Small Molecule Mesengenic Induction of Human Induced Pluripotent Stem Cells to Generate Mesenchymal Stem/Stromal Cells

YEN SHUN CHEN, a* REBECCA A. PELEKANOS, a* REBECCA L. ELLIS, a RACHEL HORNE, b ERNST J. WOLVETANG, b NICHOLAS M. FISK a

Key Words. Mesenchymal stem cells • Pluripotent stem cells • Differentiation • Induced pluripotent stem cells

INTRODUCTION

Because of their immunosuppressive properties and ability to differentiate into a wide range of mesenchymal-lineage tissues, mesenchymal stem/stromal cells (MSCs) are under intense investigation for applications in cardiac, renal, neural, joint, and bone repair, as well as in inflammatory conditions and hematopoietic cotransplantation. MSCs are typically harvested from adult bone marrow or fat, but these not only require painful invasive procedures but are low-frequency sources, with MSCs making up only 0.001%-0.01% of bone marrow cells and 0.05% in liposuction aspirates [1, 2]. Of concern for autologous use, particularly in the elderly most in need of tissue repair, MSCs decline in quantity and quality with age [3, 4]. Perinatal sources seem attractive, with greater frequency and higher proliferative-potential MSCs, but a major limitation is that MSCs can typically only be isolated from around one-third of umbilical cord blood specimens [1, 2]. Earlier fetal, placental, and amniotic sources yield abundant primitive MSCs with greater differentiative ability than later sources [5, 6], but they require invasive procedures and/or access to abortal tissue, with its attendant ethical issues. There is thus a major unmet need for new sources of MSCs for clinical application.

The established method for differentiating human embryonic stem cells (hESCs) into various cell types, including MSC-like cells, involves embryoid body (EB) formation [7, 8]. Other attempts to derive MSCs from pluripotent cells have required cumbersome or untranslatable techniques, such as murine coculture, physical manipulation, sorting, or viral transduction [9–13]. Thus there is considerable interest in developing xenogen-free, sorting-free, EB-free culture...
systems to differentiate ESCs into MSCs [14]. Epithelial-to-mesenchymal transition (EMT) has recently been implicated in the first stages of mesoderm commitment in hESCs [15], and epithelial cell adhesion molecule (EpCAM)/CD326- neural cell adhesion molecule (NCAM)/CD56 cells have been shown to be the precursors of lineage-restricted mesodermal progenitors [16]. Interestingly, the reverse process of mesenchymal-to-epithelial transition has been implicated in the reprogramming of fibroblasts to iPSCs [17, 18]. Boyd et al. used a 30-day epithelial culture to differentiate manually dissociated hESCs, which after subsequent passaging underwent an apparent spontaneous EMT, generating cells with decreased pluripotency and increased mesodermal/MSC marker expression and bilineage mesenchymal differentiation potential [19].

The fibroblast growth factor/transforming growth factor-β (TGF-β) pathways are known to maintain hESCs in a pluripotent state [20–22]. The TGF-β pathway inhibitor SB431542 induces differentiation of ESCs into several cell types, including retinal [23], endothelial [24], and neural cells [25]. SB431542 inhibits activation of the activin receptor-like kinase (ALK) receptors 4, 5, and 7, key members of the TGF-β signaling pathway, and is thought to induce EMT differentiation by inhibiting SMAD2/3 phosphorylation [20]. In support of this notion, SB431542 decreases SMAD2/3 binding to a SMAD-responsive element in the NANOG promoter controlling NANOG promoter activity, which otherwise maintains OCT4, SOX2, and NANOG expression and pluripotent cells in an uncommitted state [26].

Mahmood et al. treated hESCs with SB431542 during EB formation to upregulate paraxial mesodermal and myogenic markers and then derived MSC-like cells from EB outgrowth cultures [10]. Sanchez et al. exposed hESCs to SB431542 for 28 days to induce differentiation into multipotent progenitors [14]. After sorting for CD73-CD90-, a minority of these cells demonstrated mesenchymal characteristics. We hypothesized that shorter term exposure of human pluripotent cells to SB431542 would initiate EMT without myogenic commitment, and allow MSC-like cells to be derived without the need for EB or selection steps.

Here we report a single-step EB-free small molecule-based method that yields MSC-like cells from both human ESCs and iPSCs with high efficiency; we also report that these MSC-like cells resemble primary MSCs in terms of immunophenotype and differentiation potential. Induced pluripotent stem (IPS)-MSCs derived by this method offer a scalable, ethically unencumbered source for cell therapy and tissue engineering applications.

**MATERIALS AND METHODS**

**Cell Culture**

**Mesenchymal Cell Isolation and Culture**

Fetal tissue collection at termination of pregnancy was approved by the Human Research Ethics Committee of the Royal Brisbane and Women’s Hospital. Adult MSCs were purchased from StemCell Technologies (Vancouver, BC, Canada, http://www.stemcell.com). MSC medium consisted of Dulbecco’s modified Eagle’s medium-high glucose (DMEM-HG) supplemented with 10% non-heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 50 U/ml penicillin/streptomycin, and 20 μg/ml gentamicin (all from Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Fetal MSCs were isolated as previously described [5]. Briefly, first-trimester fetal bone marrow MSCs were prepared by flushing the bone marrow cells out of humeri and femurs using a syringe and 22-gauge needle. The resultant cells were allowed to adhere to a standard tissue culture flask for 72 hours in MSC medium before being washed and passaged with Tryple-Select (Invitrogen) upon confluence. Fetal and adult MSCs were cultured under humidified conditions in 5% CO2, and MSCs were routinely cryopreserved in 90% FCS and 10% dimethyl sulfoxide (DMSO).

**Pluripotent Cell Culture**

Human iPSCs (MR90CL2 and ES4CL1 [27]) and ESCs (Mcl1 and HEK3 [28]) were supplied by StemCore (Australian Stem Cell Centre, Brisbane, Australia; www.aibn.uq.edu.au/pluripotent-stem-cell-core-facility) under the appropriate human ethics and material transfer agreements. iPSCs and ESCs were routinely grown as bulk cultures on irradiated mouse embryonic fibroblasts (MEFs) (12,000 cells per cm²) in medium consisting of DMEM-Ham’s F-12 basal medium supplemented with 20% knockout serum replacement (KOSR), 1 mM L-glutamine, and 10 mM nonessential amino acids (all from Invitrogen). Basic fibroblast growth factor (bFGF) (100 ng/ml for iPSCs or 8 ng/ml for ESCs) was added and is required to maintain pluripotency [27, 29]. This medium is referred to as KOSR+bFGF medium. Data shown are from the iPSC line MR90CL2 and the ESC line Mel1 unless stated otherwise.

