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# Direct Differentiation of Human Embryonic Stem Cells toward Osteoblasts and Chondrocytes through an Intermediate Mesenchyme Progenitor Lineage

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## 1. Introduction

The recent establishment of in vitro cultures of human embryonic stem cells (hESC) from preimplantation embryos has provided a biologically relevant in vitro model for studying early human embryonic development and the signals involved in early stages of cellular commitment to different lineages. In addition, hESC represent a good source of an unlimited supply of cells that can generate clinical-grade, transplantable, lineage-specific cells to treat a large number of skeletal diseases including bone and cartilage diseases (e.g. localized bone and cartilage defects, non-healing fractures and systemic age-related degenerative conditions such as osteoporosis and osteoarthritis) (for review, [1], [2]). Typically, the basic in vitro methods for differentiating hESC into specific cell lineages are based on three procedures; 1) direct differentiation as a monolayer on extracellular matrix substrates, 2) differentiation in co-culture with primary tissue derived cells, and 3) the formation of 3-D spherical structures in suspension culture, termed embryoid bodies (EBs) [3-6]. However, developing clinically relevant protocols for directing the differentiation of hESC into definitive, homogenous populations of osteogenic or chondrogenic cells still faces several challenges due to the complexity of pathways that are conserved between embryonic and hESC differentiation for all cell types of the three germ layers. Our group, and others, have employed several strategies to first, generate mesenchymal progenitor cells (termed MSC) from hESCs demonstrating the characteristic phenotype of adult bone marrow-derived mesenchymal stem cells (BM-MSC), and second differentiate these cells into homogenous populations of osteoblast or chondrocytes by using protocols established for adult MSC. Thus, our objective in this chapter is to provide an overview on the majority of published protocols for derivation of osteoblasts and chondrocytes from hESC through an intermediate mesenchymal progenitor lineage.

## 2. Differentiation of hESC into mesenchymal progenitor cells

Multipotential mesenchymal stem/stromal cells (MSCs) have been isolated from diverse tissues including bone marrow (BM), adipose, muscle, periodontal ligament, umbilical cord blood and other connective tissues. MSCs are typically characterized by their adherence to plastic culture plates, expression of Stro-1, CD29, CD73, CD90, CD105, CD106, CD166, CD146, and CD44, lack of hematopoietic markers and MHC class II expression, and ability to differentiate into cells of mesenchymal origin such as bone, cartilage and adipose tissue [7]. MSC have documented evidence for their potential use in cell-based therapy for the treatment of bone and cartilage defects [8].

Derivation of MSC-like cells from hESC has previously been reported using a number of different approaches including direct differentiation employing withdrawal of feeders, addition of PDGF, or isolation and culture of spontaneous differentiation [9-13]; through an intermediate EB formation [14-16]; or by co-culturing hESC with primary bone derived cells [17], OP9 cells [18] or periodontal ligament fibroblasts [19]. In addition, methods were utilised for isolation of mesenchymal-like populations, based on cell sorting, employing a number of CD marker combinations: CD13+/-, CD71+, CD105+ [20] and CD105+, CD24- [21].

Culturing hESC as hEBs activates well-conserved cascades of genes that govern the earliest events during gastrulation and germ layer formation [5] that may be important for subsequent differentiation. Previous publications from mouse [22] and human [23] ESCs have shown that early mesoderm markers are expressed during hEB formation.

EB formation of hESC is initiated by enzymatic dissociation of hESC colonies into small clumps and differentiation is initiated by removal of bFGF and 2-mercaptoethanol, as well as by culturing the dissociated cells in ultra low adhesion culture dishes [24-26]. The length of time of EB culture is dependent upon the ultimate target cell type and EB differentiation appears to correlate well with post-implantation development of embryos [5].

