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Efficient Differentiation of Human iPSC-Derived Mesenchymal Stem Cells to Chondroprogenitor Cells

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ABSTRACT

Induced pluripotent stem cells (iPSC) hold tremendous potential for personalized cell-based repair strategies to treat musculoskeletal disorders. To establish human iPSCs as a potential source of viable chondroprogenitors for articular cartilage repair, we assessed the in vitro chondrogenic potential of the pluripotent population versus an iPSC-derived mesenchymal-like progenitor population. We found the direct plating of undifferentiated iPSCs into high-density micromass cultures in the presence of BMP-2 promoted chondrogenic differentiation, however these conditions resulted in a mixed population of cells resembling the phenotype of articular cartilage, transient cartilage, and fibrocartilage. The progenitor cells derived from human iPSCs exhibited immunophenotypic features of mesenchymal stem cells (MSCs) and developed along multiple mesenchymal lineages, including osteoblasts, adipocytes, and chondrocytes in vitro. The data indicate the derivation of a mesenchymal stem cell population from human iPSCs is necessary to limit culture heterogeneity as well as chondrocyte maturation in the differentiated progeny. Moreover, as compared to pellet culture differentiation, BMP-2 treatment of iPSC-derived MSC-like (iPSC–MSC) micromass cultures resulted in a phenotype more typical of articular chondrocytes, characterized by the enrichment of cartilage-specific type II collagen (*Col2a1*), decreased expression of type I collagen (*Col1a1*) as well as lack of chondrocyte hypertrophy. These studies represent a first step toward identifying the most suitable iPSC progeny for developing cell-based approaches to repair joint cartilage damage. J. Cell. Biochem. 114: 480–490, 2013.

KEY WORDS: HUMAN INDUCED PLURIPOTENT STEM CELLS; MESENCHYMAL STEM CELLS; CHONDROGENIC DIFFERENTIATION; HIGH-DENSITY CULTURE

Due to the limited healing capacity of articular cartilage, tissue engineering represents a promising strategy for the treatment of cartilage defects. Adult human mesenchymal stem cells (MSCs) isolated from diverse tissues such as bone marrow, synovium, and adipose tissue have been proposed as attractive autologous cell sources for articular cartilage engineering strategies due to their extensive proliferative capacity, their demonstrated chondrogenic potential and anti-inflammatory effects [Chen and Tuan, 2008]. However, results of various in vitro and animal studies showed donor and replicative age-related decline in mitogenic and differentiation potential, which significantly limit their therapeutic potential [Baxter et al., 2004; Sethe et al., 2006; Steinert et al., 2007; Stolzing et al., 2008]. Moreover, harvesting bone marrow is a highly invasive procedure and the presence of MSCs in bone marrow is extremely rare, estimated at approximately 10 MSCs per one million mononuclear cells [Jones et al., 2002]. Thus, establishing a sufficient source of autologous MSCs that retain the ability to differentiate and synthesize an articular cartilage-like extracellular matrix is a major challenge toward the development of effective cartilage repair strategies.

To overcome the limitations associated with adult somatic cells for cartilage repair [Steinert et al., 2007], alternative sources are

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necessary to procure vast quantities of autologous, chondrogenic cells that maintain a phenotype typical of the resident joint chondrocytes, without undergoing abnormal terminal maturation. Because of their unlimited self-renewal, high developmental plasticity and limited immunogenic properties, induced pluripotent stem cells (iPSCs) represent an alternative source of patient-specific cells for the repair of musculoskeletal tissues with poor regenerative capacity, such as cartilage [Nakayama and Umeda, 2011; Toh et al., 2011]. Several in vitro strategies have been developed to drive the differentiation of human pluripotent stem cells to the chondrogenic lineage [Hwang et al., 2008a; Bigdeli et al., 2009; Harkness et al., 2009; Toh et al., 2009; Gong et al., 2010; Oldershaw et al., 2010; Toh et al., 2010]. For example, high-density micromass culture of stem cells mimics the formation of prechondrogenic mesenchymal condensations in the embryo and promotes their differentiation to the chondrocytic lineage [Mello and Tuan, 1999]. The chondrogenic potential of human pluripotent stem cells in micromass culture may be augmented by stage-specific application of exogenous chondrogenic factors such as TGFβs and BMPs [Gong et al., 2010; Oldershaw et al., 2010; Toh et al., 2010]. However, many commonly used in vitro systems lead to the development of a heterogenous cell progeny, exhibiting features of transient cartilage, articular cartilage as well as fibrocartilage.

The derivation of a self-renewing human iPSC population exhibiting an intermediate stage between a pluripotent stem cell and a fully differentiated state is a critical first step toward limiting the developmental fate of iPSCs and enhancing their chondrogenic potential in vitro. Thus, to establish the necessary culture criteria for generating articular-like chondrocytes from human iPSCs, we derived multipotent MSC-like cells from a well-characterized human dermal fibroblast iPSC line (YK26 iPSC) [Martins-Taylor et al., 2011]. Using the parental precursor iPSCs and the iPSC-derived MSC-like (iPSC–MSC) population, we defined in vitro conditions for efficient differentiation to the chondrocytic lineage. Our results indicate that enrichment of the mesenchymal progenitor cell population from differentiating human iPSCs and the appropriate high density culture environment are necessary ingredients for enhancing the chondrogenic potential of iPSCs and limiting the heterogeneity of the differentiated progeny.

MATERIALS AND METHODS

iPSC CULTURE AND MAINTENANCE

Human dermal fibroblast-derived iPSC line (HDFa-YK26) [Martins-Taylor et al., 2011] were cultured on a feeder layer of irradiated mouse embryonic fibroblasts (MEF) in a humidified atmosphere at 37°C and 5% CO_2. The growth media consisted of DMEM/F-12 (Gibco), 20% Knock-out Serum Replacement (Hyclone), 8 ng/ml human recombinant basic fibroblast growth factor (bFGF; Invitrogen), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, and 1% penicillin/streptomycin.

DERIVATION OF MESENCHYMAL PROGENITOR CELLS FROM HUMAN iPSCs AND SUBSEQUENT EXPANSION

Undifferentiated iPSC colonies were treated with accutase (Innovative Cell Technologies) for 5 min at 37°C, dissociated by gentle pipetting, and then passed through 40-μm cell strainers (BD Biosciences). Cells were seeded onto gelatin-coated plates at 1×10^4 cells/cm^2 in MSC induction media consisting of DMEM-HG (Gibco), 10% defined fetal bovine serum (FBS; Hyclone), 1% nonessential amino acids, 1% penicillin–streptomycin, and 5 ng/ml human recombinant bFGF (Invitrogen) [Nakagawa et al., 2009]. Cells were propagated to 80% confluency in a humidified atmosphere at 37°C and 5% CO_2. The cultures acquired a heterogenous cell morphology, resembling a mixed population of cuboidal and elogated spindle-shaped cells. With subsequent passaging (p1–p2) onto non-coated tissue culture plates using 0.25% trypsin/EDTA (Gibco), the iPSC–MSC-like populations acquired a homogenous, fibroblast-like morphology. For routine expansion, cells were plated at 1×10^4 cells/cm^2 and maintained in MSC induction media.

