitutes from human induced pluripotent stem cells. Proc Natl Acad Sci U S A 110:8680–8685

Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, Grisanti S, Gianni AM (2002) Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 99:3838–3843

Dickhut A, Pelttari K, Janicki P, Wagner W, Eckstein V, Egermann M, Richter W (2009) Calcification or dedifferentiation: requirement to lock mesenchymal stem cells in a desired differentiation stage. J Cell Physiol 219:219–226

Ferguson CM, Schwarz EM, Puzas JE, Zuscik MJ, Drissi H, O'Keefe RJ (2004) Transforming growth factor-beta1 induced alteration of skeletal morphogenesis in vivo. J Orthop Res 22:687–696

Giordano A, Galderisi U, Marino IR (2007) From the laboratory bench to the patient's bedside: an update on clinical trials with mesenchymal stem cells. J Cell Physiol 211:27–35

Grove JE, Bruscia E, Krause DS (2004) Plasticity of bone marrow-derived stem cells. Stem Cells 22:487–500

Hatzistergos KE, Quevedo H, Oskouei BN, Hu Q, Feigenbaum GS, Margitich IS, Mazhari R, Boyle AJ, Zambrano JP, Rodriguez JE, Dulce R, Pattany PM, Valdes D, Revilla C, Heldman AW, McNiece I, Hare JM (2010) Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. Circ Res 107: 913–922

Horwitz EM, Gordon PL, Koo WK, Marx JC, Neel MD, McNall RY, Muul L, Hofmann T (2002) Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. Proc Natl Acad Sci U S A 99:8932–8937

Hwang NS, Varghese S, Elisseeff J (2008a) Derivation of chondrogenically-committed cells from human embryonic cells for cartilage tissue regeneration. PLoS One 3:e2498

Hwang NS, Varghese S, Lee HJ, Zhang Z, Ye Z, Bae J, Cheng L, Elisseeff J (2008b) In vivo commitment and functional tissue regeneration using human embryonic stem cell-derived mesenchymal cells. Proc Natl Acad Sci U S A 105:20641–20646

Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41–49

Jiang XX, Zhang Y, Liu B, Zhang SX, Wu Y, Yu XD, Mao N (2005) Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. Blood 105:4120–4126

Kaminska B, Wesolowska A, Danilkiewicz M (2005) TGF beta signalling and its role in tumour pathogenesis. Acta Biochim Pol 52:329–337

Karlsson C, Emanuelsson K, Wessberg F, Kajic K, Axell MZ, Eriksson PS, Lindahl A, Hyllner J, Strehl R (2009) Human embryonic stem cell-derived mesenchymal progenitors- Potential in regenerative medicine. Stem Cell Res 3:39–50

Kinnaird T, Stabile E, Burnett MS, Shou M, Lee CW, Barr S, Fuchs S, Epstein SE (2004) Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. Circulation 109:1543–1549

Kopher RA, Penchev VR, Islam MS, Hill KL, Khosla S, Kaufman DS (2010) Human embryonic stem cell-derived CD34+ cells function as MSC progenitor cells. Bone 47:718–728

Li O, Tormin A, Sundberg B, Hyllner J, Le Blanc K, Scheding S (2013) Human embryonic stem cell-derived mesenchymal stroma cells (hES-MSCs) engraft in vivo and support hematopoiesis without suppressing immune function: implications for off-the shelf ESMSC therapies. PLoS One 8:e55319

Liu F, Akiyama Y, Tai S, Maruyama K, Kawaguchi Y, Muramatsu K, Yamaguchi K (2008) Changes in the expression of CD106, osteogenic genes, and transcription factors involved in the osteogenic differentiation of human bone marrow mesenchymal stem cells. Journal Bone Miner Metab 26:312–320

Marolt D, Campos IM, Bhumiratana S, Koren A, Petridis P, Zhang G, Spitalnik PF, Grayson WL, Vunjak-Novakovic G (2012) Engineering bone tissue from human embryonic stem cells. Proc Natl Acad Sci U S A 109:8705–8709

Montero JA, Lorda-Diez CI, Ganan Y, Macias D, Hurle JM (2008) Activin/TGFbeta and BMP crosstalk determines digit chondrogenesis. Dev Biol 321:343–356

Mrugala D, Dossat N, Ringe J, Delorme B, Coffy A, Bony C, Charbord P, Haupl T, Daures JP, Noel D, Jorgensen C (2009) Gene expression profile of multipotent mesenchymal stromal cells: Identification of pathways common to TGFbeta3/BMP2-induced chondrogenesis. Cloning Stem Cells 11:61–76

Mueller MB, Tuan RS (2008) Functional characterization of hypertrophy in chondrogenesis of human mesenchymal stem cells. Arthritis Rheum 58:1377–1388

Nakagawa T, Lee SY, Reddi AH (2009) Induction of chondrogenesis from human embryonic stem cells without embryoid body formation by bone morphogenetic protein 7 and transforming growth factor beta1. Arthritis Rheum 60:3686–3692

Nakayama N, Duryea D, Manoukian R, Chow G, Han CY (2003) Macroscopic cartilage formation with embryonic stem-cell-derived mesodermal progenitor cells. J Cell Sci 116:2015–2028

Oldershaw RA, Baxter MA, Lowe ET, Bates N, Grady LM, Soncin F, Brison DR, Hardingham TE, Kimber SJ (2010) Directed differentiation of human embryonic stem cells toward chondrocytes. Nat Biotechnol 28:1187–1194