**SB431542 Inhibitor Differentiation Method**

To develop a feeder cell-free method to produce MSCs from ESCs or iPSCs, we first used a widely used commercially available defined medium, mTeSR1 (StemCell Technologies), which, in combination with the cell attachment matrix Matrigel (BD Biosciences, San Diego, http://www.bdbiosciences.com), maintains pluripotency of ESCs/iPSCs without the need for feeder cells or additional bFGF [27, 30].

ESC or iPSC bulk cultures were seeded into mTeSR1 medium (as per the manufacturer’s instructions) on Matrigel-coated vessels (100 μg/ml in DMEM-Ham’s F12 medium, incubated for 1 hour at room temperature). For best results, cells were seeded as large colonies at high confluence, and some cell lines required a one-passage adaptation to mTeSR/Matrigel conditions prior to MSC differentiation. Once cells were confluent, the medium was changed to inhibitor differentiation medium that consisted of KOSR medium (composition detailed above) without bFGF, supplemented with 10 μM SB431542 (SB) in DMSO (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com; referred to as KOSR+SB medium; supplemental online Fig. 1A). Once feeder-free cultures were established, the switch back to KOSR without bFGF was necessary to enhance differentiation, as it lacks the trophic factors present in mTeSR. The KOSR+SB medium was replaced daily for 10 days, with cells then passaged to a single-cell suspension using TrypleSelect (Invitrogen). At the first mesenchymal passage (MP0), single cells were reseeded at a density of 40,000 cells per cm² into MSC medium. Cells were then seeded at 20,000 cells per cm² (MP1) in MSC medium and at 10,000 cells per cm² with subsequent passages (MP2, MP3, etc.).

**Embryoid Body Differentiation Method**

iPSC and ESC colonies were cultured under standard conditions (KOSR+bFGF on MEFs, as described above) in T75 flasks, and once confluent, colonies were detached using a cell scraper and cultured 1:1 in ESC medium without bFGF (referred to above as KOSR medium) for 10 days in 10-cm non-tissue-culture-treated dishes (supplemental online Fig. 1B). EBs were then transferred...
to a standard T75 tissue culture flask containing MSC medium and allowed to adhere to the flask (typically overnight). Differentiated cells grew outward from the center of the EBs and formed a heterogeneous cell layer. After approximately 1 week, the undifferentiated cells in the center of the colony were aspirated, and the differentiated outgrowth cells were further cultured in MSC medium as per the inhibitor method above.

RNA Preparation and Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction

RT² Profiler polymerase chain reaction (PCR) array (SA Biosciences; Qiagen, Hilden, Germany, http://www1.qiagen.com) was performed on undifferentiated ESCs/iPSCs, on ESCs/iPSCs after 10 days of incubation with SB431542, and on ESC- and iP-SCs at MP2 and MP1, respectively. Total RNA was extracted using a RNeasy mini kit (Qiagen) from cells seeded into six-well plates, and 2 μg of total RNA was used to generate cDNA (First Strand Kit, SA Biosciences). The cDNA was diluted to 1.3 ml and mixed with an equal amount of RT² SYBR Green qPCR Master mix (SA Biosciences). The cDNA-SYBR Green master mix was aliquoted into each well of a 96-well PCR array plate containing predisposed gene-specific primer sets, and PCR was performed as per the manufacturer’s instructions. The thermal cycling conditions started with 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and then 56°C for 1 minute. We designed a customized array plate (SA Biosciences), selecting 91 genes associated with MSCs, EMT, and pluripotency, as well as mesodermal, endodermal, and ectodermal lineage markers. Expression data were normalized to glyceraldehyde-3-phosphate dehydrogenase, and the threshold cycle (Ct) and fold change (ΔΔCt) were calculated for each gene using proprietary software (Qiagen). Heat maps were generated by GenePattern (Broad Institute, Cambridge, MA, http://www.broadinstitute.org/cancer/software/genepattern/).

Supplemental Methods

Experimental procedures for flow cytometry, mesodermal differentiation, immunofluorescence staining, growth kinetics, Western blot, teratoma assay, and karyotypic analysis are detailed in the supplemental online data.

RESULTS

SB431542 Promotes ESC/iPSC Differentiation Toward Epithelial-like Morphology Without Embryoid Body Formation

In the absence of SB431542, undifferentiated iPSCs and ESCs cultured on Matrigel in mTeSR medium appeared as dense, multilayered colonies of tightly packed cells with a low cytoplasmic to nuclear ratio and only a few spontaneously differentiating cells around the periphery (Fig. 1A; supplemental online Fig. 1C). Incubation of cells with SB431542 for 10 days induced differentiation in both iPSCs and ESCs (Fig. 1B; supplemental online Fig. 1D), as indicated by the formation of a monolayer of large cuboidal-shaped epithelial-like cells occupying 60%–80% of the culture vessel. To enhance the proportion of cells adopting this epithelial phenotype under SB431542 treatment, we next cultured iPSCs and ESCs in KOSR medium as shown in Figure 1C and 1D and supplemental online Figure 1E and 1F. In contrast to mTeSR medium, iPSC and ESCs cultured in KOSR without bFGF differentiated more completely under the influence of SB431542 into monolayers with an epithelial-like morphology (~90%). Regions of differentiation were evident either on the periphery or the center of a colony, leaving only small islands of undifferentiated cells (Fig. 1E), and the differentiative morphology was more evident with ES4CL1, Mel1, and HES3 cultures than MR90CL2. Furthermore, 2 days after passage into MSC medium, the qualitative survival of cells to MP1 was vastly different, with only the KOSR+SB cells rapidly attaching and proliferating in MSC medium, whereas in all other test groups, very few cells attached (Fig. 1A–1D, right panels). The reason for KOSR being a more suitable differentiation medium remains unclear but may be related to different concentrations of bFGF and TGF-β in mTeSR compared with KOSR medium.

Flow cytometry was performed to examine the efficiency of the different culture conditions and determine the lineage of the intermediate cells (supplemental online Fig. 2). The early mesodermal progenitor population of EpCAM⁺ NCAM⁺ cells as described by Evseenko et al. [16] was predominant in all culture conditions (75%–79% of cells for mTeSR and KOSR – or + SB), although incubation with SB431542 increased this population (for mTeSR-SB, 75%, vs. mTeSR+SB, 97%; KOSR–SB, 89%, vs. KOSR+SB, 96%). mTeSR-SB and, to a lesser extent, KOSR–SB had a larger population of EpCAM⁺ and EpCAM⁺ NCAM⁺ cells, indicating more pluripotent cells present. Similarly, a large proportion of cells were CD24⁺ (pluripotent stem cell) and CD31⁻ (endothelial cell marker). The MSC markers CD73 and CD105 were largely negative regardless of basal medium or presence of SB431542, with the small percentage of CD73⁻ and CD105⁻ cells likely due to spontaneous differentiation. CD90 was positive in approximately 50% of cells in mTeSR, but it was not influenced by the presence or absence of SB431542.

