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Molecular characterisation of stromal populations derived from human embryonic stem cells: Similarities to immortalised bone marrow derived stromal stem cells

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ABSTRACT

Human bone marrow-derived stromal (skeletal) stem cells (BM-hMSC) are being employed in an increasing number of clinical trials for tissue regeneration. A limiting factor for their clinical use is the inability to obtain sufficient cell numbers. Human embryonic stem cells (hESC) can provide an unlimited source of clinical grade cells for therapy. We have generated MSC-like cells from hESC (called here hESC-stromal) that exhibit surface markers and differentiate to osteoblasts and adipocytes, similar to BM-hMSC. In the present study, we used microarray analysis to compare the molecular phenotype of hESC-stromal and immortalised BM-hMSC cells (hMSC-TERT). Of the 7379 genes expressed above baseline, only 9.3% of genes were differentially expressed between undifferentiated hESC-stromal and BM-hMSC. Following ex vivo osteoblast induction, 665 and 695 genes exhibited ≥2-fold change (FC) in hESC-stromal and BM-hMSC, respectively with 172 genes common to both cell types. Functional annotation of significantly changing genes revealed similarities in gene ontology between the two cell types. Interestingly, genes in categories of cell adhesion/motility and epithelial–mesenchymal transition (EMT) were highly enriched in hESC-stromal whereas genes associated with cell cycle processes were enriched in hMSC-TERT. This data suggests that while hESC-stromal cells exhibit a similar molecular phenotype to hMSC-TERT, differences exist that can be explained by ontological differences between these two cell types. hESC-stromal cells can thus be considered as a possible alternative candidate cells for hMSC, to be employed in regenerative medicine protocols.

1. Introduction

Human bone marrow stromal stem cells (BM-hMSC) are adult, multipotent stem cells present within the non-haematopoietic compartment of the bone marrow. BM-hMSC are capable of differentiating into mesoderm-type cells e.g. osteoblasts, adipocytes (Abdallah and Kassem, 2012), and chondrocytes (Puetzer et al., 2010, review) under appropriate ex vivo conditions, and can form heterotopic bone and bone marrow organ in vivo (Abdallah et al., 2005). hMSC are being introduced for regenerative therapy in a number of clinical conditions including repair of bone (Quarto et al., 2001) and cartilage defects (Wakitani et al., 2002). In addition, the BM-hMSC secrete a large number of factors that exert anti-inflammatory and immune modulatory effects and thus BM-hMSC have been used in phase I/II clinical trials of tissue regeneration following cerebral haemorrhage (Heile et al., 2009) and in graft-versus-host disease (GvHD) (Le Blanc et al., 2010). One of the limiting factors for wider use of hMSC in therapy is the inability to obtain a sufficient number of cells needed for clinical applications. This is due to the small number of hMSC recovered from bone marrow aspirates and their limited proliferative potential during ex vivo expansion (Stenderup et al., 2003).

Human embryonic stem cells (hESC) are pluripotent stem cells (PSC) that can be maintained ex vivo in a self-renewing state and therefore represent an unlimited source for generating differentiated cells for regenerative therapies (Volarevic et al., 2011). Significant progress has been made in designing and validating efficient differentiation protocols

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of hESC into a number of cell lineages including osteoblastic cells (Abdallah et al., 2011; Irion et al., 2008). Additionally, new technologies allowing the expansion of pluripotent stem cells in bioreactors, without enzymatic interventions, will allow a large number of PSC to be generated prior to differentiation and transplantation (Chen et al., 2014). While the most interesting application of hESC-derived differentiated cells is as a universal off-the-shelf product for allogenic use in cell therapy, further research about the in vivo functions of hESC-derived differentiated cells need to be conducted both in animal models and in humans.

However, concerns have been raised regarding the ability of the ex vivo differentiation of hESC to induce a differentiated phenotype similar to that observed in tissue-specific cells (Cahan and Daley, 2013). The phenotype of hESC-derived differentiated cells have traditionally been described based on a limited number of in vitro expressed lineage specific markers (D'Amour et al., 2005; Hay et al., 2008; Kroon et al., 2008). Side-by-side comparisons of hESC-derived differentiated cells and bona fide tissue resident differentiated cells have rarely been conducted (Irion et al., 2008). Four published reports have previously compared mesodermal lineage differentiation of hESC with tissue specific/resident cells. Raynaud et al. investigated cardiomyocytes derived from hESC in comparison with bone marrow mesenchymal cells (Raynaud et al., 2013); Barbet et al., Bigdeli et al. and Barberi et al. all based their comparison of mesenchymal-like (MSC-L) cells, derived from hESC, and bone marrow derived cells at baseline only (Barbet et al., 2011; Bigdeli et al., 2010; Barberi et al., 2005). Interestingly, Barbet et al. demonstrated that MSC-like cells derived from hESC clustered closer with BM-hMSC as compared to MSC-like derivatives with hESC (Barbet et al., 2011). Thus, data examining differences between MSC-like cells and BM-hMSC during osteoblastic induction are absent.

The availability of large-scale methods for gene profiling, such as DNA microarrays, offers an opportunity to define the "molecular phenotype" of ex vivo cultured cells. Additionally, data mining using bioinformatic tools can provide a functional understanding of pathways and processes enriched in differentiated cell lineages and allow comparisons between different cell populations (Klimanskaya et al., 2004; Larsen et al., 2010; Lu et al., 2007; Marei et al., 2011).