Our profiling of these cells during 20 days of EB differentiation has confirmed up-regulation of several mesoderm specific markers *MEOX1*, *MIXL1*, *ALX4*, *TBX6* and *FOXF1* in association with increasing percentages of cells expressing MSC markers including CD29, CD44, CD63, CD73 and CD166 (fig. 1) [27]. Thus, differentiation of hESC via EB formation represents a promising method for directing the hESC not only to the mesoderm lineage but also to mesenchymal progenitor cells.

Since, TGF $\beta$ /BMP signaling pathways have been shown to play a role in mesoderm induction in vertebrates [28-30], we studied the effect of several members of the TGF $\beta$ /BMP family on mesoderm induction and derivation of MSC in the hESC-EBs model. We succeeded in developing clinically relevant protocols for generating MSC from hESC by blocking TGF- $\beta$  signaling during hEB formation using SB-431542 (an inhibitor of activin receptor-like kinase (ALK)5, 4 and 7 (the TGF- $\beta$  type I receptor) [27]. This led to selective up-regulation of several markers involved in mesoderm induction and myogenic differentiation including, *TBX6* and *Myf5* genes, additionally, these findings were confirmed by microarray analysis. In another study, we showed that the treatment of hESC-derived EBs with 50ng/ml Activin B (member of TGF $\beta$  superfamily) for 10 days was sufficient to up-regulate several mesoderm specific markers (*FOXF1*, *VE-cadherin*, *KDR*) [31]. Further enrichment for MSC population was performed by establishing monolayer outgrowth culture from hEBs induced either by Activin B- or SB-induced. Our data showed that this strategy is very efficient in obtaining a homogenous MSC population with high percentage of cells expressing the characteristic surface markers for bone marrow derived-MSC including CD44, CD146, CD166, CD63, CD166 and CD73 [31].

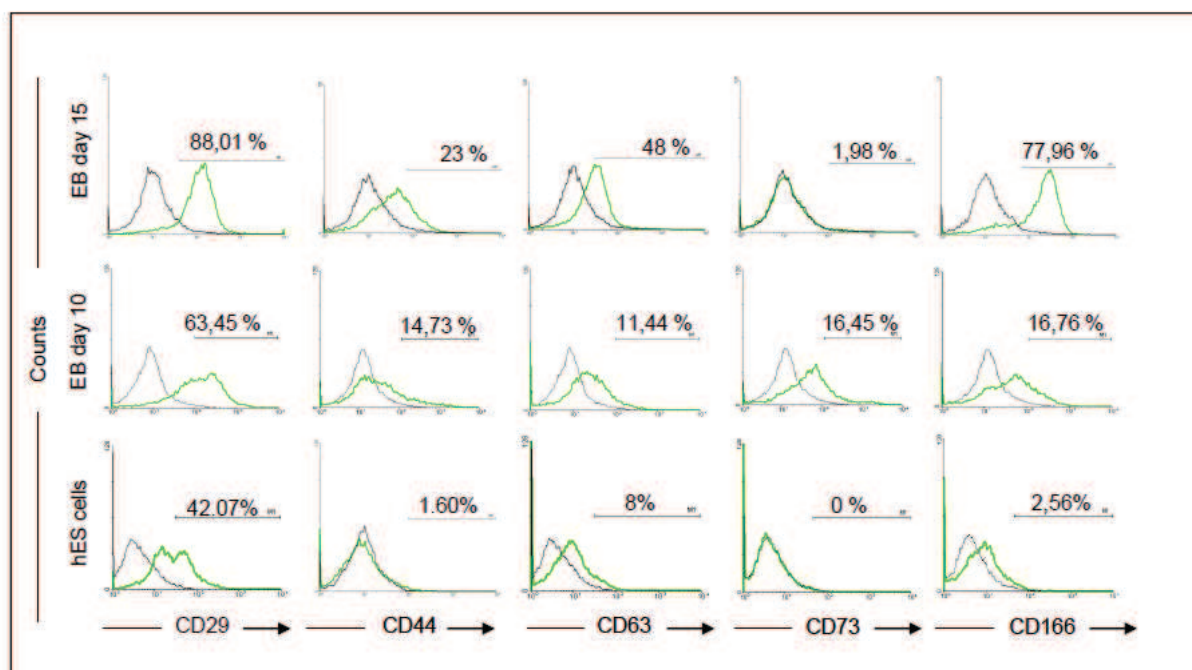


Fig. 1. FACS analysis of the MSC characteristic CD marker expression in hESC during their spontaneous differentiation as hEBs at day 10 and 15. The percentage of positive cells is indicated for each graph