SB431542-Treated iPSCs and ESCs Passaged in MSC Media Rapidly Differentiate into MSC-like Cells

When KOSR+SB iPSCs and ESCs were passaged in MSC medium (DMEM-HG + 10% fetal bovine serum), by the first or second passage the cells differentiated into spindle-shaped fibroblast-like cells (termed embryonic stem [ES]- or iP-SCs [inhibitor]), with morphology similar to that of fetal MSCs (fMSCs) and adult MSCs (Fig. 1F). In contrast, cells cultured in mTeSR with or without SB431542 treatment failed to attach and proliferate to confluence in MSC medium (Fig. 1A–1D, right panels), suggesting that the induction of the intermediate epithelial-like stage by SB431542 in KOSR is a prerequisite for MSC-like cell generation from iPSCs/ESCs under these conditions.

Derivation of Comparable MSC-Like Cells from Optimized EB Method

To compare our small molecule monolayer protocol with conventional EB-based methods, we optimized previously published protocols to generate EBs from the same human iPSCs and ESCs (supplemental online Fig. 1B). The optimized procedure did not require any coating of the tissue culture vessel, addition of growth/differentiation factors, multiple steps, or manual sorting of EBs as used in published EB protocols to derive MSCs. These were first cultured as spheres for 10 days (Fig. 2A, B) in KOSR medium without bFGF, before being transferred to standard tissue culture flasks containing MSC medium where the EB attached to the surface (Fig. 2C, D). The undifferentiated center of each EB was manually removed by aspiration, and the remaining
outgrowth cells were left in culture until confluent (~1 week). These cells initially had a mixed morphology, but those remaining in culture by MP2–MP3 possessed a comparable MSC-like morphology with a characteristic fibroblast shape (Fig. 2E, 2F). The resultant cells were termed iPS-MSCs (EB) and ES-MSCs (EB).

SB431542- and EB-Derived Cells Show MSC-Like Markers, Growth Kinetics, and Differentiation Capacity

To assess the immunophenotype of derived MSC-like cells, flow cytometry at MP6 was compared with primary fMSCs at an equivalent passage and with undifferentiated iPSCs. Figure 3 shows that iPSC-derived MSC-like cells robustly expressed typical MSC markers [31], such as CD73, CD90, and CD105, as well as CD29, CD44, and CD146 and low-level Human Leukocyte Antigen (HLA)-ABC. In agreement with standard criteria for MSCs [31], iPSC MSC-like cells derived by either method lacked expression of the macrophage and monocyte markers CD11b and CD14, human leukocyte marker HLA-DR, and the broad hematopoietic markers CD45 and CD34 (Fig. 3). In addition, iPSC-derived MSCs were negative for the endothelial marker CD31 and the primitive

Figure 1. Morphology of iPSC-derived MSCs by the SB method. Representative phase contrast images of iPSCs incubated for 10 days in mTeSR medium (A), mTeSR medium + SB (B), KOSR medium (C), or KOSR medium + SB (D) (magnification, ×4 and ×20 for each). Left panels show cells at 10 days in the designated conditions, and right panels show the same cells 2 days after passage into MSC media. Qualitative survival of cells to MP1 based on the images is indicated on the far right, with — indicating no cells attaching and surviving, + indicating a few cells, and ++++ indicating many cells. (E): Regions of SB-induced differentiation were evident as cells developing an enlarged, flattened epithelial-like morphology either on the periphery of colonies (left images) or at the center of a colony (right images, ES4CL1 cells shown). Arrows mark the boundary between undifferentiated cells and epithelial-like cells observed after 10 days SB treatment. (F): ES cell- and iPSC-derived MSCs from the SB method (MP2 and MP1, respectively) showed a fibroblast-like morphology similar to that of primary adult and fetal bone marrow MSCs (magnification, ×10). Abbreviations: ES, embryonic stem; inhib, inhibitor; iPS, induced pluripotent stem; KOSR, knockout serum replacement; MP, mesenchymal passage; MSC, mesenchymal stem/stromal cell; SB, SB431542.

Figure 2. Morphology of ESC- and iPSC-derived mesenchymal stem/stromal cells (MSCs) by the embryoid body (EB) method. Shown is the morphology of the ESCs (A) and iPSCs (B) grown for 10 days in EB suspension culture (magnification, ×4). EBs were then transferred into tissue culture flasks with MSC medium, where they rapidly attached to the vessel ([C] and [D]; images are 4 days after attachment), after which the center of undifferentiated cells was removed by aspiration, and outgrowth cells were allowed to become confluent before further passaging. (E, F): ESC- and iPSC-derived MSCs form the EB method (mesenchymal passage 2) showed a standard MSC-like fibroblastic morphology. Abbreviations: ESC, embryonic stem cell; iPS, induced pluripotent stem cell.
hematopoietic/progenitor marker CD117, as were fMSCs [31]. With the exception of CD90 and the integrins CD49b and CD29, antigens known to be expressed by ESCs [32], the parental iPSCs lacked expression of all MSC markers. Conversely, iPSC-derived MSC-like cells and fMSCs lacked expression of CD24, a marker known to be expressed by pluripotent stem cells. The immunophenotype of ES-MSCs generated by either method (inhibitor or EB) was identical to that of iPS-MSCs (data not shown). We conclude that pluripotent derived MSC-like cells displayed an immunophenotype that closely resembled that of primary MSCs and differed markedly from that of undifferentiated iPSCs.

We next subjected iPS- and ES-MSCs to standard osteogenic, chondrogenic, and adipogenic differentiation conditions. As shown in Figure 4, iPS- and ES-MSCs displayed robust osteogenic and chondrogenic differentiation under permissive conditions, as indicated by von Kossa and periodic acid-Schiff staining, respectively. However, both iPSC- and ESC-derived MSC-like cells showed little adipogenic differentiation compared with fMSCs, although some evidence of lipid droplet accumulation could be detected after extending culture to 30 days (Fig. 4). We conclude that iPS- and ES-MSCs displayed a greater propensity to undergo osteogenic and chondrogenic differentiation than adipogenic differentiation, and in this respect, our results resemble findings reported in ESC-derived MSCs [13, 19, 33], fetal, and other gestational product-derived MSCs [2, 5, 34–38].