We have developed an ex vivo culture method to isolate, propagate and differentiate stromal cells obtained from hESC (termed here hESC-stromal) into osteoblastic and adipocytic cells (Harkness et al., 2011). At a cellular level, hESC-stromal exhibited comparable cluster of differentiation (CD) surface markers, and differentiated to the osteoblastic and adipocytic lineages using the same differentiation protocol employed in hMSC-TERT (Harkness et al., 2011). In the present study, we employed DNA microarrays to compare the molecular phenotype of hESC-stromal to a bona fide bone marrow-derived, immortalised, hMSC. In addition, an in depth analysis of the differentiated phenotype of hESC derived MSC-like cells has been obtained. Our results demonstrate that these two cell populations are closely related at both the cellular and molecular levels.

2. Methods

2.1. Cell culture

hESC-stromal cells were derived as previously described (Harkness et al., 2011). Briefly, stromal fibroblast-like cells were isolated from an in house Odense3 embryonic stem cell line (Harkness et al., 2011), grown under feeder free conditions in the presence of mouse embryonic fibroblast (MEF) conditioned media, and cultured on hyaluronic acid-coated culture plates (100 μg/ml; HA; Calbiochem-Merck, Darmstadt, Germany) (Harkness et al., 2011). These cells were termed hESC-stromal cells and were routinely cultured in high glucose DMEM (Invitrogen, Taastrup, Denmark) with 10% FBS (PAA, Pasching, Austria). As a model for BM-hMSC, we employed hMSC-TERT (Simonsen et al., 2002) that exhibit a stable cellular and molecular phenotype comparable to that of primary BM-hMSC (Al-Nbaheen et al.,

2013). hMSC-TERT were routinely cultured in MEM (Invitrogen) with 10% FBS (PAA, Pasching, Austria).

Ex vivo osteoblast (OB) differentiation was performed using osteoblast induction medium containing β-Glycerophosphate (10 mM; Calbiochem-Merck), L-ascorbic acid-2-phosphate (50 μg/ml; Sigma-Aldrich, Brøndby, Denmark), dexamethasone (100 nM hESC-stromal, 10 nM hMSC-TERT; Sigma-Aldrich) and calcitriol (1,25 hydroxy-Vitamin D_3; 10nM). For samples collected for microarray analysis media were changed every 2 days until day 6 for hESC-stromal (n = 3 independent experiments, at passages 6 and 7) or day 7 for hMSC-TERT (n = 1 experiment, passage 48). Data for validation of array gene expression was generated from 6 independent experiments and plotted using the SEM (Fig. 4); data for initial comparison of hESC-stromal and hMSC-TERT undifferentiated and undergoing OB induction were calculated from 3 independent experiments (Fig. 1). The OB differentiated cells are termed: hESC-stromal-OB and hMSC-TERT-OB.

### 2.2. Alkaline phosphatase (ALP) activity measurements

ALP activity was quantified as previously described (Qiu et al., 2010) using a 1 mg/ml solution of P-nitrophenylphosphate (Sigma-Aldrich, Brøndby, Denmark) in 50 mM $NaHCO_3$ with 1 mM $MgCL_2$, pH 9.6, at 37 °C for 20 min. Activity was stopped using 3 M NaOH, absorbance of each reaction (λ_{max} = 405 nm) was measured using a FLUOstar Omega plate reader (BMG Laboratories, Ramcon A/S, Birkerod, Denmark) and ALP activity was normalised to cell number (n = 3 independent experiments, 6 wells/experiment). Cell number was determined using a CellTiter-Blue Cell Viability assay, according to the manufacturer's instructions (Promega, Nacka, Sweden). A two-tailed, paired T-Test statistical analysis was used to establish if significant differences could be determined between the normalised ALP activity of hESC-stromal-OB and hMSC-TERT-OB at 6 days of induction.

2.3. RNA isolation and real-time quantitative RT-PCR

Total RNA was isolated using TRIzol (Invitrogen) as previously reported (Harkness et al., 2011) and cDNA was generated using a revertAid H minus first strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. RT-PCR was performed on an ABI Step one PCR machine (Applied Biosystems-Life Technologies, Carlsbad, CA, USA). Data were normalised to the geometric mean of the reference genes (β-Actin, B2M, HPRT, UBC1) and analysed using a comparative Ct method where Δ-CT is the difference between the CT values of the target and the geometric mean of the reference genes. Primer sequences (Supplementary Table 1) were designed using Primer-BLAST.