### 3. Differentiation of hESC into chondrogenic lineages

Embryonic chondrogenesis is a complex biological process that plays essential roles in endochondral ossification and skeletal patterning. It involves migration of committed mesenchymal cells from the cranial neural crest, paraxial mesoderm and lateral plate mesoderm to the site of skeletogenesis to form cell mass condensations that define the morphology of the future bone [32, 33]. Inside these condensations, mesenchymal cells are differentiated into chondrocytes that proliferate and secrete an increasing amount of cartilage matrix macromolecules. Chondrogenesis is terminated through chondrocyte hypertrophy and apoptosis and is followed by blood vessel invasion and bone matrix formation by osteoblasts.

Current protocols for differentiating hESC into the chondrogenic lineage are principally based on differentiating hESC into the mesoderm/mesenchyme lineage via hEBs formation. This is followed by induction towards chondrogenesis using pellet micromass culture in combination with: TGF $\beta$ 1, TGF $\beta$ 3 BMP-2 and -4 [18, 31, 34], bovine chondrocyte conditioned medium [14] or via encapsulation in hydrogels [35], or arginine-glycine-aspartate (RGD)-modified PEGDA hydrogels [36]. Other published strategies include, co-culture of hESC-derived MSC with primary chondroprogenitor cells [37] and co-culture of enriched mesodermal cell populations (CD73<sup>+</sup>) with murine OP9 cells [18]. However, most of these protocols failed to generate clinically compliant homogenous populations of chondrocytes that could be utilized for cartilage repair due either to their complexity or use of animal products such as fetal calf serum. Table 1 provides an overview on published chondrogenic differentiation protocols for hESC.

Initial Differentiation	Chondrogenic Differentiation	In vitro Analysis	In Vivo experiments	Ref
hESC were cultured as EBs for 10days followed by outgrowth culture on fibronectin coated plates in DMEM+15%KSR+50ng/ml Activin B for mesoderm induction. MSC-like cells were FACS sorted based of expressing Dlk1/FA1+.	Pellet micromass culture of dlk1/FA1+cells induced in CDM medium supplemented with 100 nM Dex+50ug/ml VitC+1%ITS+ 10 ng/ml TGFβ1.	-FACS analysis for MSC markers. -Alcian blue staining. -Real-time PCR	n/a	[31]
hESC were cultured as EBs for 10days followed by outgrowth culture on gelatine coated plates and subculturing at high density in DMEM+10% FBS to obtain MSC-like cells.	hESC-MSC induced in Arginine-glycine-aspartic acid (RGD)-modified PEG-based hydrogels in bovine chondrocyte conditioned media: DMEM 10% FBS + 1μm dex, 200μm Indometh, 10μg/ml insulin, 0.5mM IBMX	-Immune-staining for Col type 2. -Real-time PCR	-hESC-MSC encapsulated in PEGDA hydrogels and implanted s.c. in athymic mice. -Cartilage repair capacity of hESC-MSC pellets was evaluated in rat knee defect model.	[14]
hESC were plated on mouse stromal OP9 cells in αMEM with 20% FBS, followed by FACS sorting of CD73+cells to obtain MSC-like cells.	Pellet micromass culture induced in DMEM with 10ng/ml TGFβ3+200μm Vit C in +10% FBS for 28 days.	-Alcian Blue staining. -Real-time PCR	n/a	[18]
hESC were cultured as EB for 5 days in DMEM/F12 +10% FBS, trypsinised and plated for outgrowth culture.	Cells induced in co-culture with human primary chondrocytes in DMEM + 10%FBS, +1mM L-Glut + 50μg/ml Vit C for 28days.	-Alcian Blue staining. -Immune-staining for Col type 2 and SOX9	Co-culture hESC seeded onto PDLA foam and implanted s.c. in SCID mice for 35 days.	[16]