We then compared the growth kinetics of iPS-MSCs (inhibitor and EB) with adult and fetal MSCs. Over the first 30 days, population doublings of iPS-MSCs (inhibitor) were comparable to those of fetal MSCs, whereas iPS-MSCs (EB) proliferated at a similar rate to adult MSCs (Fig. 5A). iPS-MSCs (inhibitor and EB) reached cumulative population doublings of 14–15, proliferated to passage 10, and grew in culture for 80 days before senescence was observed (Fig. 5B). However, when examined over longer time periods, these ex vivo expansion rates in the two derived iPS-MSC populations converged and were similar to those of adult MSCs [5, 39]. Nevertheless because of the superior proliferation capacity of iPS-MSCs (inhibitor) during the early expansion phases, the cumulative cell number in iPS-MSCs (inhibitor) was markedly higher than that of iPS-MSCs (EB) (≈5 × 10¹⁰ vs. 1.5 × 10¹¹, Fig. 5C).

**Immunocytochemical Correlates of Mesengenesis**

During SB431542 treatment, we observed clear evidence of differentiation into larger epithelial-like cells in the center of colonies that spread outward, as well as some differentiation at the periphery of the colony (Figs. 1E, 5D–5G). By day 10, these regions merged to form a differentiated monolayer, with only a few ridges of undifferentiated cells remaining. To determine the mechanism of SB431542-induced differentiation, immunofluorescence microscopy was used to show that both central and peripheral regions of differentiation were negative for nuclear OCT4 staining by day 4, although surrounding undifferentiated cells remained OCT4 positive (Fig. 5D, 5E). Similarly, plasma
membrane E-cadherin (ECAD) was not present in the differentiated cells but was positive in the as yet undifferentiated cells (Fig. 5F, 5G). These pluripotent stem cell markers, as well as others, including Tra 1–81 (Fig. 5H, 5I, center panels), Stella, Tra 1–60, SSEA3, and EpCAM (not shown), were also absent in MP2 cells. However, nuclear NANOG staining was seen in both differentiated and undifferentiated cells at day 4 but was markedly absent by MP2 (Fig. 5H, right panels). In parallel to losing pluripotency markers, the derived cells acquired expression of the mesodermal markers N-cadherin (NCAD) and vimentin (Fig. 5I, left and center panels, respectively) during differentiation from iPSCs to iPSC-MSCs (inhibitor), further supporting the idea that iPSCs had indeed undergone bona fide differentiation into iPSC-MSCs. Positive human nuclear antigen staining confirmed that the MSC-like cells were human in origin and not contaminating mouse embryonic fibroblasts used as a feeder layer for the undifferentiated pluripotent stem cells (Fig. 5I, right panel).

**Gene Expression Analysis of SB431542-Induced MSC Differentiation from Human iPSCs and ESCs Implicates EMT**

To investigate the effect of SB431542-induced differentiation under various culture conditions, mRNA expression levels were analyzed using a customized quantitative real-time PCR (qPCR) array. The expression of 91 genes associated with pluripotency, mesodermal, endodermal and ectodermal lineages, as well as markers of MSCs, EMT and the TGF-β pathway was compared between undifferentiated cells, cells treated with SB431542 for 10 days, and iP- and ES-MSCs. The 53 genes showing substantial change in expression level are listed in supplemental online Table 1 and displayed as a heat map and scatter plot (Fig. 6). These indicate clear differences in gene expression between undifferentiated iPSCs/ESCs, the resultant cells cultured in KO5/SB431542 (Fig. 6B, 6C), and the iPSC/ESC-derived MSCs (Fig. 6D, 6E).

As expected, expression of genes involved in maintaining pluripotency, such as OCT4 (Pou5F1), was decreased in SB-treated cells and MSC-like cells in comparison with the undifferentiated iPSCs/ESCs (Fig. 7A; supplemental online Fig. 3A). Interestingly, iPSCs showed increased expression of classic pluripotency markers SOX2 and ECAD in the presence of SB431542, but expression decreased once cells were transferred to MSC medium (Fig. 7B, 7C).

Following SB431542 treatment, we detected upregulation not only of ectodermal lineage genes, such as PAX6 and CDX2 (Fig. 7D, 7E; supplemental online Fig. 3D, 3E; supplemental online Table 1), but also mesodermal genes, such as NCAM, BMP4, and MSX2 (Fig. 7F–7H; supplemental online Fig. 2F–2H), and endodermal genes SOX7 and SOX17 (Fig. 7I, 7J; supplemental online Fig. 3I, 3J), suggesting that TGF-β kinase inhibition led to multilineage differentiation outcomes without a particular bias toward one of the germ layers. The expression of mesodermal genes LEFTY1 and LEFTY2 (Fig. 7K, 7L; supplemental online Fig. 3K, 3L) was also downregulated, and the SMAD2/3 complex, which is specifically dephosphorylated by SB431542, is a known upstream factor for LEFTY2 gene expression [40]. We confirmed that SB431542 treatment does decrease phosphorylated SMAD2 (pSMAD2) at the protein level in our system for both iPSCs and ESCs in both mTeSR and KO5 culture media at day 10 (Fig. 6F).

In SB431542-treated iPSCs/ESCs, MSC and EMT marker genes were not highly expressed after 10 days of SB431542 treatment. However, when 10-day SB-treated ESCs and iPSCs were subsequently cultured in MSC medium, expression of the MSC marker genes CD73, CD105, CD29, and CD44 (Fig. 7M–7P; supplemental online Fig. 3M–3P) was upregulated after one or two passages, concomitant with the marked change in morphology to a fibroblast-like shape (Fig. 1F). Our data further demonstrate that ES- and iPSC-MSCs cultured in MSC medium showed a substantial increase in expression of the mesodermal-linked EMT marker genes NCAD, Zeb1, SNAI1, and TWIST2 (Fig. 7Q–7T; supplemental online Fig. 3Q–3T). This induction of EMT under MSC culture conditions may be the mechanism by which SB431542-induced monolayer multipotent progenitor cells differentiate toward CD73$^+$, CD105$^+$, CD29$^+$, CD44$^+$ MSC-like cells.