2.4. Illumina® bead chip microarray

Following RNA extraction, samples were purified using a GenElute mammalian total RNA miniprep kit (Sigma-Aldrich; according to the manufacturer's instructions). Five hundred nanograms of total RNA per sample were used for biotin-labelled cRNA production using a linear amplification kit (Ambion). Hybridizations, washing, Cy3-streptavidin staining, and scanning were performed on the Illumina BeadStation 500 platform (Illumina®) according to the manufacturer's instruction. cRNA samples were hybridised onto Illumina® human-8 BeadChips version 3. All basic gene expression data analyses were carried out using the BeadStudio® software 3.0. Raw data were background-subtracted, normalised using the "rank invariant" algorithm and then filtered for significant expression on the basis of negative control beads. To demonstrate the relationship between the different samples, hierarchical clustering was performed using the Illumina BeadStudio® software. Correlation was used as the distance measure, and clustering was performed with the average linkage method.
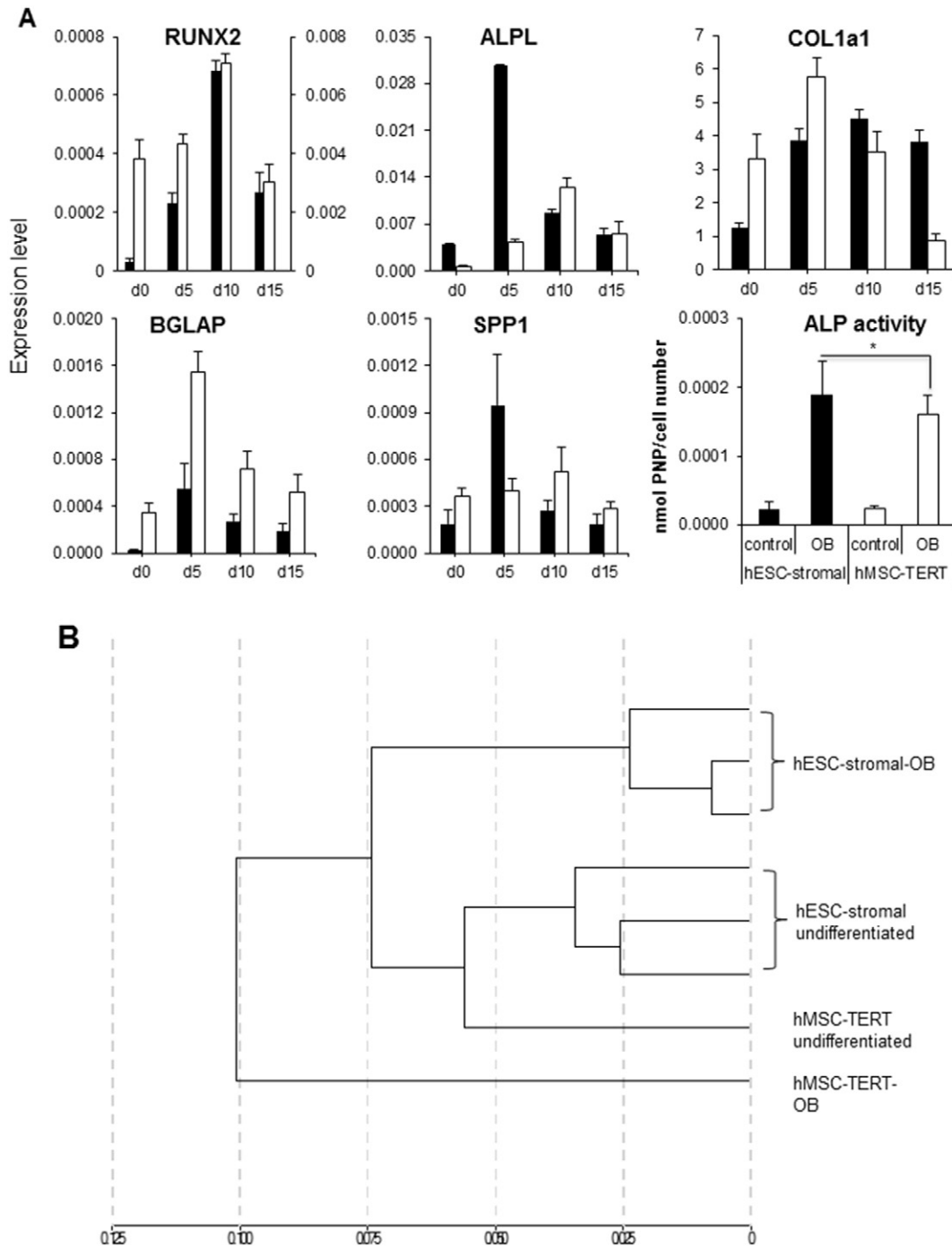
Fig. 1. Comparison of undifferentiated and in vitro osteoblast (OB) differentiated hESC-stromal and hMSC-TERT. (A) Gene expression of osteoblast lineage markers in hESC-stromal-OB ■ and hMSC-TERT-OB □ (n = 3 independent experiments) and ALP activity normalised for cell number in hESC-stromal-OB and hMSC-TERT-OB (n = 3 independent experiments; *p < 0.05); (B) Hierarchical clustering of cell samples following Illumina bead array.

2.5. Functional gene enrichment analysis

Data analysis for functional enrichment was performed using the **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery (DAVID) (Huang et al., 2008, 2009). Genes were selected for comparison if they had a detection threshold p value < 0.01. Under OB induction, genes that had a fold change (FC) of ≤− 2 or ≥2 compared to the corresponding control were employed in the analysis. Fold change from undifferentiated hESC-stromal over hMSC-TERT, hMSC-TERT over hESC-stromal and fold change of OB induced/non induced for each cell line were analysed using DAVID. Data from DAVID was plotted using the one tail Fisher

exact probability value calculated by DAVID. MetaCore® v6.14 was used to perform pathway enrichment analysis on genes significantly expressed (p < 0.01). Gene Ontology (GO) ossification data were downloaded from the GO database (http://www.geneontology.org/GO.downloads.ontology.shtml). Ossification genes, relative to undifferentiated cells, differentially regulated during OB differentiation were identified (>2 FC or <− 2 FC) in either hMSC-TERT-OB (n = 17), hESC-stromal-OB (n = 15) or in both hESC-stromal-OB and hMSC-TERT-OB (n = 11).

