Mesenchymal differentiation propensity of a human embryonic stem cell line

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Received 11 October 2010; revision accepted 17 November 2010

Abstract

Objectives: To characterize basal differentiation tendencies of a human embryonic stem (hES) cell line, KCL-002.

Materials and methods: *In vitro* specification and differentiation of hES cells were carried out using embryoid body (EB) cultures and tests of pluripotency and *in vivo* differentiation were performed by teratoma assays in SCID mice. Real-time PCR, immunohistochemistry, flow cytometry and histological analyses were used to identify expression of genes and proteins associated with the ectodermal, endodermal and mesodermal germ layers.

Results: Undifferentiated KCL-002 cells expressed characteristic markers of pluripotent stem cells such as Nanog, Sox-2, Oct-4 and TRA 1-60. When differentiated *in vitro* as EB cultures, expression of pluripotency, endodermal and ectodermal markers decreased rapidly. In contrast, mesodermal and mesenchymal markers such as VEGFR-2, α -actin and vimentin increased during EB differentiation as shown by qPCR, immunostaining and flow cytometric analyses. Teratoma formation in SCID mice demonstrated the potential to form all germ layers *in vivo* with a greater proportion of the tumours containing mesenchymal derivatives.

Conclusions: The data presented suggest that the KCL-002 hES cell line is pluripotent and harbours a bias in basal differentiation tendencies towards mesodermal and mesenchymal lineage cells. Characterizing innate differentiation propensities of hES

cell lines is important for understanding heterogeneity between different cell lines and for further studies aimed at deriving specific lineages from hES cells.

Introduction

Human embryonic stem (hES) cells are pluripotent cells that represent an unlimited source of cells for *in vitro* analysis of early developmental pathways and cell differentiation studies as well as for potential replacement of cells and tissues damaged in disease. hES cell potential in regenerative medicine is widely acknowledged, and progress towards derivation of clinically useful differentiated cell types from hES cells is advancing rapidly (1–13). Pluripotency, the ability of hES cells to differentiate into cell types representing ectodermal, endodermal and mesodermal germ layers, underpins all studies aimed at understanding the molecular basis of and directing differentiation of, these cells to specific lineages. Upon initial hES cell line derivation, pluripotency of the cells is typically verified through induction of spontaneous differentiation following cell transplantation into immunocompromised mice, resulting in formation of teratomas (14–20). Teratomas often have phenotypes from all three germ layers that are usually identified based on their morphological and molecular characteristics. Pluripotency may also be ascertained *in vitro* through employment of differentiation of embryoid body (EB) cell approaches, whereby culture of hES cells in adherence-independent, three-dimensional aggregates induces germ layer differentiation of the cells, and these can be identified within these structures using molecular markers (21,22).

Recent studies by several groups have suggested that, whilst different hES cells express similar stem cell markers and can form teratomas efficiently, they are pre-programmed with innate developmental biases, perpetrated through as yet unclear mechanisms (23–26).

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This results in a heterogeneous spectrum of differentiation potentials between independently isolated hES cell lines. It is important to identify and characterize these basal differentiation tendencies, as progress towards eventual attainment of clinically useful cell types from hES cells may be augmented by careful initial choice of the hES cell line. We have previously derived a panel of hES cell lines, including one harbouring a common mutation found in cystic fibrosis (27,28). Here, we describe characterization of an hES cell line derived in our laboratory, KCL-002, using EB and teratoma differentiation studies to address the basal differentiation potential of this line, and show that KCL-002 cells display propensity towards mesenchymal differentiation tendencies both *in vitro* and *in vivo*.

Materials and methods

hES cell maintenance and differentiation

KCL-001, -002 and -003 hES cells were maintained in the undifferentiated state, on a layer of mitotically inactivated mouse embryonic fibroblasts, in basic hES medium, DMEM, supplemented with 2 mM Glutamax, 0.1 mM non-essential amino acids and 0.1 mM beta-mercaptoethanol (all from Invitrogen, Paisley, UK), 20% ES-FCS (Autogen Bioclear, Wiltshire, UK) and 8 ng/ml FGF-2 (Peprotech, Rocky Hill, NJ, USA). KCL-003 line is homozygous for the $\Delta F508$ mutation responsible for human cystic fibrosis (27). All the hES cell types were passaged mechanically once weekly as described previously (27–29), and were used from comparable passage numbers, between passages 20 and 40. There were no differences between any of these cell lines in terms of morphology and proliferation rates. For differentiation studies, undifferentiated hES cell colonies were cut into an average of 6–8 pieces and cultured in suspension as embryoid bodies (EBs) in basic hES medium in 60 mm ultra low adherence Petri dishes (Appleton Woods, Birmingham, UK). EBs were harvested after 4, 8 and 16 days of differentiation for analysis.