Interestingly, there were several examples of differential expression of key pluripotency and differentiation makers between ESCs and iPSCs in this system. Treatment of ESCs with SB431542 induced large increases in the expression of trophoblast markers MSX2 (164-fold), GATA2 (375-fold), and CDX2 (41-fold), whereas iPSCs displayed a more modest increase in these genes (MSX2, 14-fold; GATA2, 9-fold; and CDX2, 4-fold). These
genes decreased almost to basal levels in the MSC culture, suggesting that the intermediate cells may be similar to early trophoblasts. Although OCT4 expression was dramatically reduced in cells treated with SB431542 for 10 days, we noticed that NANOG expression was unchanged in SB431542-treated iPSCs and actually increased 15-fold in ESCs incubated long-term in SB431542, before decreasing to baseline after passaging in MSC medium. In agreement with our qPCR and immunostaining data (Fig. 5F–5H; supplemental online Table 1), persistent NANOG expression through the bFGF-mediated MEK-ERK pathway was...
recently shown to bias BMP4-induced human ESC differentiation into mesendoderm [41]. Because bFGF is present in the culture medium and RNA extracted after one or two passages, data are categorized into markers of pluripotency, germ layers (ectoderm, mesoderm, and endoderm), hematopoietic cells, MSCs, and EMT. Expression levels were normalized to GAPDH and compared with those in mTeSR culture conditions. The heat map profile is presented as follows: red, high expression; white, medium expression; blue, low expression relative to overall expression over the three samples (time points) for a given probe. (B–E): Scatter plot comparisons of the gene expression profiles of the undifferentiated iPSC mTeSR condition with iPSC KOSR + SB (B) and iPSC-derived MSCs (iPS-MSCs) (D) at mesenchymal passage 1, and similarly of gene expression profiles of undifferentiated ESCs in mTeSR with ESC KOSR + SB (C) and ESC-derived MSCs (ES-MSCs) (E) at mesenchymal passage 2. Pink lines indicate boundaries of fourfold difference in gene expression, and highly expressed marker genes are indicated. Gene expression levels are depicted on a log₁₀ scale. The scatter plots show the fold changes calculated using the $2^{-\Delta\Delta Ct}$ formula. (F): iPSCs and ESCs were cultured on a Matrigel-coated plate in mTeSR medium (lanes 1 and 4) and treated with 10 μM SB in either mTeSR medium (lanes 2 and 5) or KOSR medium (lanes 3 and 6). After 10 days of treatment, the proteins in the cell lysates were subjected to Western blot analysis for SMAD2 (upper panel) and pSMAD2 (middle panel), and equal loading was assessed by Western blotting of the cell lysates for actin (bottom panel). Abbreviations: EMT, epithelial-to-mesenchymal transition; ESC, embryonic stem cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iPSC, induced pluripotent stem cell; KOSR, knockout serum replacement; MSC, mesenchymal stem/stromal cell; SB, SB431542.

The mRNA levels of differentiation-specific markers determined by the qPCR array correlated empirically to protein expression determined by flow cytometry on samples before (mTeSR), during (KOSR + SB day 10), and after (iPS-MSC MP1– MP2) SB431542-induced differentiation of iPSCs (supplemental online Fig. 4). CD73 and CD105 gene and protein expression was negligible in mTeSR (undifferentiated) and high in KOSR + SB, low in MSC medium. NCAM expression was moderate to high in mTeSR, high in KOSR + SB, and low in MSC medium. CD34 and CD45 protein and RNA expression was negative/low for all conditions.
Although surface protein expression did not completely correlate with RNA expression, which may be due to rapid protein internalization in response to treatment, in most cases it was analogous. Differential expression of these cell surface markers during SB431542-induced differentiation supports insights from the array data into mechanism of mesodermal commitment.

**iPS-MSCs Have Normal Karyotype and Do Not Form Teratomas**

To test whether our monolayer SB-induced MSC differentiation protocol allowed the persistence of undifferentiated, potentially tumorigenic cells, we injected iPS-MSCs intramuscularly into NOD/SCID mice (n = 3/cell line) and assessed the formation of teratomas. Nine weeks after injection, no palpable teratoma had formed in any of three mice injected with iPSC-derived MSCs. In contrast, in two of three animals injected with undifferentiated iPSCs, teratoma formation was observed, which, as expected, displayed the presence of tissues derived from all three germ layers (supplemental online Fig. 5). Karyotype analysis of iPS-MSC-derived cultures at intermediate (5–6) and high (11–12) passage numbers showed the presence of 46 normal chromosomes, indicating that iPS-MSCs are genetically stable in culture (supplemental online Fig. 5).
**Discussion**

In this study, we devised a one-step method for generating ESC/iPSC-derived MSCs, which exhibit typical MSC characteristics that conform to the criteria of the International Society for Cell Therapy for classification of MSCs. These ES- and iPSC-MSCs lack teratoma-forming ability, display a normal stable karyotype in culture, and exhibit growth and differentiation characteristics that closely resemble those of primary MSCs. Because iPSCs can be harvested free of ethical constraints and culture can be expanded indefinitely, the simple EB-free differentiation protocol described has considerable potential for the in vitro scale-up required to enable a wide range of MSC-based therapies.

Our one-step, small molecule-based method allows for a substantially shorter culture period (20 days) to generate MSC-like cells compared with ~30 days of differentiation required in our EB-based protocol and 40+ days with other monolayer-based protocols [19]. A further advantage is that it avoids the initial heterogeneity of cell populations seen with the EB method when placed in MSC media and thus an apparent lower propensity for unwanted cell types remaining in the MSC culture. Indeed, our method allows rapid and complete MSC generation without the need for immortalization, coculture with mouse MSCs, epitope selection, or physical selection procedures. Similar to primary MSCs but in contrast to undifferentiated iPSCs and ESCs, the iP- and ES-MSCs did not require costly or cumbersome attachment factors, such as gelatin, fibronectin, or Matrigel, nor did they require a feeder layer or added growth factors to support growth. Therefore, the SB431542 protocol seems a promising strategy for driving differentiation of human iPSCs into MSCs destined for regenerative medicine applications.

SB431542 inhibits the activin/TGF-β pathways by blocking phosphorylation of ALK4, ALK5, and ALK7 receptors. Our Western blot data (Fig. 6F) confirmed that SB431542 specifically inhibits SMAD2 phosphorylation and causes a downregulation of LEFTY1 and LEFTY2 expression in iPSCs (~50-fold) and in ESCs (~20-fold), similar to that previously observed by Galvin et al. [43]. After 10 days of SB431542 exposure, iPSCs/ESCs presented a flattened monolayer morphology (Fig. 1), with increased expression of the trophoblast markers CDX2, MSX2, and GATA2 and a marked increase in BMP4. Xu et al. previously reported that addition of BMP4 to human ESCs causes upregulation of trophoblast markers and differentiation into early trophoblasts with an enlarged, flattened morphology [44].

The SB431542 inhibitor has been shown to enhance differentiation of ESCs or iPSCs into neural cells [25, 45, 46], retinal cells [23], endothelial cells [24, 47], and hematopoietic cells [16], depending on modulation of TGF-β pathway activity under different culture conditions. The increased mesodermal (BMP4, MSX2, GATA4, RUNX1), ectodermal (PAX6, CDX2), and endodermal (SOX7, SOX17) marker gene expression levels (Fig. 7; supplemental online Fig. 3), concomitant with the decreased expression of pluripotency-related genes (OCT4, SOX2, DMNT3B, LEFTY1, LEFTY2, and MYST2), in ESCs and iPSCs treated with the SB431542 inhibitor in KOSR medium for 10 days confirmed that SB431542-treated cells indeed display multilineage differentiation potential.