Studies published here did not require ethical approval further to that published in the original derivation articles (Simonsen et al., 2002; Harkness et al., 2010).

3. Results

3.1. Cellular phenotype

Cultured hESC-stromal and hMSC-TERT exhibited a fibroblast-like morphology at baseline and cuboidal morphology following ex vivo OB differentiation, respectively (data not shown). Both hESC-stromal and hMSC-TERT responded to OB induction by upregulation of OB marker genes: *RUNX2*, *ALPL*, *COL1A1*, *BGLAP* and *SPP1* as well as ALP activity (Fig. 1A). Both cell types formed heterotopic bone and bone marrow organ when implanted subcutaneously in immune deficient mice as previously reported (Harkness et al., 2011).
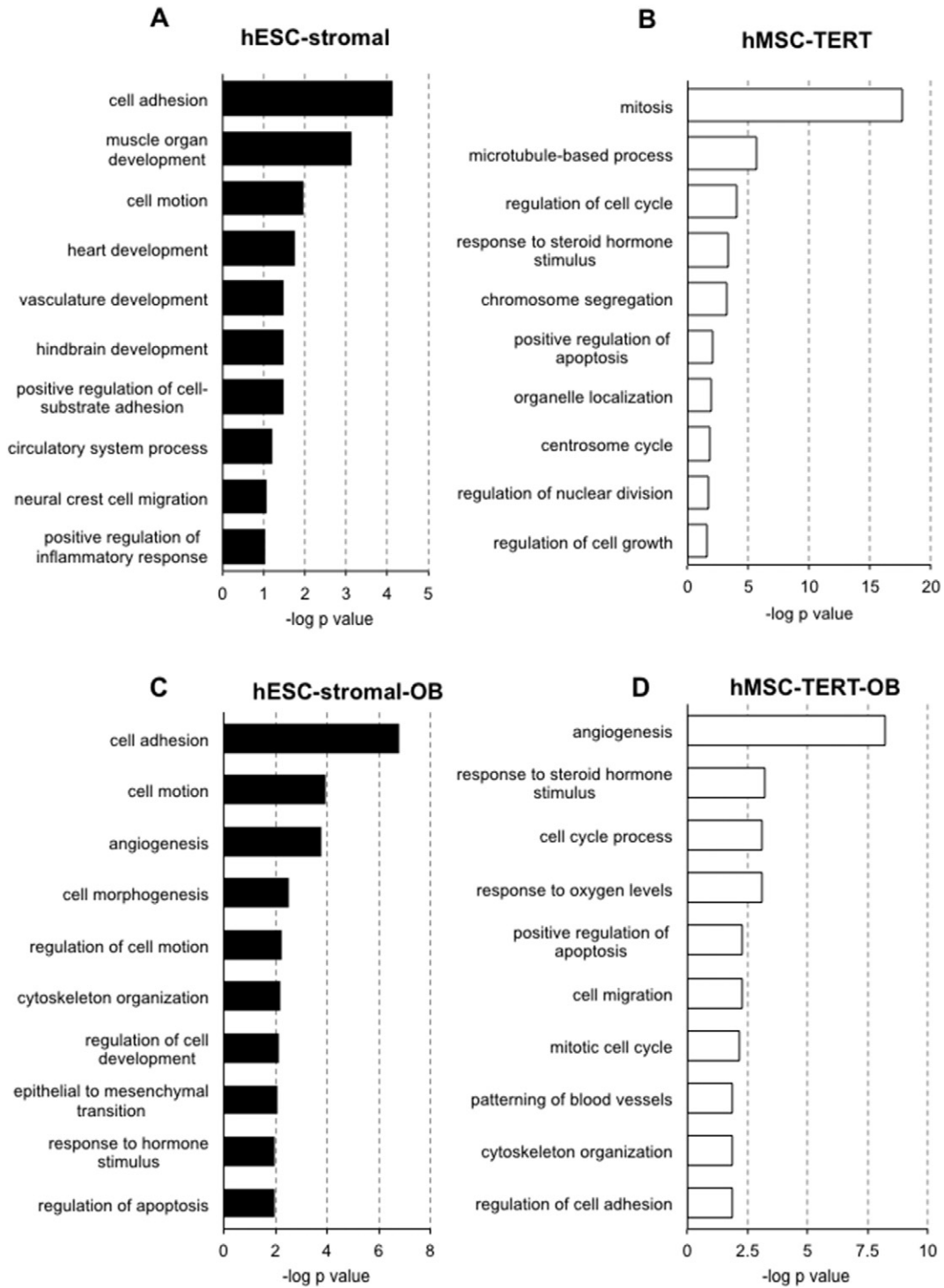
Fig. 2. GO functional enrichment of hMSC-TERT and hESC-stromal cells over 2 FC (detection threshold p ≤ 0.01). (A) GO biological process categories of undifferentiated hESC-stromal cells/hMSC-TERT show an increased annotation to developmental genes suggesting an increased capacity for multi-lineage differentiation as compared to hMSC-TERT; (B) in comparison undifferentiated hMSC-TERT/hESC-stromal demonstrate an increased GO BP annotation to cell cycle/mitosis categories; (C) GO functional enrichment of genes up and down regulated during osteogenic differentiation unique to hESC-stromal-OB (n = 493); (D) GO functional enrichment of up and down regulated genes unique to hMSC-TERT-OB (n = 523).