FLOW CYTOMETRIC ANALYSIS

Human bone marrow derived MSCs (Lonza) and iPSC–MSC-like cells (passage 5–7) were grown to confluence, harvested by 0.25% trypsin/EDTA, washed with PBS, and resuspended in staining solution consisting of 2% FBS and 2% HEPES in PBS. Cell suspensions (1×10^6 cells) were mixed with PE mouse anti-human CD90 (BD Pharmingen), PE mouse anti-human CD73 (BD Pharmingen), PE muose anti-human CD29 (BD Pharmingen), PE mouse anti-human HLA-DR (BD Pharmingen), FITC mouse anti-human CD44 (BD Pharmingen), FITC mouse anti-human HLA-ABC (BD Pharmingen), FITC mouse anti-human CD105 (BD Pharmingen), FITC mouse anti-human CD45 (BD Pharmingen), and FITC mouse anti-human CD31 (BD Pharmingen). Nonspecific fluorescence was determined by incubation of cell aliquots with isotype-matched monoclonal antibodies (IgG1-PE and IgG2b-FITC). Samples were run on a Becton–Dickinson LSR II Flow Cytometer (BD Biosciences) instrument using FACS Diva software (Becton–Dickinson). For each analysis, a minimum of 10,000 cells was assayed. Data was analyzed using FloJo Software (Tree Star, Inc.).

OSTEOGENIC AND ADIOPOGENIC DIFFERENTIATION ASSAYS

To induce osteogenesis, iPSC–MSC-like cells (passage 4–6) were seeded at 60,000 cells/cm^2 and cultured in DMEM containing 10% FBS (Hyclone), 1 mM sodium pyruvate, 10^{-7} M dexamethasone, 50 μg/ml ascorbic acid 2-phosphate, 10 mM β-glycerophosphate, and 1% penicillin/streptomycin for 14 and 21 days. After 14 and 21 days, cultures were fixed in formalin and stained for 10 min in Alkaline Phosphatase solution (Sigma, St. Louis, MO), or Alizarin Red solution (Sigma) to visualize calcium deposits. Adipogenic differentiation was induced by treating cells (passage 4–6) seeded at 60,000 cells/cm^2 in DMEM-HG containing 10% FBS (Hyclone), 1 mM sodium pyruvate, 10^{-6} M dexamethasone, 10 μg/ml insulin, 0.5 mM isobutylmethylxanthine, 200 μM indomethacin and 1% penicillin/streptomycin. After 21 days in culture, formalin-fixed adipogenic cultures were rinsed in 60% isopropanol and then stained in Oil red O in 60% isopropanol for detection of lipid accumulation.

HIGH DENSITY MICROMASS AND MICROPELLET CULTURES

Chondrogenic differentiation of iPSC–MSC-like (passage 5–6) was induced by culturing accutase-dispersed cells in high-density micromass (2×10^5 cells/10 μl drop) as previously described [Soung et al., 2007]. Twenty-four hours after the onset of micromass formation, the culture media was replaced with chondrogenic media consisting of DMEM-HG media (Gibco) supplemented with 1% KSR (Invitrogen), 1% ITS$^+$ premix (containing human recombinant insulin, human transferrin, selenous acid, BSA, and linoleic acid; BD Biosciences), 40 μg/ml L-proline, 1 mM sodium pyruvate, 1% non-essential amino acids, 2 mM Glutamax, 50 μg/ml ascorbic acid 2-phosphate, and 10^{-7} M dexamethasone and penicillin/streptomycin [Toh et al., 2010]. On Day 2 of micromass formation and for the duration of the differentiation assay, select cultures were treated with human recombinant BMP-2 (100 ng/ml, RnD Systems). Media and growth factor were replenished every other day. Formalin-fixed micromass cultures were stained with 1% Alcian Blue in acetic acid, pH 2.5 (Polysciences Inc.).

High-density micropellet cultures were formed by sedimenting 2.5×10^5 cells (passage 4–6) in 15-ml conical tubes at 200*g* for 5 min. The micropellets were incubated in 0.5 ml of chondrogenic media described above and human recombinant BMP-2 (100 ng/ml, RnD Systems) was added on Day 2 of micropellet formation. Media and growth factor were replenished every other day. Micropellets were fixed in 10% formalin, embedded in paraffin, sectioned using a Leica microtome (Leica Microsystems), and then subjected to Alcian Blue staining for detection of glycosaminoglycan.

REAL-TIME PCR ANALYSES

Total RNA from undifferentiated or differentiated YK26-iPSCs at different timepoints was harvested by TRIzol reagent (Invitrogen), treated with DNaseI (Biorad) and reverse transcribed to cDNA with iScript reverse transcriptase (QIAGEN). cDNA samples synthesized from 1 μg of total RNA were subjected to Q-PCR with SYBR Green I Master Mix kit (Roche) using a Real-Time PCR System (AB 7900, Applied Biosystems). Values are expressed as 2^DeltaDeltaCt, with DeltaDeltaCt defined as the difference in crossing threshold (C_t) values between experimental and control samples as described, using *Gapdh* as an internal standard. Oligonucleotide primers are listed in Table I.

TABLE I. Q-RT-PCR Primers

Gene (accession no.)	Forward primer	Reverse primer
Gapdh (NM_002046)	aattccatggcaccgtcaag	agggatctcgctcctggaag
Oct3/4 (NM_203289)	tgtactcctcggtccctttc	tccaggttttctttccctagc
Nanog (NM_024865)	cagtctggacactggctgaa	ctcgctgattaggctccaac
Klf4 (NM_004235)	tatgacccacactgccagaa	tgggaacttgaccatgattg
ALP (NM_001177520)	gacaagaagcccttcactgc	agactgcgcctggtagttgt
L-Sox5 (NM_006940)	atcccaactaccatggcagct	tgcagttggagtgggccta
Sox6 (NM_017508)	gcagtgatcaacatgtggcct	cgctgtcccagtcagcatct
Sox9 (NM_000346)	agacagccccctatcgactt	cggcaggtactggtcaaact
Aggrecan (NM_013227)	tcgaggacagcgaggcc	tcgagggtgtagcgtgtagaga
Col2a1 (NM_001844)	ggcaatagcaggttcacgtaca	cgataacagtcttgccccactt
Col2B (NM_033150)	agggccaggatgtccggca	gggtcccaggttctccatct
Col1a1 (NM_000088)	gtgctaaaggtgccaatggt	accaggttcaccgctgttac
ColXa1 (NM_000493)	caaggcaccatctccaggaa	aaagggtatttgtggcagcatatt
Runx2 (NM_004348)	gccttcaaggtggtagccc	cgttacccgccatgacagta
Runx1 (NM_001754)	aaccctcagcctcagagtca	caatggatcccaggtattgg

STATISTICS

Data are expressed as mean ± SEM of at least three independent samples. Statistical comparisons between two groups (untreated vs. BMP-2) were performed using a two-tailed Student's *t*-test. Statistical comparisons among groups (>two groups) were performed by one-way analysis of variance (ANOVA) using Bonferroni's Multiple Comparison test. Significance was denoted at $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