Undifferentiated ESCs display epithelial markers and can spontaneously differentiate into mesenchymal or fibroblast-like cells [21, 48] in a process similar to the type 1 EMT that occurs during embryogenesis, a process distinct from the type 2 that occurs during fibrosis and the type 3 that occurs during cancer progression [49, 50]. Our qPCR and immunofluorescence data indeed show high expression levels of typical epithelial markers (ECAD, EpCAM) in undifferentiated ESCs and iPSCs, which then decrease during SB431542 treatment and fall more dramatically in MSC culture. Furthermore, known biomarkers of EMT, including SNAI2, TWIST, ZEB1, and ZEB2, were upregulated by SB431542 treatment, whereas differentiated mesodermal markers, such as NCAD and vimentin, were upregulated only in MSC medium (Fig. 7; supplemental online Fig. 3) [16]. This ECAD-Ncad switching observed in our MSC differentiation protocol was not observed by Boyd et al. [19] during the generation of MSCs from hESCs and iPSCs with an EMT differentiation method, suggesting either that cadherin switching is not required for in vitro type 1 EMT or that the two protocols generate two distinct MSC-like cell populations. These data and the strong induction of recognized inducers of EMT, such as BMP4, ZEB1, and ZEB2, suggests that SB431542 triggers both intrinsic and autocrine mechanisms in iPSCs that collectively prime a subset of cells for a mesenchymal stromal cell fate. Paradoxically, SB431542 also enhances reprogramming of mouse fibroblasts into iPSCs [51, 52], a process that commences with a mesenchymal-to-epithelial transition and generates a population of highly plastic intermediate cells with epithelial characteristics that exhibit increased ECAD and EpCAM expression and downregulation of mesenchymal genes, such as SNAI1 and NCAD [17, 18], a subset of which transition into iPSCs [51, 52]. We note that the exogenous transcription factors used to induce iPSC generation (OCT4, SOX2, and cMyc) typically suppress TGF-β superfamily signaling and KLF4, directly activating epithelial gene expression [17] and inhibiting mesodermal factors [18]. However, the role of TGF-β superfamily members in pluripotent stem cell maintenance, differentiation, and reprogramming is a very complex issue that is dependent on cell type, species, and concentrations relative to various ligand, receptor, and inhibitor complexes [21, 26, 42, 51, 53, 54].

It will be interesting to determine how similar the intermediate epithelial-like cells generated during the early steps of SB431542-induced MSC generation are to the early intermediates in iPSC cell generation. We speculate that altered expression of more EMT/pluripotency developmental genes not identified with the qPCR array during SB431542 treatment may be due to alterations in posttranslational modification at the protein level or altered microRNA production rather than purely gene expression [50, 55].

MSC genes were not highly expressed by ESCs/iPSCs after 10 days of SB431542 treatment, but they were highly upregulated after one or two passages in MSC medium, when the cells adopted an MSC morphology, phenotype, and functionality. SB431542 treatment has previously been used to generate mesodermal progenitors from hESCs [10, 14], Sanchez et al. [14], however, found that hESCs, but not iPSCs, underwent differentiation into CD90+ CD73+ MSCs after treatment with SB431542. We also found temporal differences in MSC gene expression between iPSCs and ESCs during the differentiation period. For example, iPSCs did not express MSC markers after 10 days of SB431542 treatment, whereas ESCs expressed high levels of MSC markers (CD29, CD73, CD44) after 10 days of SB431542 treatment. In contrast to that of Sanchez et al. [14], however, our protocol produced MSC-like cells from both iPSCs and ESCs without the need for selection. This difference may be due to the fact
that Sanchez et al. used SB431542 for an extended period (28 days for ESCs, 35 for iPSCs) and used standard ESC/iPSC growth medium (20% KOSR + MEF-conditioned medium + bFGF) instead of MSC culture medium. Indeed, our data show that CD73 expression in SB-treated iPSC is sharply upregulated only after SB431542 exposure, iPSCs/ESCs presented a flattened monolayer morphology (Fig. 1), with increased expression of the trophoblast markers, CDX2, MSX2, and GATA2 and a marked increase in BMP4. Studies have shown that under differentiation conditions, BMP4 signaling promotes differentiation of ESCs. Xu et al. reported that addition of BMP4 to human ESCs causes up-regulation of trophoblast markers and differentiation into early trophoblasts with an enlarged, flattened morphology [44]. This suggests that SB431542-treated ESCs and iPSCs intermediate cells may be similar to trophoblasts. Although a homogeneous population of MSC-like cells was then obtained after 1–2 passages in MSC medium, this maturation process was accompanied by extensive cell death, suggesting that culture selection allows selective amplification of a subset of cells with an MSC phenotype.

Despite showing trilineage differentiation in vitro, both iPScs and ES-MSCs derived by either the inhibitor method or the EB method displayed more limited adipogenic differentiation compared with their osteogenicity and chondrogenicity (Fig. 4). Similarly, primary deciduoplacental MSCs [34], umbilical cord blood [38], Wharton’s jelly [35, 36], and fetal MSCs [37, 56] have been reported to have less adipogenicity than adult bone marrow MSCs. Consistent with this, the ESC-derived MSC literature to date either reports cells displaying low adipogenesis [13, 19, 33] or avoids commenting on relative adipogenic capability [8–10, 12, 14, 16]. There are few reports as yet of iPSC-MSCs, although Zhang et al. performed a semiquantitative analysis of adipogenic and osteogenic differentiation to claim that differentiation was similar in bone marrow MSCs, fetal MSCs, and ES- and iPSC-derived MSCs [57]. Although all cell types differentiated, their data instead show that the degree to which each cell line differentiated was different and support our findings and those in the literature that fetal, ES-, and iPSC-MSCs have less adipogenicity than adult bone marrow MSCs. The reasons for this are not known, although ontological differences in inhibitory DLK-1/PREF1 expression is one possibility [37, 58]. It is also well known in the field that MSC trilineage differentiation is also dependent on intrinsic factors, including age of donor [3, 4], genotype of donor [59], and tissue of origin [34, 39, 60], as well as in vitro parameters, including culture medium, substrate, and two-versus three-dimensional culture [61–63].

It is now timely to test in vivo the efficacy and long-term safety of iPScs derived MSCs generated by this method. This testing should be done in a range of in vivo models (vascular, bone repair, sepsis, cotransplantation, etc.) and with a sizeable cohort of iPSCs of identical provenance. We report our findings at this stage to allow others to appraise this innovative method independently.