3.2. Comparison of molecular phenotype of undifferentiated hESC-stromal vs. hMSC-TERT cells at baseline

Microarray analysis identified 7379 expressed genes (a gene was considered to be expressed if the p-value of detection threshold is ≤0.01). Gene lists, used for GO BP and MetaCore® analyses as well as comparison with GO database, were established by the following criteria: undifferentiated genes regulated ≥2 FC of hESC-stromal/hMSC-TERT with a detection p-value of ≤0.01; OB induced gene lists were established for each cell line of OB induced/undifferentiated ≥2 FC with a detection p-value of ≤0.01. Hierarchical clustering demonstrated a close relationship between undifferentiated hESC-stromal and hMSC-TERT (Fig. 1B). The majority of genes demonstrated similar expression levels in both cell types with 9.3% of total expressed genes differentially regulated (353 genes differentially up-regulated (FC ≥ 2) and 334 down-regulated (FC ≤ −2)) between the two cell lines. Functional enrichment analysis for gene ontology (GO) biological processes (BP) revealed, in hESC-stromal the highest enrichment scores in categories of cell adhesion, mesodermal tissue developmental and cell motion (Fig. 2A). In comparison, GO BP categories for cell division, response to steroid hormone stimulus and positive regulation of apoptosis were highly enriched in hMSC-TERT (Fig. 2B). An overview demonstrating the distribution of genes (non-induced and OB induced) is shown in the Venn diagrams in Supplementary Fig. 1A–D.

3.3. Comparison of molecular phenotype of hESC-stromal-OB vs. hMSC-TERT-OB

Prior to selecting a time point during OB induction for microarray analysis, hMSC-TERT and hESC-stromal, undergoing differentiation induction, were compared using ALP activity and ALP gene expression as a measure for osteoblast lineage differentiation. From these preliminary experiments d6 of hESC-stromal-OB and d7 of hMSC-TERT-OB were selected as being the most comparable time points (data not shown). In order to detect whether hESC-stromal and hMSC-TERT employ similar biological processes during ex vivo OB differentiation, we compared hESC-stromal-OB and hMSC-TERT-OB utilising the following four bio-informatic approaches.

First, osteoblast differentiation regulated genes were compared between hESC-stromal and hMSC-TERT. Comparison of fold induction (OB induced/undifferentiated) identified a comparable number of genes both up and down regulated: 695 genes differentially regulated (FC ≤ −2 or ≥2) in hMSC-TERT-OB and 665 genes in hESC-stromal-OB. Among these, 172 genes (≈30%) were common to both cell types following differentiation suggesting a common OB differentiation program. Employing the DAVID tool for GO functional annotation of BP, the highest enriched GO categories of these 172 genes included mitosis, response to estradiol stimulus, insulin receptor signalling and regulation of apoptosis (Supplementary Fig. 1E). In addition, the top 10 enriched GO categories for each cell type exhibited similarities e.g. cell adhesion, angiogenesis, cytoskeletal organisation, response to hormone stimulus and regulation of apoptosis (Fig. 2C and D). Conversely, differences in GO categories were also observed. GO categories for epithelial-to-mesenchymal (EMT) transition and cell morphogenesis were unique for hESC-stromal-OB (Fig. 2C) whereas hMSC-TERT-OB (Fig. 2D) were enriched in GO BP categories for cell cycle processes, mitotic processes and response to oxygen levels. Data lists detailing genes annotated to the top 10 categories are presented in Supplementary Table 2.

Second, we examined the separate annotations for genes that were up- or down-regulated during OB differentiation in the two cell types. hESC-stromal-OB demonstrated 231 genes and hMSC-TERT showed 335 genes that were up-regulated ≥2 FC during OB differentiation, and among these 91 genes were common between the two cell types (Supplementary Fig. 2). Common GO categories were present e.g. proliferation, response to hormone stimulus, regulation of apoptosis and

regulation of cell adhesion. For genes down-regulated during OB differentiation, 262 genes and 188 genes were found in hESC-stromal-OB (Supplementary Fig. 3B) and hMSC-TERT-OB (Supplementary Fig. 3C), respectively. Among these 81 genes were common between the two cell types (Supplementary Fig. 3A) and were enriched for GO categories: mitosis and regulation of phosphorylation. The list of differentially up-regulated or down-regulated genes in hESC-stromal-OB and hMSC-TERT-OB are given in Supplementary Table 2.

Third, to test for the presence of a common "osteoblastic differentiation signature" we curated an 'ossification' GO dataset that identified 293 genes. Of these 293 genes, we found that 135 did not pass the p-value detection threshold of ≤0.01 in either hESC-stromal-OB or hMSC-TERT-OB. Of those genes that were expressed (p ≤ 0.01), 115 (73%) were expressed equally in both cell types, 25 (16%) genes were uniquely expressed in hESC-stromal-OB and 18 (11%) genes uniquely expressed in hMSC-TERT-OB (Supplementary Table 3).

