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Selective isolation and differentiation of a stromal population of human embryonic stem cells with osteogenic potential

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ABSTRACT

The derivation of osteogenic cells from human embryonic stem cells (hESC) has been hampered by the absence of easy and reproducible protocols. hESC grown in feeder-free conditions, often show a sub population of fibroblast-like, stromal cells growing between the colonies. Thus, we examined the possibility that these cells represent a population of stromal (mesenchymal) stem cells (hESC-stromal). Two in house derived hES cell lines (Odense3 and KMEB3) as well as an externally derived cell line (Hues8) were transitioned to feeder-free conditions. A sub population of fibroblast-like cells established between the hESC colonies were isolated by selective adherence to hyaluronic acid-coated plates (100 μg/ml) and were characterized using a combination of FACS analysis and staining. The cells were $CD44^+$, $CD29^+$, $CD73^+$, $CD166^+$, $CD146^+$, and $CD105^+$; and, $Oct4^-$, $CD34^-$, $CD45^-$ and $CXCR4^-$. When cultured in osteogenic differentiation media, up regulation of osteoblastic lineage markers (*DLX5, MSX2, RUNX2, SPARC, ALP, COL1a1, BGLAP, IBSP, DCN, LOX-L4*) and production of *in vitro* mineralized matrix was detected. hESC-stromal cells loaded on a carrier and implanted either subcutaneously or in a critical size calvarial defect in immune deficient mice for 10 weeks, resulted in new bone formation and partial repair of the calvarial defect. In conclusion, hESC-stromal can be isolated from hESC cultures and represent a good source for obtaining cells with osteogenic differentiation potential suitable for regenerative medicine protocols.

Introduction

Human bone marrow stromal stem cells (hBMSC) (also known as bone marrow mesenchymal cells, multipotent mesenchymal stem cells, skeletal stem cells) are considered an accessible source of cells that can differentiate into osteogenic cells, however their *in vitro* proliferation ability is finite and the number of cells retrievable from patients is limited [1]. In contrast to hBMSC, human embryonic stem cells (hESC) are pluripotent cells that can differentiate into cells of all three germ layers (endoderm, mesoderm, ectoderm) [2]. hESC have several advantages relevant for studies of bone biology. hESC are a suitable model for studying the molecular mechanisms of stem cell differentiation to mesoderm and osteo-progenitor cells. This information is highly relevant since pathways, important for osteoblast generation during development, are similar to osteoblast regeneration following injury or disease in adult life [3]. In addition, hESC can potentially provide a large number of cells with specific characteristics needed for regenerative medicine protocols e.g. treatment of bone defects and non-healed fractures.

Three methods have been described to differentiate hESC into an osteogenic phenotype: via an intermediate stage using embryoid body (EB) formation [4–9], via induction through outgrowth culture [10–17], or through co-culture with a more differentiated cell type [18–21]. However, the ability of these culture methods to provide homogeneous, efficient and reproducible differentiation of hESC cells into osteogenic cells has been limited. The efficiency of differentiation is usually unreported, and resultant cell cultures are morphologically and functionally heterogeneous and thus there is a concern that these cells may form teratoma if used in clinical applications [22]. Furthermore, initiation of differentiation through EB leads to a heterogeneous cell population and co-culture, and using cells from a different species, could lead to potential contamination from animal

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products [23]. Finally, the osteogenic potential of the cells have generally been based on *in vitro* criteria except in four reports where the bone forming capacity was demonstrated *in vivo* either in heterotopic subcutaneous bone formation following implantation in immune deficient mice [4,7,21] or in diffusion chambers implanted in the peritoneal cavity of the nude mice [9]. However, these studies have not demonstrated that the bone observed was formed by the implanted human cells and not due to a dystrophic calcification reaction of the recipient murine cells.

Hyaluronan (also called hyaluronic acid (HA) or hyaluronate) is a non-sulfated glycosaminoglycan (GAG) of high molecular weight which is widely distributed throughout connective, epithelial, skin and neural tissues. It is one of the chief components of the extracellular matrix and can bind to aggrecan, along with type II collagen, which together form a major structural component of cartilage [24]. HA has also been reported to play a role in early human preimplantation embryogenesis [25].Within skeletal biology, HA has multiple functions including migration and condensation of BMSC [26], joint cavity formation and long bone growth [24]. HA is expressed in osteoblastic cells, osteocytes, osteoclasts and is a major component in bone marrow suggesting a role in bone remodelling [24,27]. One of HA's receptor's, CD44, has 21 variant isoforms due to extensive alternative splicing. However, all isoforms of CD44 contain a HA binding site in the extracellular domain [24,28] and binding of HA to CD44 is implicated in migration, proliferation and cell adhesion to extra cellular matrices [29].

The aim of this study was therefore to isolate and characterise an osteoprogenitor cell population from hESC. We report here the possibility of employing HA as a substrate to enrich for a stromal cell population (called here hESC-stromal) with osteogenic differentiation potential based on side-by-side comparison with bone marrow-derived human stromal stem cells (hBMSC) and phenotypic characterization both *in vitro* and *in vivo*. We have also demonstrated the functional ability of hESC-stromal cells in participating in bone healing in a critical calvarial defect in mice.