Conclusion

We conclude that SB431542 in two-dimensional culture conditions induces an EMT-like transition in both human iPSCs and human ESCs that produces MSC-like cells in culture. This method substantially improves on current methods by inducing rapid and uniform MSC conversion of pluripotent stem cells under adherent culture conditions without the need for embryoid body formation or feeder cell coculture. Our protocol provides a robust, clinically applicable, and efficient system for generating MSCs from human iPSCs. Apart from allogeneic applications under development, this suggests that patient-specific iPSC-MSCs may also be considered as an alternative cell source for future tissue-engineering applications.

Acknowledgments

This work was supported by an Australian Stem Cell Centre grant (to N.M.F. and E.J.W.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. R.A.P. holds a National Health and Medical Research Council Postdoctoral Training Fellowship. We are grateful to Dr. Liza Raggatt for advice, Dr. Jennifer Ryan for supplying fetal MSCs, Samuel Jesuadian for technical assistance, and Professor Anne-Marie McNicol for teratoma histology.

Author Contributions

Y.S.C. and R.L.E.: collection and/or assembly of data, data analysis and interpretation, manuscript writing; R.A.P.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, submission of manuscript; R.H.: collection and/or assembly of data, data analysis and interpretation; E.J.W. and N.M.F.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

References

1. Bieback K, Kern S, Kocaomer A et al. Comparing mesenchymal stromal cells from different human tissues: Bone marrow, adipose tissue and umbilical cord blood. Biomed Mater Eng 2008;18:571–576.
2. Kern S, Eichler H, Stoeve J et al. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 2006;24:1294–1301.
3. Stolzing A, Jones E, McGonagle D et al. Age-related changes in human bone marrow-derived mesenchymal stem cells: Sequences for cell therapies. Mech Ageing Dev 2008;129:163–173.
4. Zhou S, Greenberger JS, Epperly MW et al. Age-related intrinsic changes in human bone-marrow-derived mesenchymal stem cells and their differentiation to osteoblasts. Aging Cell 2008;7:335–343.
5. Guillot PV, Gotherstrom C, Chan J et al. Human first-trimester fetal MSC express pluripotency markers and grow faster and have longer telomeres than adult MSC. Stem Cells 2007; 25:646–654.
6. Zhang H, Fazel S, Tian H et al. Increasing donor age adversely impacts beneficial effects of bone marrow but not smooth muscle
myocardial cell therapy. Am J Physiol Heart Circ Physiol 2005;289:H2089–H2096.
7 Brown SE, Tong W, Krebsbach PH. The derivation of mesenchymal stem cells from human embryonic stem cells. Cells Tissues Organs 2009;189:225–230.
8 Hwang NS, Varghese S, Lee HI et al. In vivo commitment and functional tissue regeneration using human embryonic stem cell-derived mesenchymal cells. Proc Natl Acad Sci U S A 2008;105:20641–20646.
9 Barberi T, Willis LM, Socci ND et al. Derivation of multipotent mesenchymal precursors from embryonic stem cells. PLoS Med 2005;2:e161.
10 Mahmood A, Harkness L, Schroder HD et al. Enhanced differentiation of human embryonic stem cells to mesenchymal progenitors by inhibition of TGF-β/activin/nodal signaling using SB-431542. J Bone Miner Res 2010;25:1216–1223.
11 Olivier EN, Rybicki AC, Bouhassira EE. Differentiation of human embryonic stem cells into bipotent mesenchymal stem cells. Stem Cells 2006;24:1914–1922.
12 Trivedi P, Hematti P. Simultaneous generation of CD34+ primitive hematopoietic cells and CD73+ mesenchymal stem cells from human embryonic stem cells cocultured with murine OP9 stromal cells. Exp Hematol 2007;35:146–154.
13 Xu C, Jiang J, Sottile V et al. Immortalized fibroblast-like cells derived from human embryonic stem cells support undifferentiated cell growth. Stem Cells 2004;22:972–980.
14 Sanchez L, Gutierrez-Arandas I, Ligero G et al. Enrichment of human ESC-derived multipotent mesenchymal stem cells with immunosuppressive and anti-inflammatory properties capable to protect against experimental inflammatory bowel disease. Stem Cells 2010;29:251–262.
15 Xu J, Lamouille S, Derynck R. TGF-β-induced epithelial to mesenchymal transition. Cell Res 2009;19:156–172.
16 Evesenko D, Zhu Y, Schenke-Layland K et al. Mapping the first stages of mesoderm commitment and differentiation of human embryonic stem cells. Proc Natl Acad Sci U S A 2010;107:13742–13747.
17 Li R, Liang J, Ni S et al. A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts. Cell Stem Cell 2010;7:51–63.
18 Samavarchi-Tehrani P, Golipour A, David L et al. Functional genomics reveals a BMP-driven mesenchymal-to-epithelial transition in the initiation of somatic cell reprogramming. Cell Stem Cell 2010;7:64–77.
19 Boyd NL, Robbins KR, Dhara SK et al. Human embryonic stem cell-derived mesoderm-like epithelium transitions to mesenchymal progenitor cells. Tissue Eng Part A 2009;15:1897–1907.
20 Hannan NR, Jamshidi P, Pera MF et al. BMP-11 and myostatin support undifferentiated growth of human embryonic stem cells in feeder-free cultures. Cloning Stem Cells 2009;11:427–435.
21 Vallier L, Pedersen RA. Human embryonic stem cells: An in vitro model to study mechanisms controlling pluripotency in early mammalian development. Stem Cell Rev 2005; 1:119–130.
22 Vallier L, Touboul T, Brown S et al. Signaling pathways controlling pluripotency and early cell fate decisions of human induced pluripotent stem cells. Stem Cells 2009;27:2655–2666.
23 Osakada F, Jin ZB, Hirami Y et al. In vitro differentiation of retinal cells from human pluripotent stem cells by small-molecule induc- tion. J Cell Sci 2009;122:3169–3179.
24 Watabe T, Nishihara A, Mishima K et al. TGF-β receptor kinase inhibitor enhances growth and integrity of embryonic stem cell-derived endothelial cells. J Cell Biol 2003;163:1303–1311.
25 Chambers SM, Fasano CA, Papapetrou EP et al. Highly efficient neural conversion of human ES and iP cells by dual inhibition of SMAD signaling. Nat Biotechnol 2009;27:275–280.
26 Xu RH, Sampsell-Barron TL, Gu F et al. NANOG is a direct target of TGFβ/activin-mediated SMAD signaling in human ESCs. Cell Stem Cell 2008;3:196–206.
27 Yu J, Vodyanik MA, Smuga-Otto K et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007;318:1917–1920.
28 Reubinoff BE, Pera MF, Fong CY et al. Embryonic stem cell lines from human blasto- cysts: Somatic differentiation in vitro. Nat Biotechnol 2000;18:399–404.
29 Costa M, Sourris K, Hatzistavrou T et al. Expansion of human embryonic stem cells in vitro. Curr Protoc Stem Cell Biol. 2008;1C.1–1C.1.7.
30 Ludwig T, Thomson J. Defined, feeder- independent medium for human embryonic stem cell culture. Curr Protoc Stem Cell Biol. 2007;Chapter 1:Unit 1C 2.
31 Dominici M, Le Blanc K, Mueller I et al. Minimal criteria for defining multipotent mesenchymal stromal cells: The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315–317.
32 Adewumi O, Afitatoon B, Ahrlund-Richter L et al. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nat Biotechnol 2007;25:803–816.
33 Karlsson C, Emanuelsson K, Wessberg F et al. Human embryonic stem cell-derived mesenchymal progenitors: Potential in regenera- tive medicine. Stem Cell Res 2009.
34 Barlow S, Brooke G, Chatterjee K et al. Comparison of human placenta- and bone marrow-derived multipotent mesenchymal stem cells. Stem Cells Dev 2008;17:1095–1107.
35 Hsieh JY, Fu YS, Chang SJ et al. Functional module analysis reveals differential osteogenic and stemness potentials in human mesenchymal stem cells from bone marrow and Wharton’s jelly of umbilical cord. Stem Cells Dev 2010;19:1895–1910.
36 Kim MJ, Shin KS, Jeon JH et al. Human chorionic-plate-derived mesenchymal stem cells and Wharton’s jelly-derived mesenchymal stem cells: A comparative analysis of their potential as placenta-derived stem cells. Cell Tissue Res 2011;346:53–64.
37 Morganstein DL, Wu P, Mane MR et al. Human fetal mesenchymal stem cells differentiate into brown and white adipocytes: A role for ERα in human UCP1 expression. Cell Res 2010;20:434–444.
38 Rebelatto CK, Aguiar AM, Moretao MP et al. Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue. Exp Biol Med (May- wood) 2008;233:901–913.
39 Zang ZY, Teoh SH, Chong MS et al. Superior osteogenic capacity for bone tissue en- gineering of fetal compared with perinatal and adult mesenchymal stem cells. Stem Cells 2009;27:126–137.
40 Dvash T, Sharon N, Yanuka O et al. Molecular analysis of LEFTY-expressing cells in early human embryoid bodies. Stem Cells 2007;25:465–472.
41 Yu P, Pan G, Yu J et al. FGFR sustains NANOG and switches the outcome of BMP4- induced human embryonic stem cell differen- tiation. Cell Stem Cell 2011;8:326–334.
42 Jiang J, Ng HH. TGFβ and SMADs talk to NANOG in human embryonic stem cells. Cell Stem Cell 2008;3:127–128.
43 Galvin KE, Travis ED, Yee D et al. Nodal signaling regulates the bone morphogenetic pro- tein pluriptoty pathway in mouse embryonic stem cells. J Biol Chem 2010;285:19747–19756.
44 Xu RH, Chen X, Li DS et al. BMP4 initiates human embryonic stem cell differentiation to trophoblast. Nat Biotechnol 2002;20:1261–1264.
45 Kim DS, Lee JS, Leem JW et al. Robust enhancement of neural differentiation from human ES and iP cells regardless of their in- nate difference in differentiation propensity. Stem Cell Rev 2010;6:270–281.
46 Smith JR, Vallier L, Lupo G et al. Inhibition of Activin/Nodal signaling promotes specification of human embryonic stem cells into neu- roectoderm. Dev Biol 2008;313:107–117.
47 James D, Nam HS, Seandel M et al. Expansion and maintenance of human embry- onic stem cell-derived endothelial cells by TGFβ inhibition is ID1 dependent. Nat Biotechnol 2010;28:161–166.
48 Ullmann U, In’t Veld P, Gilles C et al. Epithelial-mesenchymal transition process in human embryonic stem cells cultured in feede- r-free conditions. Mol Hum Reprod 2007;13:21–32.
49 Kalluri R, Weinberg RA. The basics of ep- ithelial-mesenchymal transition. J Clin Invest 2009;119:1420–1428.
50 Zeisberg M, Neilson EG. Biomarkers for epithelial-mesenchymal transition. J Clin Invest 2009;119:1429–1437.
51 Ichida JK, Blanchard J, Lam K et al. A small-molecule inhibitor of TGF-β signaling replaces sox2 in reprogramming by inducing nanog. Cell Stem Cell 2009;5:491–503.
52 Lin T, Ambasudhan R, Yuan X et al. A chemical platform for improved induction of human iPSCs. Nat Methods 2009;6:805–808.
53 Harrison CA, Gray PC, Vale WW et al. Antagonists of activin signaling: Mechanisms and potential biological applications. Trends Endo- crino Metab 2005;16:73–78.
54 Pera MF, Tam PP. Extrinsic regulation of pluripotent stem cells. Nature 2010;465:713–722.
55 Acloque H, Adams MS, Fishwick K et al. Epithelial-mesenchymal transitions: The im- portance of changing cell state in development and disease. J Clin Invest 2009;119:1438–1449.
56 Guillot PV, Abass O, Bassett JH et al. Intrauterine transplantation of human fetal mesenchymal stem cells from first-trimester blood repairs bone and reduces fractures in osteogenesis imperfecta mice. Blood 2008;111:1717–1725.