Finally, we compared pathways enriched in hESC-stromal-OB and hMSC-TERT-OB using MetaCore®. All expressed genes (detection p value ≤ 0.01) in hMSC-TERT-OB and hESC-stromal-OB were employed. The top 10 enriched pathways for hESC-stromal-OB and hMSC-TERT-OB are shown in Fig. 3A and B, respectively. While common genetic pathways for both cell types included EMT and CSF signalling, their function may differ. For example, hESC-stromal-OB demonstrated enrichment for GM-CSF with a role in formation and release of inflammatory cytokines whereas hMSC-TERT-OB was enriched in G-CSF with a role in formation and release of granulocytes from bone marrow. In addition, both cell types demonstrated enrichment of the EMT pathway through induction of TGFB signalling. Interestingly, hMSC-TERT-OB demonstrated additional EMT pathway induced via MAPK and SMAD signalling.

3.4. Validation of microarray data of hESC-stromal-OB and hMSC-TERT-OB

We chose 24 genes (FC ≤ −2 or ≥2) that were found among the core "ossification" gene category for validating the microarray data using real-time qRT-PCR (Fig. 4A–C). We included genes present in both hESC-stromal-OB and hMSC-TERT-OB: CLEC3B, SRGN, IFITM1, PTN, MN1, EXT1, CBS and IGFBP5, genes expressed uniquely in hESC-stromal OB: DCN, COMP, MGP, P2RX7, SOX9, GLI2, PTGS2, IL6 or genes expressed uniquely in hMSC-TERT-OB: TGBF3, TGBF2, PRKD1, CEBPB, ID1, ECM1, GJA1, MMP2. As seen in Fig. 4A–C, qRT-PCR revealed a close correlation of qRT-PCR measurements with those from DNA microarray data.

4. Discussion

In the present manuscript, we have compared the molecular phenotype of hMSC either derived from hESC or from bone marrow. While there were some quantitative differences between the gene expression and cellular responses, the two cell populations exhibited a high degree of homology in relation to undifferentiated gene expression and qualitative responses to OB differentiation induction. In addition, we identified a common molecular signature and common core of genes and genetic pathways that are employed by both cell types during in vitro OB differentiation.

We used an immortalised cell line (hMSC-TERT) to compare with hESC-stromal cells undifferentiated and under osteoblastic induction. The hMSC-TERT cell line has been extensively characterised by our group and has been demonstrated as a good predictive model for the biological behaviour of primary hMSC (Abdallah et al., 2005; Simonsen et al., 2002). In addition, publications from other groups comparing immortalised versus primary cells have also demonstrated retention of basic cell characteristics (Boerma et al., 2006; Ouellette et al., 2000). However, the expression of ectopic hTERT, and thus immortalisation, is indicative that genes associated with cell cycle will be up-regulated in comparison with primary cell lines. Thus, over-representation of cell cycle categories in undifferentiated hMSC-TERT biological processes

A

hESC-stromal-OB

B
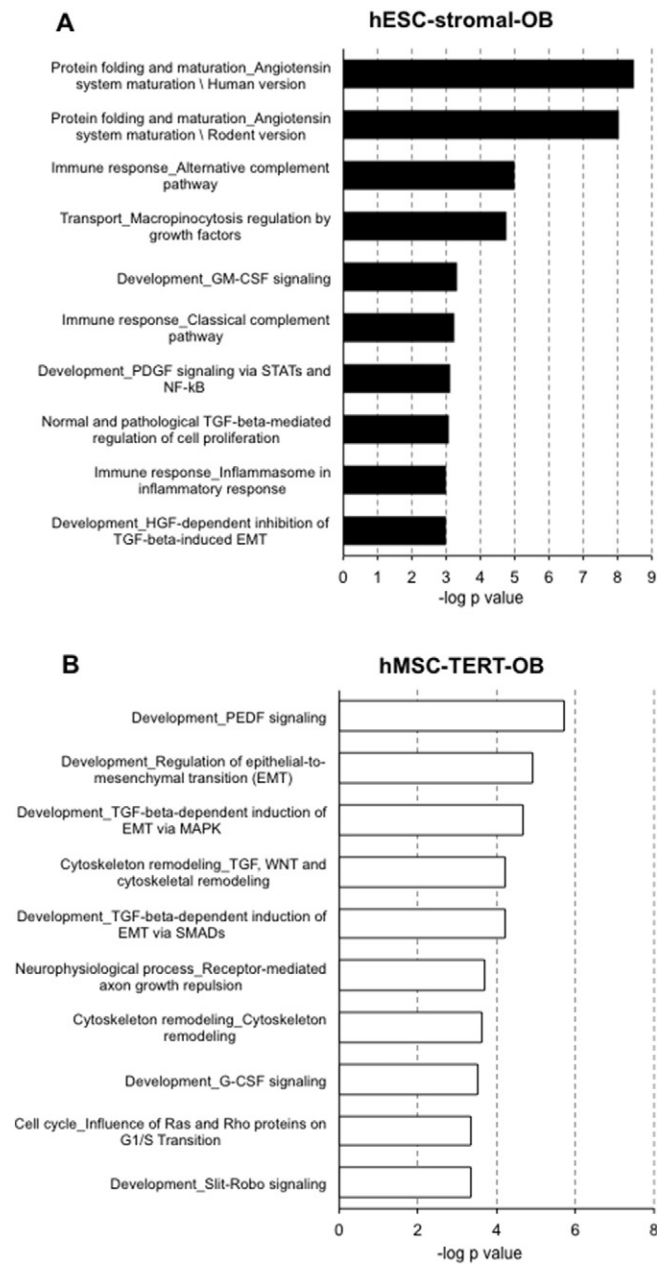
hMSC-TERT-OB

Fig. 3. Enrichment analysis (MetaCore®) for unique and common pathways: (A) pathways unique to hESC-stromal-OB; (B) pathways unique to hMSC-TERT-OB. Both annotations demonstrate categories involved in EMT and TGFβ pathways suggesting these are important to both cell types during differentiation.