RESULTS

CHONDROGENIC INDUCTION, MATURATION, AND HETEROGEOUS DIFFERENTIATION OF BMP-2 TREATED iPSC MICROMASS CULTURES

High-density micromass culture of pluripotent stem cells models the formation of prechondrogenic mesenchymal condensations and their differentiation into the chondrogenic lineage [Daniels et al., 1996]. We assessed the in vitro chondrogenic potential of human dermal fibroblast-derived iPSCs (YK26-iPSCs) using our well-established micromass culture assay [Zhang et al., 2004; Wang et al., 2005; Bradley and Drissi, 2010]. In the absence of growth factor stimulation, Alcian blue staining of Day 7–21 YK26-iPSC micromass cultures showed temporal accumulation of a glycoprotein-rich matrix throughout the control, untreated micromasses as compared to Day 1 micromass cutltures (Fig. 1A). In cultures treated with human recombinant BMP-2, the majority of cells appeared to coalesce or compact within the center of the micromass within 2–3 days of initial growth factor stimulation. As shown in Figure 1A and B, Alcian blue staining of micromass cultures treated with BMP-2 for 7 days showed a densely-stained central core surrounded by a diffusely-stained outer cellular layer. These observations are consistent with previous ex vivo imaging studies demonstrating that BMP-2 signaling promotes tight mesenchymal cell–cell interactions through cellular compaction, prior to the mesenchyme to cartilage transition in limb bud micromass cultures [Barna and Niswander, 2007]. In contrast, micromass cultures treated for 7 days with TGFβ1 were similar in appearance to the control, untreated micromasses (data not shown). With progressive differentiation (Days 14 and 21), Alcian blue positive cellular outgrowths and cartilaginous nodules were observed in the BMP-2 treated micromasses, whereas staining of untreated cultures revealed less extensive nodule formation at Days 14 and 21. Similar results were observed using a separate human dermal fibroblast-derived iPSC line (data not shown).

Using parallel micromass cultures, we further characterized the phenotype of untreated control and growth factor-stimulated YK26-iPSCs by assessing the relative expression of discrete genes representative of the chondroprogenitor lineage (*Sry-type HMG box*, *Sox9*, and *type II collagen*) or fully differentiated chondrocytes (*Col2b*, *aggrecan*, *Runx2*, *type X collagen*) from Days 7 to 21. Temporal regulation of *Sox9*, an essential transcription factor for chondrogenesis and cartilage-specific gene expression was observed in both control and BMP-2 stimulated YK26-iPSC micromass cultures over the indicated time course (Fig. 2A). A significantly greater induction of *Sox9* transcript expression was detected in response to BMP-2 treatment, however this effect was limited to the

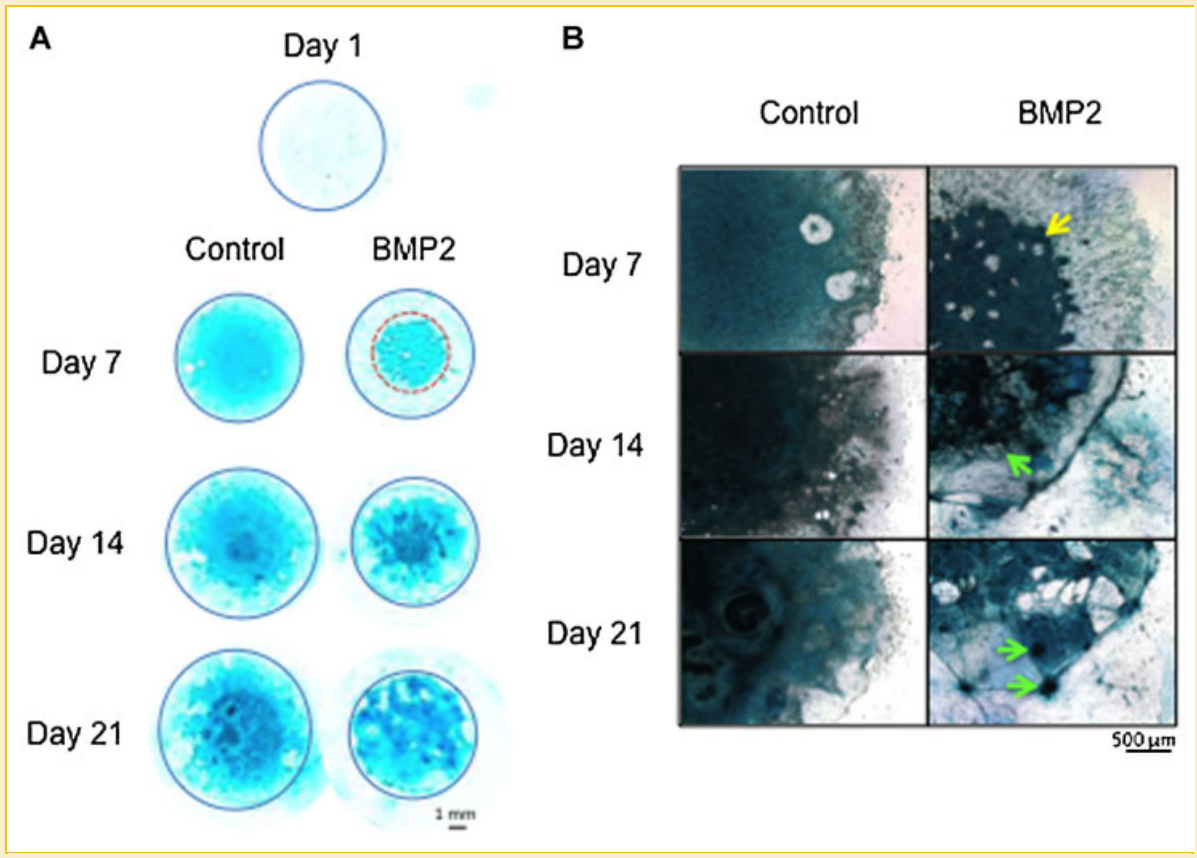

Fig. 1. Proteoglycan-rich matrix accumulation in chondrogenic iPSCs micromass cultures. A: Phenotypic chondrogenic differentiation of YK26-iPSCs cultures was assessed by Alcian blue histochemical staining of Day 1 micromass cultures and control and BMP-2 (100 ng/ml) treated YK26-iPSC high-density micromasses at Days 7, 14, and 21. Alcian blue staining revealed temporal accumulation of sulfated proteoglycans in both control and BMP-2 treated cultures. iPSC micromasses cultured in the presence of BMP-2 showed enhanced compaction of the cells within the central core of the micromass. Black circles demarcate the perimeter of each micromass. Red circle indicates area of cellular compaction at the core of the Day 7 iPSC micromass culture treated with BMP-2. Scale bar, 1 mm. B: High magnification images further demonstrated enhanced cellular compaction (yellow arrow) in BMP-2 treated cultures at the early phase of in vitro differentiation (Day 7). BMP-2 treatment promoted the development of cartilaginous nodules (green arrows) at later timepoints of iPSC micromass differentiation (Days 14 and 21). Scale bar, 500 μm.

early phase of differentiation (Days 7–14; Fig. 2A). Consistent with these observations, transcript expression of *type II collagen* (*Col2a1*), the primary constituent of the articular cartilage matrix, was significantly up-regulated in BMP-2 treated cultures at Day 7, then down-regulated between Days 14 and 21 relative to controls (Fig. 2B). Transcript expressions of *aggrecan* (Fig. 2C) and *Col2B* (Fig. 2D), a definitive marker of fully differentiated chondrocytes [McAlinden et al., 2005], were markedly increased in the BMP-2 treated YK26-iPSC micromass cultures, thus providing further evidence of progression toward a more advanced differentiation status. Notably, transient exposure of human YK26-iPSCs to BMP-2 at the early stages of micromass culture (Days 2–4) elicited a similar effect on cartilage gene expression as in conditions where BMP-2 was administered continuously throughout the differentiation assay (data not shown). The down-regulation of the definitive cartilage markers (*Col2a1*) in BMP-2 treated micromass cultures at Day 14 was concomitant with increased expression of pre-hypertrophic (*Runx2*; Fig. 2E) and hypertrophic chondrocyte genes (*Type X collagen*; Fig. 2G). To corroborate these observations, histological analyses of high density YK26-iPSC micropellets also showed the presence of hypertrophic chondrocytes in response to BMP-2 treatment (Fig. 2H). As shown in Figure 2F, significantly enhanced transcript expression of *type I collagen (Col1a1)*, a major component of connective tissue and osteoblasts, was observed in BMP-2 treated micromasses between Days 14 and 21 relative to control cultures. Taken together, these data indicate that high density micromass formation alone was sufficient to drive chondrogenic differentiation of human YK26-iPSCs and treatment with BMP-2 promoted chondrocyte maturation as well as culture heterogeneity.