<p>hESC were cultured as EB for 10 days and plated on gelatine coated plates in DMEM 10% FBS.</p>	<p>Pellet micromass cultures induced in DMEM +1mM L-glut, 100nm Dex+ 50µg/ml Vit C+ 1mM Na pyruvate + 1% ITS with 10ng/ml TGFβ1 or 25ng/ml BMP2 for 2 weeks .</p>	<p>-Safranin o – staining . -PCR</p>	<p>n/a</p>	<p>[36]</p>
<p>Undifferentiated hESC cells were co-cultured with irradiated neonatal or adult articular chondrocytes in high-density pellet mass cultures for 14 days, followed by monolayer expansion.</p>	<p>-Pellet micromass cultures of co cultured hESC were induced in DMEM with 10%FCS and 10ng/ml TGFβ3. -Co-cultures induced in hyaluronan-based Hyaff-11 scaffolds</p>	<p>-Safranin o –and Alcian Blue staining</p>	<p>n/a</p>	<p>[44]</p>
<p>hESC were cultured as EBs for 5 days in DMEM +10% FBS. Single cells were obtained either from the hESC aggregates or EBs day5.</p>	<p>hESC or 5-day EB-derived cells were resuspended at a high density in DMEM + ITS, 1.25mg/ml BSA+ 5.35µg/ml linoleic acid, 1% KSR+ 40µg/ml L-proline, 50µg/ml Vit C, 1% NEAA, 100nM Dex+100ng/ml BMP2 and cultured in micromass on gelatin coated plates.</p>	<p>-Immune-staining for Col type 2.</p>	<p>n/a</p>	<p>[45]</p>
<p>Dissociated single human ESCs were cultured and passaged on gelatin-coated plates in DMEM + 10% FBS, 5ng/ml FGF2.</p>	<p>Pellet micromass culture of hESC aggregates induced in DMEM + 0.1µM dex+ 50µM Vit C+ 40µg/ml proline, 1mM Na pyruvate+ 1% ITS+ premix, and/or 10ng/ml TGFβ1 or 300ng/ml BMP7 for 14days.</p>	<p>-Toluidine blue staining, -Col type 2 staining -RT-PCR</p>	<p>n/a</p>	<p>[46]</p>

Used the same protocol as [45]	Used the same protocol as [45] except used 10ng/ml of TGFβ1 or 1 μM SB431542 instead of BMP2 in chondrogenic media to study the effect of TGFβ signaling pathway.	Real-time PCR Col2 immune-staining	n/a	[47]
hESC were cultured on Matrigel and differentiated as EBs in chondrogenic media consisting of DMEM with 100nM Dex+1% ITS+ 40 μg/ml L-proline, 50 μg/ml VitC+ 100 μg/ml sodium pyruvate and 1 ng/ml TGFβ1. EBs were cultured in this medium for 3 weeks.		col1, 2 and GAG staining. Mechanical testing using unconfined creep cytocompression	n/a	[48]

Abbreviations used, EB: embryoid bodies; KSR:knockout serum replacement; FBS :fetal bovine serum; CDM: chemically defined medium; Dex: dexamethasone; VitC: ascorbic acid/vitamin C; RGD: arginine-glycine-aspartic acid; PEG: poly ethylene glycol; Indometh: indomethacin; IBMX: 3-isobutyl-1-methylxanthine; NEAA: non essential amino acids; PEGDA: Poly (Ethylene Glycol)-Diacrylate; s.c: subcutaneously; PDLLA: Poly-DL-lactic acid