GO is not unexpected. We expected that the most up-regulated genes observed in non-induced hMSC-TERT would be associated with mitosis, however, while cell cycle genes were overrepresented, the majority of the upregulated genes, annotated to mitosis, were between 2–3 FC with only 3 genes demonstrating >3 FC upregulation when compared to hESC-stromal (INCEP, CCNF, NEK2). These 3 genes were not found within the top 50 hMSC-TERT genes which were differentially annotated in hESC-stromal. Functional clustering of the top 50 genes demonstrated enrichment in categories: response to hormone stimulus and skeletal system development. In comparison functional clustering of the top 50 genes in hESC-stromal revealed enrichment for categories of: smooth muscle proliferation and muscle organ development. This suggests that these differences reflect differences of the ontology of

the two cell types (adult versus embryonic) rather than immortalisation caused by overexpression of hTERT in hMSC-TERT.

Data analysis determined that 90.3% of genes expressed were similar between undifferentiated hESC-stromal and hMSC-TERT. Our data, thus, corroborates previously published reports comparing the molecular phenotype of hESC-derived MSC-like cells and bone marrow-derived hMSC (Raynaud et al., 2013; Barbet et al., 2011; Bigdeli et al., 2010; Barberi et al., 2007). The authors demonstrated a close relationship between hESC-MSC-like cells and BM-hMSC employing principal component analysis (PCA) and hierarchical clustering (Raynaud et al., 2013). Barbet et al. identified differentially expressed genes utilising a Taqman low-density array and reported that hESC-MSC clustered more closely with BM-hMSC than with undifferentiated hESC (Barbet et al., 2011). Barberi et al. utilised affymetrix oligonucleotide arrays on a CD73^{+} sorted population derived from hESC and compared these with BM-hMSC (Barberi et al., 2005). Similar to our data the authors found enrichment for genes associated with vascular development and inflammatory response/wound healing. Finally, Bigdeli et al. (2010) reported similarities in the enrichment of osteoblastic genes in undifferentiated hESC-MSC and BM-MSC.

Other studies have examined the molecular phenotype of osteoblastic cells differentiated from hESC i.e. hESC-MSC-OB as compared with hiPSC-MSC-OB derived from induced pluripotent stem cells (iPSC) (de Peppo et al., 2013) and demonstrated the ability of both cell types to undergo in vitro osteoblastic differentiation. Global DNA microarray analysis of hESC-MSC and hiPSC-MSC demonstrated similarities and differences in both undifferentiated cells and in cells undergoing OB differentiation. However, the limitation of these studies is the absence of comparison with bona fide osteoblastic cell populations e.g. osteoblastic cells derived from BM-hMSC as we did in our current study. Our comparison with a Gene Ontology (GO) derived osteogenic gene set demonstrated that 73% of osteogenic genes found in hESC-stromal-OB and hMSC-TERT-OB were similar. Within this common list, 11 genes were regulated over 2 FC (p ≤ 0.01) of which only 1 gene (STC1) was differentially regulated. In addition, of the 172 genes in the OB-induced cells (FC ≤ −2 or ≥2; p ≤ 0.01) which were commonly expressed in both cell types, 86% of the genes were similarly expressed. Analysis of genes which were differentially regulated during OB induction (FC ≤ −2 or ≥2; p ≤ 0.01) in hMSC-TERT-OB (n = 523) and in hESC-stromal-OB (n = 493), showed that the majority (96.7% hMSC-TERT-OB; 97% hESC-stromal-OB) did not demonstrate annotations for GO ossification. Data presented here therefore demonstrates a common set of genes for OB induction shared between hESC-stromal and hMSC-TERT. The large gene sets not exhibiting osteogenic annotation demonstrates that osteogenesis involves an activation of a complex network of genes related to basal biological processes.

A large number of genes in both hESC-stromal-OB and hMSC-TERT-OB were annotated as related to epithelial-to-mesenchymal transition (EMT). EMT is a mechanism for generation of primary mesenchyme during embryogenesis (Kalluri and Weinberg, 2009) and is orchestrated through Wnt signalling (Kalluri and Weinberg, 2009; Ullmann et al., 2008). Among the 130 genes regulated during EMT (Groger et al., 2012), 68 genes were present in hESC-stromal and 65 genes in hMSC-TERT. Our study corroborates findings from previous studies reporting that development of MSC-like cells in hESC cultures was mediated through EMT as evidenced by differential regulation of E-cadherin and N-cadherin (Ullmann et al., 2007), expression of EMT-associated genes (Hwang et al., 2008) and flow cytometric analysis of E-Cadherin (Boyd et al., 2009).