Teratoma assay

The undifferentiated hES cells were harvested and transplanted into the testis of immunocompromised SCID mice, at 50 000 cells/100 μ l as described previously (20). Mice were monitored weekly for presence of tumours by palpation, and teratomas were harvested approximately 3 months after implantation for analysis by paraffin wax-embedded histology for light microscopy or by quantitative PCR (qPCR), flow cytometry and immunostaining as described below.

Quantitative PCR

RNA was extracted from undifferentiated hES cell colonies and EBs at times indicated, using the RNeasy Kit (Qiagen, West Sussex, UK). Real-time qPCR was performed using Rotor-Gene 3000 Quantitative multiplexing system and Rotor-Gene 6 (Version 6.0) software. Specific primer pairs were designed using Primer3 website (<http://frodo.wi.mit.edu/>) as follows: Nanog sense 5'-AATACCTCA GCCTCCAGCAGATG-3'; Nanog anti-sense 5'-TGCGTC ACACCATGCTATTCTTC-3'; Sox-2 sense 5'-TGGAC AGTTACGCGCACAT-3'; Sox-2 anti-sense 5'-CGAGT AGGACATGCTGTAGGT-3'; neural cellular adhesion molecule 1 (NCAM-1) sense 5'-AGGAGACAGAAACG AAGCCA-3'; NCAM-1 anti-sense 5'-GGTGTGGAAG TGCTCTGGT-3'; glial fibrillary acidic protein (GFAP) sense 5'-CCTCTCCCTGGCTCGAATG-3'; GFAP anti-sense 5'-GGAAGCGAACCTTCTCGATGTA-3'; α -feto protein (AFP) sense 5'-TCGACCACATTTACCGACA AG-3'; AFP anti-sense 5'-TTGCCAGTAACCAGGA AGAT-3'; forkhead boxA2 (FoxA2) sense 5'-AAGACCT ACAGGCGCAGCTA-3'; Foxa2 anti-sense 5'-GGCACC TTCAGGAAACAGTC-3'; vascular endothelial growth factor receptor 2 (VEGFR-2) sense 5'-CACCACCTAAA CGCTGACATGTA-3'; VEGFR-2 anti-sense 5'-GCTCGT TGGCGCACTCTT-3'; α -actin sense 5'-GTACCACAG GCATTGTTCTGG-3'; α -actin anti-sense 5'-AGAAGCA TACAGGGATAGCACT-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense 5'-GAAGGTGAAGGTC GGAGTC-3'; GAPDH anti-sense 5'-GAAGATGGTGAT GGGATTTC-3'. Expression of all genes in undifferentiated (Day 0) KCL-002 colonies was used to denote 100% level of expression.

Flow cytometry

Undifferentiated KCL-002 hES cell colonies and differentiating EBs were dissociated with TrypLE Express (Invitrogen) to generate single cell suspensions, fixed in 4% PFA and permeabilized. Cells were incubated with anti-vimentin primary antibody (1:200; V9 clone, GE Life Sciences, Buckinghamshire, UK) followed by goat α -mouse IgG Alexa Fluor 488 (Invitrogen) secondary antibody (1:1000). Samples were analysed for immunofluorescence using FACS Aria cell sorting system (BD Biosciences, Oxford, UK), and processed using FACSDiva and Weasel software.