Oshimori N, Fuchs E (2012) The Harmonies Played by TGF-beta in Stem Cell Biology. Cell Stem Cell 11:751–764

Pelttari K, Winter A, Steck E, Goetzke K, Hennig T, Ochs BG, Aigner T, Richter W (2006) Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. Arthritis Rheum 54:3254–3266

Rivera FJ, Aigner L (2012) Adult mesenchymal stem cell therapy for myelin repair in Multiple Sclerosis. Biol Res 45:257–268

Rui YF, Du L, Wang Y, Lui PP, Tang TT, Chan KM, Dai KR (2010) Bone morphogenetic protein 2 promotes transforming growth factor beta3-induced chondrogenesis of human osteoarthritic synovium-derived stem cells. Chin Med J (Engl) 123:3040–3048

Schmierer B, Hill CS (2007) TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. Nat Rev Mol Cell Biol 8:970–982

Schmitt B, Ringe J, Haupl T, Notter M, Manz R, Burmester GR, Sittinger M, Kaps C (2003) BMP2 initiates chondrogenic lineage development of adult human mesenchymal stem cells in high-density culture. Differentiation 71:567–577

Seebach C, Henrich D, Wilhelm K, Barker JH, Marzi I (2012) Endothelial Progenitor Cells Improve Directly and Indirectly Early Vascularization of Mesenchymal Stem Cell-Driven Bone Regeneration in a Critical Bone Defect in Rats. Cell Transplant 21(8):1667–1677

Shen B, Wei A, Whittaker S, Williams LA, Tao H, Ma DD, Diwan AD (2010) The role of BMP- 7 in chondrogenic and osteogenic differentiation of human bone marrow multipotent mesenchymal stromal cells in vitro. J Cell Biochem 109:406–416

Sotiropoulou PA, Perez SA, Gritzapis AD, Baxevanis CN, Papamichail M (2006) Interactions between human mesenchymal stem cells and natural killer cells. Stem Cells 24:74–85

Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L (2006) Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood 107: 1484–1490

Tolar J, Le Blanc K, Keating A, Blazar BR (2010) Concise review: hitting the right spot with mesenchymal stromal cells. Stem Cells 28:1446–1455

Toubai T, Paczesny S, Shono Y, Tanaka J, Lowler KP, Malter CT, Kasai M, Imamura M (2009) Mesenchymal stem cells for treatment and prevention of graft-versus-host disease after allogeneic hematopoietic cell transplantation. Curr Stem Cell Res Ther 4:252–259

Trivedi P, Hematti P (2008) Derivation and immunological characterization of mesenchymal stromal cells from human embryonic stem cells. Exp Hematol 36:350–359

Varga N, Vereb Z, Rajnavolgyi E, Nemet K, Uher F, Sarkadi B, Apati A (2011) Mesenchymal stem cell like (MSCl) cells generated from human embryonic stem cells support pluripotent cell growth. Biochem Biophys Res Commun 414:474–480

Varghese S, Hwang NS, Ferran A, Hillel A, Theprungsirikul P, Canver CA, Zhang Z, Gearhart J, Elisseeff JH (2010) Engineering musculoskeletal tissue with human embryonic germ cell derivatives Stem Cells 28:765–774

Vodyanik MA, Yu J, Zhang X, Tian S, Stewart R, Thomson JA, Slukvin II (2010) A mesodermderived precursor for mesenchymal stem and endothelial cells. Cell Stem Cell 7:718–729

Wagner W, Horn P, Castoldi M, Diehlmann A, Bork S, Saffrich R, Benes V, Blake J, Pfister S, Eckstein V, Ho AD (2008) Replicative senescence of mesenchymal stem cells: a continuous and organized process. PLoS One 3:e2213

Wagner W, Bork S, Horn P, Krunic D, Walenda T, Diehlmann A, Benes V, Blake J, Huber FX, Eckstein V, Boukamp P, Ho AD (2009) Aging and replicative senescence have related effects on human stem and progenitor cells. PLoS One 4:e5846

Wan M, Cao X (2005) BMP signaling in skeletal development. Biochem Biophys Res Commun 328:651–657

Wang G, Bunnell BA, Painter RG, Quiniones BC, Tom S, Lanson NA Jr, Spees JL, Bertucci D, Peister A, Weiss DJ, Valentine VG, Prockop DJ, Kolls JK (2005) Adult stem cells from bone marrow stroma differentiate into airway epithelial cells: potential therapy for cystic fibrosis. Proc Natl Acad Sci U S A 102:186–191

Xu D, Gechtman Z, Hughes A, Collins A, Dodds R, Cui X, Jolliffe L, Higgins L, Murphy A, Farrell F (2006) Potential involvement of BMP receptor type IB activation in a synergistic effect of chondrogenic promotion between rhTGFbeta3 and rhGDF5 or rhBMP7 in human mesenchymal stem cells. Growth Factors 24:268–278

Yen BL, Chang CJ, Liu KJ, Chen YC, Hu HI, Bai CH, Yen ML (2009) Brief report–human embryonic stem cell-derived mesenchymal progenitors possess strong immunosuppressive effects toward natural killer cells as well as T lymphocytes. Stem Cells 27:451–456

Yen ML, Hou CH, Peng KY, Tseng PC, Jiang SS, Shun CT, Chen YC, Kuo ML (2011) Efficient derivation and concise gene expression profiling of human embryonic stem cell-derived mesenchymal progenitors (EMPs). Cell Transplant 20:1529–1545

Yoon BS, Lyons KM (2004) Multiple functions of BMPs in chondrogenesis. J Cell Biochem 93:93–103