DERIVATION OF AN EXPANDABLE PROGENITOR CELL POPULATION FROM THE YK26 iPSC LINE

To generate human iPSC-derived progenitors exhibiting an intermediate stage of development between a stem cell and a fully differentiated state, we dispersed undifferentiated YK26-iPSC colonies and cultured the cells under feeder-free conditions in serum and human recombinant bFGF (5 ng/ml) supplemented media. These culture conditions promoted the selection of highly proliferative, adherent cells [Karlsson et al., 2009; Nakagawa et al., 2009] and led to the initial generation of a heterogenous population of cuboidal and elongated spindle-shaped cells. First confluence occurred after approximately 3 weeks in culture and a more uniform

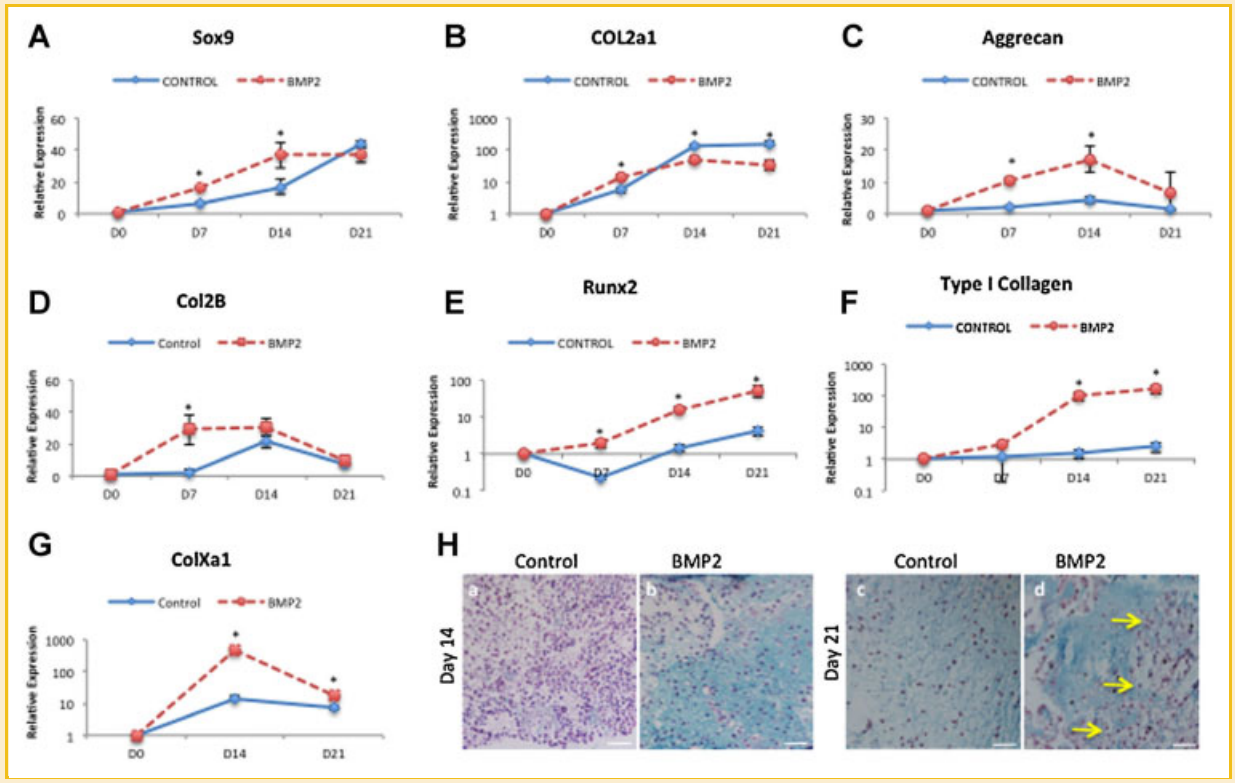

Fig. 2. BMP-2 treatment enhanced and accelerated iPSC chondrogenic differentiation. Quantitative PCR analyses of the relative transcript levels of (A) *Sox9*, (B) *Col2a1*, (C) *Aggrecan*, (D) *Col2b*, (F) *Runx2*, and (E) *Col1a1* in Day 7, 14, and 21 control and BMP-2 treated micromass cultures. *Gapdh* served as the housekeeping gene and internal control. Values represent mean fold induction relative to undifferentiated iPSCs (Day 0). Blue lines represent control, untreated cultures; red, dashed lines indicate BMP-2 treated cultures. Error bars indicate SEM ($n = 5$–9) Asterisks indicate values that are statistically different ($P < 0.05$) from untreated samples at each timepoint. G: Relative expression of *ColXa1* transcripts in Days 14 and 21 untreated control and BMP-2 (100 ng/ml) treated iPSC micromass cultures. Error bars indicate SEM ($n = 3$) Asterisks indicate values that are statistically different ($P < 0.05$) from the untreated samples. H: Histological appearance of untreated and BMP-2 treated iPSC micropellets at Days 14 and 21. Alcian blue/Nuclear red staining of representative sections (5 μm) through a paraffin-embedded high-density iPSC micropellets treated with or without human recombinant BMP-2 (100 ng/ml). Hypertrophic cell morphology is identified by arrows. Scale bar, 50 μm.

population of spindle-shaped cells (Fig. 3A) was attained with subsequent passaging (p2–p3). These cells grew at high density in a fingerprint whorl pattern and phenotypically resembled human bone marrow-derived MSCs. Profiling by Q-PCR confirmed significant suppression of stem cell genes such as *Oct4*, *Nanog*, *Alkaline phosphatase (ALP)*, and *Klf4* in YK26-iPSC derived MSC-like cells as compared to the parental undifferentiated YK26-iPSCs (Fig. 3B), suggestive of a loss of pluripotency.

PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF iPSC–MSC-LIKE CELLS

To gain insight into the progenitor properties of the iPSC-derived population, we surveyed the expression of gene markers associated with the mesenchymal and chondroprogenitor lineage by Q-PCR analyses. *Twist1*, an epithelial to mesenchymal transition (EMT) related gene, was significantly induced in iPSC–MSC-like cells (17.86 ± 0.89 fold) as compared to the pluripotent parental cells and was also highly expressed in human bone marrow-derived MSCs (BM-MSC; 38.29 ± 2.98 fold; Fig. 3C). As shown in Figure 3C, *Type I collagen (Col1a1)*, an extracellular matrix molecule synthesized by MSCs [Prockop, 1997], was highly expressed in the bone marrow derived-MSCs and iPSC–MSC-like cells (51.24 ± 2.37 and 31.43 ± 1.2 fold, repectively), while minimally expressed in the parental YK26-iPSCs. As compared to the pluripotent cells, bone marrow derived-MSCs and iPSC–MSC-like cells showed significantly higher transcript expression of *Sox9* (3.75 ± 0.24 and 7.12 ± 0.42 fold, respectively), a key molecular determinant of chondrogenesis (Fig. 3C). Co-expression of *Sox* auxillary factors (*Sox5* and *Sox6*) are essential for *Sox9* activity and the specification of the chondrogenic lineage [Lefebvre et al., 1998, 2001]. These factors were variably expressed in the human BM-MSCs and iPSC–MSC-like cells, thus indicative of a less differentiated, progenitor state (Fig. 3C). *Runx1*, a transcription factor expressed in mesenchymal progenitors during early embryonic development with key roles in skeletal development [Smith et al., 2005], was also induced in the iPSC–MSC-like population (81.2 ± 3.0 fold) relative to the pluripotent parental cell and highly expressed in bone marrow derived MSCs (140.6 ± 5.3 fold).

We further characterized the iPSC–MSC-like cells by fluorescence activated cell sorting (FACS) analyses of the CD surface antigen marker expressions. We compared the immunophenotype of the MSC-enriched population from YK26-iPSCs with that of human MSCs derived from adult bone marrow. As shown in Figure 4A, the immunophenotype of the iPSC–MSC-like cells was highly similar to

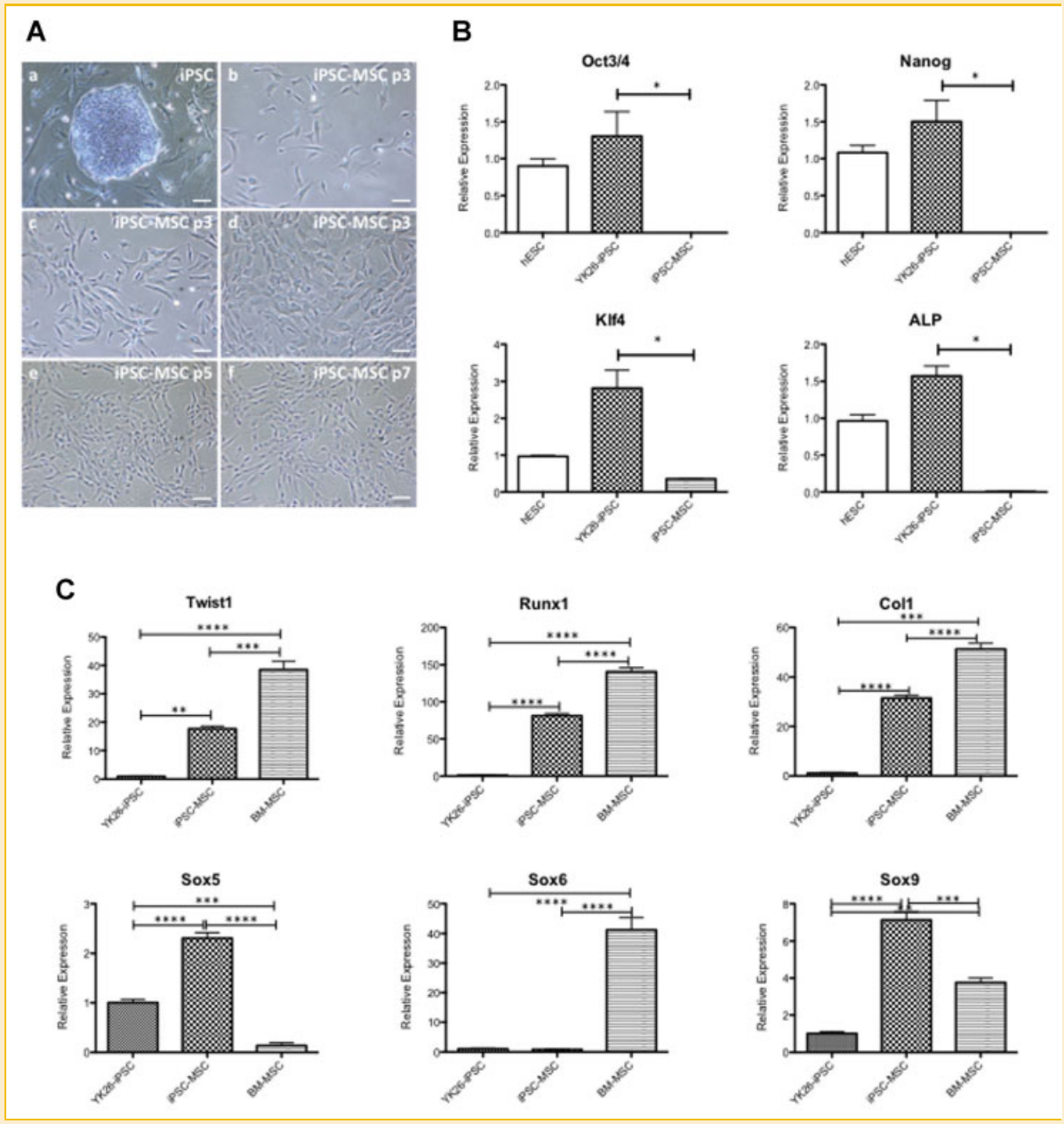

Fig. 3. Derivation of a mesenchymal progenitor population from YK26-iPSC line. A: Typical morphology of an undifferentiated human YK26-iPSC colony (a) cultured on a feeder layer of irradiated MEFs, visualized by phase contrast microscopy. The morphology of the iPSC-MSC-like cells (iPSC-MSC) (b–f) resembled elongated spindle shaped cells and differed significantly from the undifferentiated iPSCs. Representative images are shown for iPSC-MSC-like cells at passage 3 at low density (b), medium density (c) and high density (d). Representative images are also shown for iPSC-MSC-like cells at higher passages (e, passage 5) and (f, passage 7). Scale bar, 100 μm. B: Gene expression analyses by Q-PCR showed significant suppression of pluripotent markers *Oct3/4*, *Nanog*, *Klf5*, and *ALP* in the iPSC-MSC-like population (passage 3) relative to the parental, undifferentiated iPSCs. Undifferentiated human H9 ESCs served as the standard for comparison of gene expression. Expression data is represented as fold induction relative to H9 ESCs, set at 1.0 ($n = 3$). Asterisk (*) denotes significance versus parental undifferentiated human YK26-iPSCs at $P < 0.05$. C: Comparative expression of mesenchymal genes (*Twist1*, *Cola1*) and chondroprogenitor genes (*Sox5*, *Sox6*, *Sox9*, and *Runx1*) in iPSC-MSC-like cells (passage 3) and human bone marrow-derived mesenchymal stem cells (BM-MSCs) by qPCR. Gene expression data is represented as fold induction relative to the undifferentiated parental YK26-iPSC line, set at 1.0. Error bars indicate SEM ($n = 3$). Asterisk denotes statistical significance at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

that of human BM-MSCs. Analyses of the typical MSC markers in an unsorted population of iPSC–MSC-like cells confirmed high level expression of CD29 (99.4%) CD44 (84%), CD73 (99.9%), CD90 (70.6%), CD105 (80%), CD166 (92.2%), and HLA-ABC (81.6%). The iPSC–MSC-like cells largely lacked expression of the definitive hematopoietic lineage marker CD45 (7.9%), the endothelial marker CD31 (8.8%) as well as the MHC class II cell surface receptor HLA-DR (2.2%).