Table 1. hESC chondrogenic differentiation protocols

We have recently identified *dlk1/FA1* as a surface marker for chondrogenesis that indicates transition of immature proliferating to pre-mature hypertrophic chondrocytes during mouse embryonic limb development as well as in vivo hESC-derived teratoma formation. Our data showed that *Dlk1* is a dynamic surface marker and responds to some external stimuli controlling chondrogenesis. *dlk1/FA1* (delta-like 1 protein, fetal antigen 1, also named preadipocyte factor 1 [Pref-1]) is a membrane-associated protein belonging to Notch/Serrata/Delta family [38]. *Dlk1/FA1* plays an important role in controlling the cell fate decisions during embryogenesis [39] and extends its function to regulate many mesoderm differentiation processes in the post-natal organism including adipogenesis [40], myogenesis [41] and osteoblastogenesis [42].

We have designed a clinically relevant protocol for directing the differentiation of hESC into the definitive early chondrogenic lineage based on tracking the expression of *dlk1/FA1* as a mesoderm/chondroprogenitor surface marker. In this protocol, treatment of hESC-EB cultures with Activin B is shown to markedly up-regulate *dlk1/FA1* expression in association with increasing several mesoderm induction markers (i.e. *FOXF1*, *KDR* and *MEOX1*) [31]. By establishing an hEB-outgrowth culture we were able to enrich for cells expressing *Dlk1/FA1* and obtain a homogenous population of mesenchymal/chondroprogenitor cells that could be further differentiated as micromass cultures into chondrocytes in serum free medium containing TGFβ1 [31]. Thus, there is a need to identify novel surface markers that define early stages of hESC commitment to chondrocytes. Tracking these markers during the progression of hESC-EBs formation will be valuable for developing well-defined and efficient protocols for directing hESC differentiation into the chondrogenic lineage in vitro.

#### 4. Differentiation of hESC into osteogenic lineages

Osteoblasts originate from mesenchymal stem cells in bone marrow through a differentiation process that is controlled by numerous hormones and growth factors. Osteoblasts are characterized by expressing various phenotypic markers such as high alkaline phosphatase (ALP) activity and synthesizing collagenous and noncollagenous bone matrix proteins including osteocalcin. The most important function of osteoblasts is to form mineralized bone [43].

Initial Differentiation	Osteogenic Induction	In vitro assays	In Vivo assays	Ref
hESC were cultured as EBs for 10 days followed by outgrowth culture on fibronectin coated plates in DMEM+15%KSR+ 10 $\mu$ M SB421543. hEBs-outgrowth were passaged and maintained in CDM medium for enrichment for MSC.	hESC-MSC induced in $\alpha$ MEM medium with 10%FCS+10mM $\beta$ -glyc+ 100 $\mu$ g/ml Vit C for 20 days.	-FACS analysis for MSC surface markers. -Alzarin Red and ALP staining. -Real-time PCR.	hESC-MSC were mixed with HA/TCP s.c. into Nod/SCID mice for 8 weeks.	[27]
hESC were cultured as EBs for 10 days and plated for outgrowth culture on gelatine coated plates in DMEM+10% FBS+2mM L-Glut to obtain MSC-like cells.	hESC-MSC induced in DMEM media with 50 $\mu$ M Vit C + 10mM $\beta$ -glyc + 100nM Dex in for 14 d	-Alzarin Red and ALP staining. -Real-time PCR.	hESC-MSC were seeded onto the polymer scaffold of PLLA/PLGA, cultured for 10 days and implanted s.c. in athymic mice for 8 weeks.	[14]
hESC were plated on mouse stromal OP9 cells in $\alpha$ MEM with 20% FBS, followed by FACS sorting of CD73 <sup>+</sup> cells.	CD73 <sup>+</sup> cells induced in $\alpha$ MEM medium with 10% FBS+10mM $\beta$ -glyc+ 0.1 $\mu$ M Dex+ 200 $\mu$ M Vit C for 3 to 4weeks.	-Alzarin Red and - Von Kossa staining. -Real-time PCR.	n/a	[18]
hESC were cultured as EBs for 3 to 4 days, trypsinised and plated onto gelatin plates in KODMEM + 10% FBS, L-Glut, NEAA, BME	Cells induced in KODMEM + 10% FBS+ BME +50 $\mu$ M Vit C, 10mM $\beta$ -glyc +100nM Dex	-Von Kossa staining	Cells induced for 4d, injected into diffusion chamber and implanted into nude mice for 7 weeks.	[15]