Methods and materials

Cell culture

Two in-house hESC lines, Odense3 and KMEB3, were derived and used in accordance with Danish stem cell guide lines and approval from the local Scientific Ethical committee. The characterisation of Odense3 and KMEB3 has previously been reported [30,31]. The Hues8 cell line was kindly provided by D. Melton (Howard Hughes Medical Institute, Harvard University, Boston, Mass) [2]. All cell lines were transitioned from routine culture on inactivated MEF feeders to standardised *in vitro* feeder free conditions [30]. Briefly, hESC were cultured in MEF-conditioned Melton's media (Knockout DMEM, 15% KOSR, 1% NEAA, 1 mM Glutamax, 0.1 mM β-mercaptoethanol (all Invitrogen, Taastrup, Denmark) and 0.5% human serum albumin (HSA; CSL Behring GmbH, Marburg, Germany)) and supplemented with 5 ng/ml hbFGF (Invitrogen, Taastrup, Denmark). Reduced growth factor Matrigel® (Becton Dickinson, Brøndby, Denmark) was used according to the manufacturer's instructions. Within two to three passages of transition a sub population of fibroblast-like, stromal cells developed between the hESC colonies. These cells have been referred to here as hESC-stromal cells. The hESC-stromal cells were isolated by plating both the colony and stromal cells onto plates pre-coated with 100 μg/ml hyaluronic acid (Calbiochem/Merck, Darmstadt, Germany) in DMEM (Invitrogen, Taastrup, Denmark) supplemented with 10% FBS (PAA, Pasching, Austria). Very few colony cells attached to the hyaluronic acid, and within two passages of culture on hyaluronic acid no residual colony cells were found.

Flow cytometry

Flow cytometry was carried out to establish a phenotypic profile of the hESC-stromal cells and to compare that profile with a well characterized hBMSC line established through over expression of human telomerase gene (TERT) and called hMSC-TERT [32]. OD3-, Hues8-, KMEB3-stromal cells as well as hMSC-TERT cells were stained for the pre-conjugated markers CD29-FITC (AbCam, Cambridge, UK); CD34-PE (Dako, Glostrup, Denmark); CD44-PE, CD45-PE, CD73-PE, CD146-PE and CD166-PE (all BD Pharmingen, Brøndby, Denmark); CXCR4-PE and Oct3/4-FITC (all R&D Systems, Abingdon, UK); Tra1-81-PE and CD105-APC (both eBioscience, Hatfield, UK). Cells, trypsinised to a single cell suspension, were blocked in 2% BSA before incubation with the above mentioned pre-conjugated antibodies, or matched isotype controls, for 45 min on ice. All samples were then analysed on a FACScan (BD Biosciences, Brøndby, Denmark) and data evaluated using WinMdi (http://www.cyto.purdue.edu/flowcyt/software/Winmdi.htm).

Differentiation to osteoblast, adipocyte and chondrocyte cells

hESC-stromal cells were plated, on tissue coated plastics (pre-coated, for 30 min at RT, with 100 μg/ml hyaluronic acid in PBS) in high glucose DMEM supplemented with 10% FBS (PAA, Pasching, Austria). Once 80% confluence had been achieved, cells were cultured in osteoblastic induction mixture containing 100 nM dexamethasone (Sigma-Aldrich, Brøndby, Denmark), 50 μg/ml L-ascorbic acid-2-phosphate (Sigma-Aldrich, Brøndby, Denmark) and 10 mM β-glycerophosphate (Calbiochem-Merck, Darmstadt, Germany). For adipocyte differentiation, the cells were incubated at 100% confluency in adipocyte induction mixture: DMEM plus 10% FBS media supplemented with 10% horse serum (Sigma-Aldrich, Brøndby, Denmark), 100 nM dexamethasone (Sigma-Aldrich, Brøndby, Denmark), 450 μM 1-methyl-3-isobutyl-xanthine (IBMX, Sigma-Aldrich, Brøndby, Denmark), 1 μM Rosiglitazone (BRL49653, Cayman Chemical, Ann Arbor, Michigan) and 5 μg/ml insulin (Sigma-Aldrich, Brøndby, Denmark). For the chondrocyte lineage, 2×10^5 cells were pelleted at 900 g for 5 min, and left overnight in CDM (chemically defined media: DMEM:F12 (Invitrogen, Taastrup, Denmark), 0.5% fraction V BSA (Sigma-Aldrich, Brøndby, Denmark), 1% lipid concentrate (Invitrogen, Taastrup, Denmark) and 1 mM glutamax (Invitrogen, Taastrup, Denmark)) supplemented with 10 nM dexamethasone (Sigma-Aldrich, Brøndby, Denmark), 50 μg/ml L-ascorbic acid-2-phosphate (Sigma-Aldrich, Brøndby, Denmark), 40 μg/ml L-proline (Sigma-Aldrich, Brøndby, Denmark), 0.91 mM sodium pyruvate (Invitrogen, Taastrup, Denmark) and 1% insulin-transferrin-selenium +1 (ITS+, BD Biosciences, Brøndby, Denmark). Cells were incubated overnight to form a pellet prior to the addition of 10 ng/ml TGFβ1 (Peprotech, London, UK).

Quantitative real time PCR (qRT-PCR) analysis

All collected samples underwent total RNA extraction using TRIzol (Invitrogen, Taastrup, Denmark; according to manufacturer's instructions), phase separation was carried out using chloroform and the resultant pellets washed in isopropanol and 70% ETOH, before air drying and resuspension in DEPC water. cDNA was constructed using a revertAid H minus first strand cDNA synthesis kit (Fermentas, St Leon-Rot, Germany) according to the manufacturer's instructions. qRT-PCR was accomplished using a MyIQ detection system (Bio-Rad, Copenhagen, Denmark) in a 20 μl volume with 20 pmol of each primer, 2× SYBERgreen (Bio-Rad, Copenhagen, Denmark) and 15 μg cDNA. Using the ICycler optical system software (Bio-Rad, v3.1) and Microsoft Excel, relative gene expression data was calculated. Following normalisation to the reference gene β-actin, quantification of gene expression was carried out using a comparative Ct method