To establish the multilineage potential of the iPSC–MSC-like cells, we assessed their ability to differentiate into adipogenic, osteogenic, and chondrogenic lineages in vitro. Following 21 days of culture in adipogenic media, the iPSC–MSC-like cells acquired a

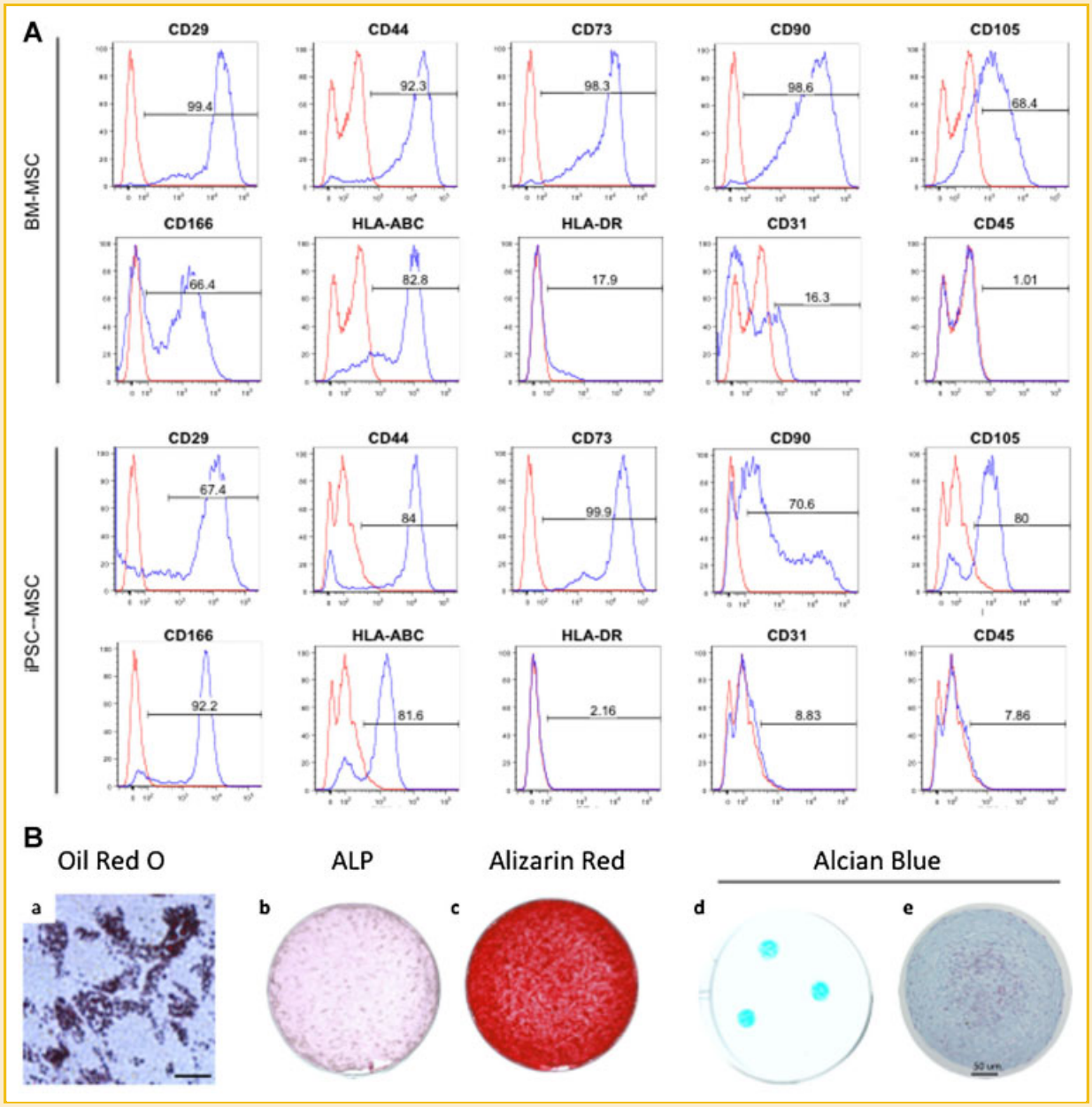

Fig. 4. iPSC–MSC-like cells display features of mesenchymal stem cells. A: Expression of surface antigens in human bone marrow-derived mesenchymal stem cells (BM-MSCs) and iPSC–MSC-like cells by fluorescence activated cell sorting (FACS) analysis. Representative FACS profiles show iPSC–MSC-like cells (passages 5–7) express markers associated with the mesenchymal phenotype (positive for CD29, CD44, CD73, CD90, CD105, CD166, and HLA-ABC; negative for HLA-DR, CD31, and CD45). Blue histogram indicates antibody stained population; red profile indicates negative isotype stained population. Fluorescence intensity considered as positive signal obtained from isotype controls. B: iPSC–MSC-like cells exhibit multilineage differentiation. Adipogenesis was induced by culturing the iPSC–MSC-like cells in media containing 3-isobutyl-1-methylxanthine, indomethacin, insulin, and serum. After 14 days in adipogenic media, lipid vesicles (a) were detected in iPSC–MSC-like cells by staining with Oil Red O. Scale bar, 25 μm. To demonstrate differentiation to the osteoblast lineage, iPSC–MSC-like cells were cultured in osteogenic media, then stained with (b) Alkaline phosphatase (ALP) at Day 14; or (c) alizarin red at Day 21. Chondrocyte differentiation was shown by (d) Alcian blue staining of micromass cultures in serum free chondrogenic media for 14 days with 100 ng/ml BMP-2. Alcian blue staining of thin sections of micropellets treated with TGFβ1 for 28 days is shown in (e). Scale bar, 50 μm.

typical adipocyte-like morphology and showed abundant accumulation of lipid vacuoles that stained positive with Oil Red O (Fig. 4Bi and ii). Culturing of iPSC–MSC-like cells in osteogenic conditions led to the production of alkaline phosphatase (ALP; Day 14; Fig. 4Biii), an early maker of osteogenesis. The formation of calcium deposits was detected in more advanced cultures (Day 21) by robust Alizarin Red staining (Fig. 4B). The undifferentiated iPSC–MSC-like cultures did not display positive staining for Oil Red O, alkaline phosphatase or alizarin red when cultured in control growth media (data not shown). As positive controls for our histochemical analyses, we confirmed the adipogenic and osteogenic potential of human bone marrow derived mesenchymal stem cells (data not shown). To demonstrate their chondrogenic potential, the cells were cultured as high density micromass (Fig. 4Bv) or micropellets

(Fig. 4Bvi) in serum-free media supplmented with TGFβ1 (10 ng/ml). These conditions led to the accumulation of an Alcian blue-stained matrix, indicative of chondrogenic differentiation (Fig. 4B). Together, these data demonstrate the successful generation of functional mesenchymal progenitor cells from human YK26-iPSCs, which exhibited the potential to differentiate into adipocytes, osteoblasts, and chondrocytes.

ENHANCED CHONDROGENIC POTENTIAL OF iPSC–MSC-LIKE CELLS IN MICROMASS VERSUS MICROPELLET CULTURES

Next, we sought to detemine the optimal culture conditions necessary to promote the generation of articular-like chondrocyte cells from the human iPSC–MSC-like cells. To this end, we compared the effects of different high density culture systems on the chondrogenic differentiation potential of the newly-derived mesenchymal progenitors. iPSC–MSC-like cells were cultured as either adherent micromass or suspension micropellets in chondrogenic media with or without human recombinant BMP-2 for up to 21 days. Q-PCR analyses of key chondrogenic genes was used to evaluate the chondrogenic efficiency of the YK26-iPSC cells in micromass versus micropellet culture at 7, 14, and 21 days of differentiation.