hESC were cultured as EBs for 7 days, and plated onto gelatin coated plates in $\alpha$ MEM with 10% FBS+ 200mM L-glut+10mM NEAA+4ng/ml FGF for up to 2 weeks.	hESC-MSC were induced as described [18]	-Von Kossa staining	n/a	[49]
hEBs day3 were plated with inactivated human primary bone derived cells (hPBDs).	The co-culture of hEBs-derived cells with hPBDs was continued for 2 weeks.	-Real Time PCR	Cells seeded on PLGA/HA scaffolds implanted with BMP2 s.c. into SCID mice for 4 & 8 weeks.	[17]
Removal of MSC-like cells from long term culture of hESC colonies in feeder free system.	hESC-MSC induced in $\alpha$ MEM with 20% FBS +10nM Dex+ 0.2 mM VitC+ 10 mM + $\beta$ -glyc for 3 weeks	-Alzarin Red staining.	n/a	[20]
hESC aggregates were removed from the MEF and placed onto a tissue culture dish for 24 hours, followed by single-cell suspensions and subsequent plating.	Cells were induced in $\alpha$ MEM with FBS was supplemented with 10nM DEX+ 50 $\mu$ g/ml VitC + 5 mM $\beta$ -glyc	-Von Kossa and ALP staining .	n/a	[50]
hESC single cell suspension plated on gelatin coated plate in DMEM-HG, L-Glut+ 10% FBS+ 10ng/ml FGF. Continuous passaging to obtain hESC-MSC.	hESC-MSC induced in DMEM + 1% PEST, L-glutamine (2mM), 10% FCS+ 45 nM VitC+ 100nM dex+ 20mM $\beta$ -glyc for 6 weeks.	-Von Kossa staining	n/a	[51]
hESC were cultured as EBs for 10 days, and plated onto gelatin plates in $\alpha$ MEM + 20% FBS, NEAA, L-Glut, 1ng/ml FGF. hESC-MSC outgrowth cells taken to p4	hESC-MSC induced in DMEM, 10%FBS, 50 $\mu$ g/mL Vit C, 10mM $\beta$ -glyc +100nM Dex or 50ng/ml BMP7 or Dex+BMP7 for 4 weeks. Cells induced also on Scaffolds (6 weeks), matrices or film cultured with Dex+BMP7.	-Von Kossa and ALP staining	n/a	[52]

<p>hESC were cultured as EBs for 5 days in DMEM/F12, 10% KSR, NEAA, BME. EBs were placed on Matrigel coated plated .</p>	<p>Attached EBs were induced in medium with 0.1 mM VitC+ 10mM <math>\beta</math>-glyc, 0.1 mM dex +/- 5<math>\mu</math>M LY294002+ 0.5<math>\mu</math>M AKT inhibitor,+1nM Rapamycin or 10nM FK506 (PI3K/ AKT/mTOR inhibitors) for 3 weeks.</p>	<p>-Von Kossa and ALP staining. -Real time-PCR.</p>	<p>n/a</p>	<p>[53]</p>
<p>Prolonged in vitro cultivation and starvation for hESC mesenchymal differentiation based on method used by [20].</p>	<p>Medias: #1 KODMEM + 20% KSR, with/without FBS (hi or non hi) or 5% hPL + 40u/ml heparin; + 1% ITS+, and/or 10<sup>-8</sup> M Dex, and 10<sup>-4</sup> M L-ascorbic acid phosphate magnesium salt n-hydrate (AscP). 32-45days #2 aMEM +10% FBS +/- Dex/ AscP 45-69days #3 KODMEDM + 20 KSR, +/- FGF or media #2, 53 days</p>	<p>-Real time-PCR.</p>	<p>Differentiated hESC using different cocktails were mixed with HA/TCP and implanted s.c. in immunodeficient mice.</p>	<p>[54]</p>