57 Zhang J, Lian Q, Zhu G et al. A human iPSC model of Hutchinson Gilford Progeria reveals vascular smooth muscle and mesenchymal stem cell defects. Cell Stem Cell 2011;8:31–45.

58 Kluth SM, Buchheiser A, Houben AP et al. DLK-1 as a marker to distinguish unrestricted somatic stem cells and mesenchymal stromal cells in cord blood. Stem Cells Dev 2010;19:1471–1483.

59 Leskelä HV, Olkku A, Lehtonen S et al. Estrogen receptor α genotype confers inter-individual variability of response to estrogen and testosterone in mesenchymal-stem-cell-derived osteoblasts. Bone 2006;39:1026–1034.

60 Guillot PV, De Bari C, Dell’Accio F et al. Comparative osteogenic transcription profiling of various fetal and adult mesenchymal stem cell sources. Differentiation 2008;76:946–957.

61 Frith JE, Thomson B, Genever PG. Dynamic three-dimensional culture methods enhance mesenchymal stem cell properties and increase therapeutic potential. Tissue Eng Part C Methods 2010;16:735–749.

62 Hudson JE, Mills RJ, Frith JE et al. A defined medium and substrate for expansion of human mesenchymal stromal cell progenitors that enriches for osteo- and chondrogenic precursors. Stem Cells Dev 2011;20:77–87.

63 Karlsen TA, Mirtaheri P, Shahdadfar A et al. Effect of three-dimensional culture and incubator gas concentration on phenotype and differentiation capability of human mesenchymal stem cells. J Cell Biochem 2011;112:684–693.

www.StemCellsTM.com