As compared to the undifferentiated MSC population (Day 0), transcript induction of *Sox9* was significant in both Day 7 micromass (7.38 ± 1.46 fold) and micropellet cultures (16.55 ± 3.28 fold) in response to BMP-2 treatment (Fig. 5A). Over the time course of in vitro differentiation, the highest induction of *Sox9* expression was observed in Day 14 micropellets treated with BMP-2 (19.72 ± 3.15 fold). As shown in Figure 5B, minimal to no induction of *Col2a1* mRNA expression was detected in untreated micropellet cultures over 7–21 days, relative to undifferentiated iPSC-derived progenitors (Day 0). BMP-2 treatment was necessary to induce *Col2a1* gene expression in the micropellets (150.45 ± 16.20 fold induction at Day 21). Superior chondrogenic differentiation was observed in BMP-2 treated micromass cultures of iPSC–MSC-like cells, shown by the progressive accumulation of *Col2a1* transcripts through 7–21 days (240–55,125 fold induction; Fig. 5B). BMP-2 treated iPSC–MSC-like micromass cultures expressed reduced levels of *Col1a1* and elevated levels of *Col2a1*, reflected by a higher overall *Col2a1* to *Col1a1* expression ratio (226,128 fold at Day 21), as compared to BMP-2 treated micropellet cultures (12.78 fold at Day 21; Fig. 5D). The higher overall *Col2a1* to *Col1a1* expression ratio was indicative of a more chondrocytic versus fibroblastic gene expression. Consistent with the effects of BMP-2 treatment on *Col2a1* expression, enhanced expression of

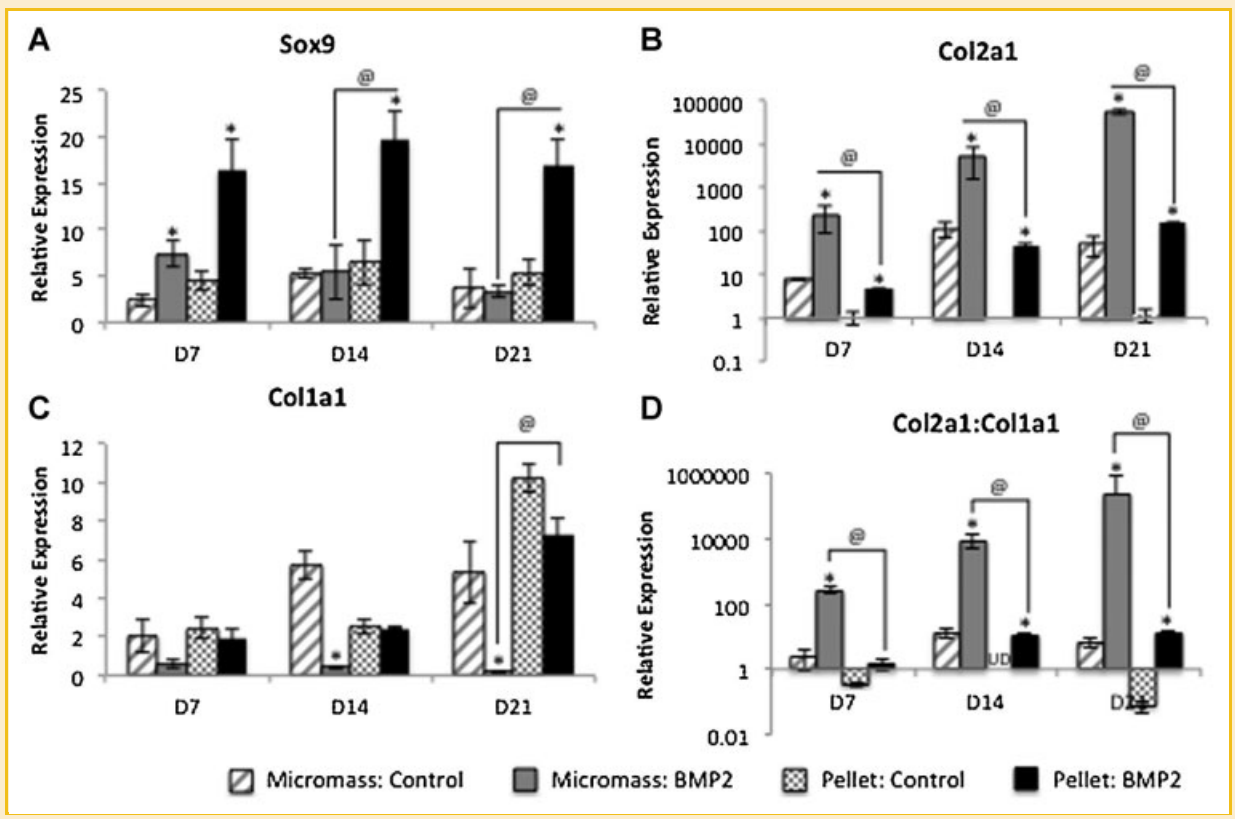

Fig. 5. High density micromass conditions influence the chondrogenic potential of iPSC–MSC-like cells. Profile of temporal gene expression determined by Q-PCR in YK26-MSC-like micromass versus micropellet cultures treated with and without BMP-2. Genes analyzed included (A) *Sox9*, (B) *Col2a1*, and (C) *Col1a1*. The ratio of *Col2a1* to *Col1a1* transcript expression is shown in (D). *Gapdh* served as the housekeeping gene and internal control. Gene expression is represented as fold induction relative to the undifferentiated iPSC–MSC-like cells (Day 0), set at 1.0. Asterisk (*) denotes significance versus untreated iPSC–MSC-like cells for each timepoint at $P < 0.05$. @ denotes significant differences between BMP-2 treated iPSC–MSC-like micromass versus BMP-2 treated pellet cultures for each timepoint at $P < 0.05$.

aggrecan was observed in both BMP-2 treated micromass and micropellet cultures, with the highest level of induction observed in BMP-2 treated micromass cultures (data not shown). Notably, *type X collagen* gene expression was not detected in either BMP-2 treated micromass or micropellet cultures of iPSC-derived mesenchymal progenitors, indicating a lack of chondrocyte hypertrophy over the course of the differentiation assays. Thus, based on gene expression analyses, micromass cultures of iPSC–MSC-like cells treated with BMP-2 provided a superior platform for chondrogenic induction as compared to the pellet culture method.

DISCUSSION

The aim of this study was to evaluate the chondrogenic potential of human iPSCs versus iPSC-derived progenitors exhibiting an intermediate stage of development between a stem cell and a fully differentiated state. Using standard high density culture systems commonly employed for the chondrogenic induction of human mesenchymal and pluripotent stem cells, we established that the chondrogenic efficiency of iPSCs was influenced by the progenitor properties of the cells and as well as the high density cell culture environment.