Abbreviations used,  $\beta$ -glyc: beta glycerophosphate; KODMEM: Knockout DMEM; BME: BME amino acids; HA/TCP: Hydroxyapatite tri calcium phosphate; hi: heat inactivated

Table 2. hESC osteogenic differentiation protocols

A number of cell culture conditions have been used to induce differentiation of hESCs into the osteogenic lineage with and without EB formation. Osteoblastic differentiation of hESCs has principally been achieved by differentiating hESC into MSC-like cells followed by induction of the hESC-MS to the osteogenic lineage using a well known osteogenic cocktail containing ascorbic acid,  $\beta$ -glycerophosphate, and dexamethasone, or through co-culture with primary bone derived cells (more detailed protocols are shown in table 2). However, the majority of these protocols still show some limitations in providing homogeneous cell populations which are efficient in differentiating into osteoblasts and could be used in tissue engineering for skeletal regeneration. These limitations include; 1) insufficient data comparing the phenotype of hESC-derived osteoblasts with well-established osteoblastic cells; 2) using in vitro assays as the main criteria for evaluation of the osteogenic differentiation protocols; 3) small numbers of published in vivo studies examining the capacity of hESC-derived osteoblasts for bone regeneration; 4) little or no available data on the reproducibility of a specific protocol on different hESC cell lines.

Recently, we have reported an efficient protocol for directing hESC into mesenchymal osteo-progenitors by inhibiting TGF- $\beta$  signaling with SB-431542 (SB) during hEBs formation.

Detailed cellular and molecular analysis revealed the differentiation of SB-treated hEBs into muscle progenitor cells (MPC)[27]. Successive outgrowth cultures of the MCP, in the presence of 10% fetal bovine serum (FBS), demonstrated a step-wise progression from a heterogeneous EB outgrowth cell population, through an MCP population, to a homogeneous population of mesenchymal progenitors that expressed CD markers characteristic of mesenchymal stem cells (MSCs): CD44 (100%), CD73 (98%), CD146 (96%), and CD166 (88%). Lastly these mesenchymal progenitors verified their ability to differentiate into osteoblasts in vitro and could form ectopic bone upon subcutaneous implantation with hydroxyl-apatite/tricalcium phosphate ceramic powder (HA/TCP) in immune deficient mice [27].

## 5. Conclusions

Due to the unlimited self-renewal and differentiation capacity of hESC-MS over adult MSC the derivation of MSC from hESC for skeletal regeneration demonstrates a promising alternative to the use of adult stem cells. Despite the increasing number of published protocols for differentiating hESC into definitive osteogenic and chondrogenic lineages, the use of hESC in tissue engineering for bone/cartilage repair in pre-clinical studies is elusive due to either the complexity of available protocols or lack of data on testing the functional activity of cells in vivo. More work is needed, to design robust xeno-free protocols that can provide large numbers of homogenous and efficiently differentiated osteoblastic /chondrocytic cells that work on multiple hESC cell lines. To achieve this goal, new strategies need to be developed to improve the in vitro and in vivo assessment assays, to develop a predictive molecular signature for each particular cell type at different differentiation stages, and to identify new and dynamic surface markers that recognize early chondro-progenitor and osteo-progenitor cells during hESC differentiation.

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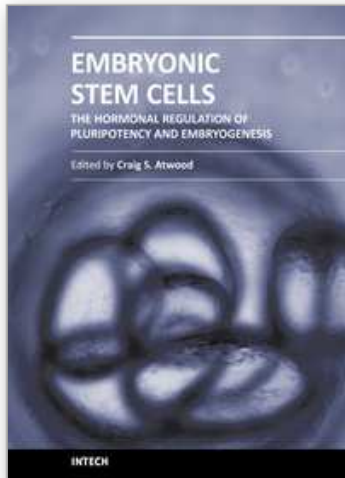
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