We observed that the differentiated progeny of hESC, hESC-stromal cells, did not express pluripotent markers e.g. *POU5F1, SOX2, NANOG, DNMT3B, GABRB3,* and *TDGF1* suggesting the loss of pluripotency and providing strong evidence that the hESC-derived differentiated cells will not form teratomas or tumours when implanted in vivo. In support of this, tumour formation was absent when hESC-stromal were subcutaneously implanted in immune compromised mice (Harkness et al., 2011).
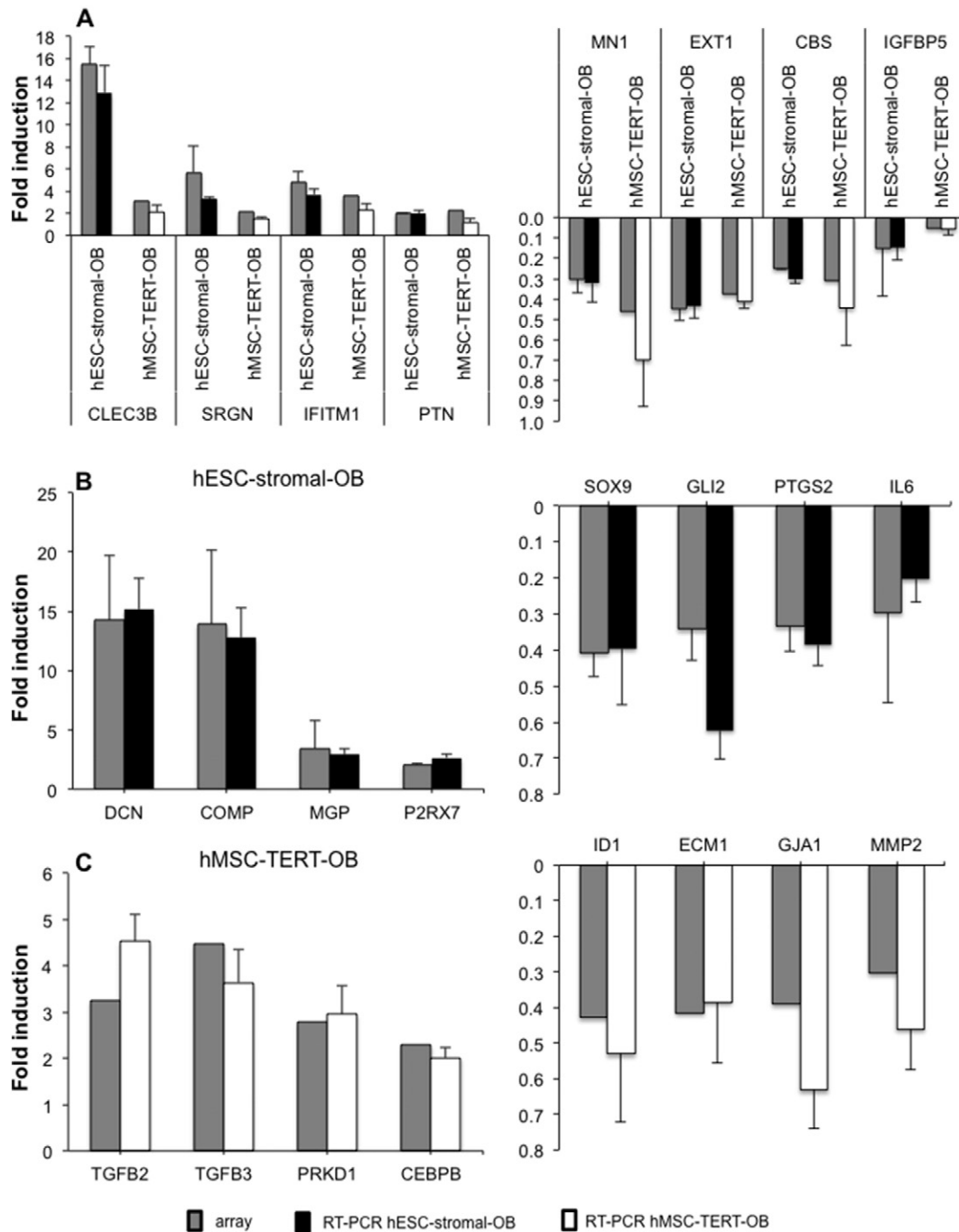
Fig. 4. Validation of microarray data by RT-PCR (n = 6 independent experiments): (A) genes common to hESC-stromal-OB (array—grey bar; PCR—black bar) and hMSC-TERT-OB (array—grey bar; PCR—white bar) up or down regulated over 2 FC (p ≤ 0.01); (B) OB genes unique to hESC-stromal-OB; (C) OB genes unique to hMSC-TERT-OB. Data is presented as fold change.

Our data demonstrate similarities in the molecular phenotype of undifferentiated and OB differentiated hESC-stromal cells when compared with hMSC encouraging the use of hESC-stromal cells as an alternative source of hMSC for regenerative medicine protocols.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bonr.2015.07.002.

Competing interests

The authors have no competing interests.

Author contributions

LH, MK, AA, JA were responsible for the experimental concept and design; LH and AJ carried out the laboratory experiments and data analysis; RAD and JA performed the microarray and data analysis; NAT and MRW carried out the bioinformatic analysis; LH and NAT prepared the manuscript; all authors read and approved the manuscript.

Acknowledgements

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