Controlled, uniform differentiation to the chondrogenic lineage is essential for procuring iPSC-derived chondroprogentitor cells for autologous cell-based cartilage repair. Various growth factor cocktails including TGFßs and BMPs have been used to enhance the chondrogenic potential of high-density cultures of human ESCs and iPSCs in vitro. It is well established that BMP signaling exerts multiple stage-specific effects on cartilage development [Kramer et al., 2000; Pizette and Niswander, 2000; Yoon and Lyons, 2004]. In the early stages of skeletal development, signaling by BMP is critical for the compaction and tight association of mesenchyme cells, and establishes the prechondrogenic condensations that shape the cartilage template [Pizette and Niswander, 2000; Barna and Niswander, 2007]. At later stages, BMP signaling induces collagen and proteoglycan production, and is necessary for differentiation into chondrocytes [Yoon et al., 2005]. In the present study, the direct plating of human iPSC into micromass, without prior EB formation, was sufficient to induce chondrogenic differentiation of the cells. Treatment with BMP-2 significantly enhanced their chondrogenic potential, as shown by increased cartilage specific gene expression. Interestingly, the transient exposure to BMP-2 (Days 2–4) elicited similar effects on cartilage-specific gene induction as chronic BMP-2 treatment. These findings suggest that signaling by exogenous BMP-2 provides an initial stimulus to direct tight mesenchymal cell–cell associations through compaction and the recruitment of iPSCs to the chondrogenic lineage [Fischer et al., 2002; Barna and Niswander, 2007]. However, BMP-2 treatment of iPSC-derived micromass was not sufficient to restrict chondrocyte differentiation to an articular-like phenotype in vitro, but rather favored progression toward chondrocyte maturation. This was shown by significant down-regulation of *Col2a1*, concomittant with the induction of markers of chondrocyte maturation, *Runx2* and *type X collagen*. Moreover, based on induction of *type I collagen (Col1a1)*, a major component of fibrous tissue, substantial culture heterogeneity was observed with BMP-2 treatment of iPSC micromass cultures. Together, these in vitro data indicate that the culture conditions typically used to promote chondrogenic induction of human pluripotent stem cells may not be optimal for the generation of articular-like chondrocytes. In addition to their chondrogenic effects, growth factors such as BMPs elicit pleiotrophic responses in many different cell types [Sieber et al., 2009]. Thus, it is unlikely that a scaleable, homogenous population of chondrocytic cells suitable for articular cartilage repair may be obtained by growth factor-mediated differentiation of human pluripotent stem cells [Nakayama and Umeda, 2011].

The enrichment of the relevant musculoskeletal progenitors constitutes a necessary first step toward the bulk production of a chondrolineage-restricted progeny and also for reducing or eliminating the inherent risk of contaminating pluripotent cells. Various methodologies have been described for the derivation of functional, multipotent MSCs from human ESCs and iPSCs, including (i) manual dissection of embyroid body (EB) outgrowths [Hwang et al., 2008b]; (ii) co-culture with murine OP9 cells [Barberi et al., 2005]; and (iii) fluorescence activated cell sorting of specific cell populations [Lian et al., 2007]. Using a relatively straightforward direct plating strategy [Nakagawa et al., 2009] without the requirement of EB formation or cell selection, we derived a self-renewing population of mesenchymal progenitors from human iPSCs. A combination of gene analyses and FACS characterization of cell surface antigen expression was used to demonstrate the mesenchymal stem cell-like properties of the derived YK26-iPSC population. Moreover, we confirmed the potential of iPSC–MSC-like progenitors to generate osteoblasts, adipocytes, and chondrocytes in vitro. We observed notable differences in the overall pattern of gene expression in chondrogenic iPSC micromass cultures as compared to iPSC–MSC-like micromass cultures treated with BMP-2. Chondrogenic micromass cultures derived from iPSC–MSC-like cells displayed a progressive induction of *Col2a1* transcript expression in response to BMP-2 over 21 days (Fig. 5B) that was not observed in iPSC micromass cultures treated with BMP-2 over the same time course (Fig. 2B). Whereas Col1a1 transcript expression was progressively induced in iPSC micromass cultures in response to BMP-2 over 7–21 days in culture (Fig. 2F), BMP-2 treatment repressed the accumulation of Col1a1 transcripts in iPSC–MSC-like micromass cultures (Fig. 5C). Moreover, BMP-2 treatment of iPSC micromass resulted in the induction of ColXa1 transcripts that was not observed in iPSC–MSC-like micromass cultures. These chondrogenic assays indicated that derivation of a mesenchymal stem cell population from human iPSCs was necessary to limit the extent of cellular heterogeneity in micromass cultures. Moreover, in contrast to the hypertrophic chondrocyte differentiation observed in BMP-2 treated chondrocytic micromass cultures generated from undifferentiated iPSC, there was no evidence of chondrocyte maturation in MSC cultures treated with BMP-2.

The high density culture systems used to induce chondrogenesis of human pluripotent and multipotent stem cells are thought to recapitulate the cell–cell interactions within the pre-cartilage condensations of the developing embryo [Pizette and Niswander, 2000; Barna and Niswander, 2007]. One of the most relevant findings of our study is that superior chondrogenic differentiation of

human iPSC–MSC-like cells was achieved using the micromass culture system as opposed to the pellet culture system. These observations are in agreement with previous studies demonstrating that the chondrogenic activity of human bone marrow-derived MSCs was superior in micromass versus micropellet cultures. Studies by Zhang et al. [2010] showed enhanced Col2a1 protein expression, decreased chondrocyte maturation and as well as decreased fibrocartilage-like formation in MSC micromass cultures as compared to micropellet cultures [Zhang et al., 2010]. Similarly, we demonstrated greater enrichment of cartilage-sprecific *Col2a1* and decreased expression of *Col1a1* in BMP-2 treated iPSC-derived MSC micromass cultures as compared to micropellet cultures. Interestingly, we found that increased induction of *Col2a1* and *aggrecan* transcripts in iPSC–MSC-like micromass culture did not correlate with enhanced *Sox9* expression. This phenomenon was also reported in other chondrogenic cell culture systems [Murdoch et al., 2007; Zhang et al., 2010], and suggests the involvement of additional factors in promoting cartilage extracellular matrix induction in micromass. It is suggested that superior chondrogenic differentiation capacity in micromass may be attributed to improved cell–cell contact and nutrient diffusion as compared to the pellet system [Zhang et al., 2010]. Furthermore, micromass cultures represent a more convenient culture system for obtaining large numbers of chondrogenic cells necessary for in vitro analyses and in vivo studies, and may further provide the ideal platform for future translational studies.

The generation of a self-renewing, homogenous population of MSCs from human pluripotent stem cells may offer distinct advantages over the use of adult MSCs for the treatment of cartilage defects, as well as other musculoskeletal disorders. Cellular reprogramming may overcome the age-related changes that arise in human MSC populations, as functional reprogramming of human aged donor cells mediated telomere elongation and resulted in the ''rejuvenation'' of telomeres, with iPSCs acquiring the epigenetic marks typical of human ESCs [Marion et al., 2009]. Lian et al. [2010] also demonstrated enhanced survival and engraftment of the iPSC-derived MSCs in mice, resulting in superior attenuation of hindlimb ischemia as compared to adult bone marrow-derived MSCs. Future studies in animal models of cartilage defects will evaluate the phenotypic stability and the repair capacity of iPSC-derived MSCs in vivo.

Our data suggest that the degree of chondrocyte maturation and culture heterogeneity in differentiating iPSC cultures may be modulated by the enrichment of a mesenchymal progenitor population and by the appropriate choice of high density culture system. Overall, BMP-2 treatment of micromass cultures of progenitor cells derived from human iPSCs generated a phenotype more typical of articular-like chondrocytes and may represent an appropriate strategy for future tissue engineering applications.

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