Histology and immunostaining

All material for histological processing was fixed in 4% PFA, processed to paraffin wax, sectioned at 8 μ m and stained with haematoxylin and eosin. For

immunohistochemistry, undifferentiated hES colonies, and sections of EBs and teratomas were treated with antigen retrieval and permeabilization where necessary. Incubations in blocking solutions containing the following primary antibodies were performed at 4 °C overnight: TRA 1-60 (1:120; Millipore (UK) Ltd, Watford, UK); Oct-3/4 (1:100; Santa Cruz Biotechnology, Heidelberg, Germany); neurofilament (1:200; Abcam, Cambridge, UK); pan-cytokeratin (1:200; AE1/AE3 clone, Dako, Glostrup, Denmark) and vimentin (1:200; V9 clone, GE Life Sciences). Control reactions lacking either primary or secondary antibodies were performed in parallel. Secondary antibodies used were HRP-conjugated goat α -mouse IgG or IgM, and AP-conjugated goat α -mouse IgG (both 1:250; Stratech, Suffolk, UK), followed by either diaminobenzidine or alkaline phosphatase chromagen systems respectively (Vector Labs, Peterborough, UK). Images were captured using N.I.S. Version 3.0 Software and Nikon Eclipse E800 bright field microscope (Nikon UK Ltd, Surrey, UK).

Immunopositivity was quantified using a point-counting method; a grid with known number of intersections was placed over each image. Number of intersections overlying an immunopositive area was counted and expressed as percentage of total intersections. Data were integrated over several sections.

Statistical analysis

Student's *t*-test, Kruskal–Wallis non-parametric statistical test and Mann–Whitney *U*-post-hoc test were employed to analyse qPCR data.

Results

KCL-002 cells displayed characteristics typical of hES cells

Potential utility of hES cells in regenerative medicine is underpinned by pluripotency of the cells. As *in vitro* expression of several genes and proteins is widely used to infer pluripotency of a putative hES cell line, we sought to confirm expression of such markers by our KCL-002 cells. Expression was compared to that in the control hES cell line, KCL-003. qPCR analysis confirmed that two pluripotency-associated genes, Nanog and Sox-2, were expressed in both KCL-002 and KCL-003 hES cell lines at significantly higher levels than those of a fibroblast cell negative control (Fig. 1a). Expression of pluripotency markers was also confirmed at the protein level as undifferentiated KCL-002 and KCL-003 colonies displayed immunoreactivity for pluripotency-associated proteins TRA 1-60 and Oct-3/4 (Fig. 1b–e).

In vitro analysis of KCL-002 differentiation

EBs, three-dimensional aggregates of hES cells cultured in suspension, induce spontaneous differentiation of the cells and represent a convenient method of assessing their differentiation potential *in vitro*. To confirm the pluripotent nature of KCL-002 cells and investigate their differentiation propensity, we examined kinetics of germ layer specification in EB differentiation cultures *in vitro*. Genes indicative of each embryonic germ layer were selected, as well as markers of pluripotency, Nanog and Sox-2; their expression was examined by qPCR over time-course of differentiating KCL-002 EBs compared to undifferentiated KCL-002 colonies.

Expression of transcripts of both Nanog and Sox-2 decreased rapidly within 4d of the differentiation time-course compared to undifferentiated KCL-002 cells, consistent with the expected loss of pluripotency during EB differentiation (Fig. 2a). Expression of marker genes representing both ectodermal and endodermal germ layers, such as neural cell adhesion molecule (NCAM)-1 and alpha fetoprotein (AFP) respectively, also decreased by 4d of differentiation (Fig. 2a). In contrast, mesoderm marker vascular endothelial growth factor receptor (VEGFR)-2 demonstrated marked increase in expression during EB differentiation. This was confirmed with a second marker, α -actin (Fig. 2a).

To characterize further changes in germ layer markers, undifferentiated KCL-002 hES cell colonies and cryostat sections of differentiating 8 day KCL-002 EBs were immunostained using antibodies directed against proteins indicative of each germ layer. Immunostaining using an antibody directed against ectodermal marker protein neurofilament, demonstrated weak immunoreactivity in undifferentiated colonies which was absent by 8d EB differentiation (Fig. 2b). Cytokeratin immunopositivity, indicative of endodermal and ectodermal cell types, was detected in KCL-002 colonies and also decreased 8d post-differentiation (Fig. 2b). In contrast, vimentin immunopositivity was observed at higher levels in 8d EBs compared to undifferentiated cells, suggesting an inclination towards mesenchymal differentiation (Fig. 2b). This was quantified further by flow cytometric analyses which demonstrated that expression of vimentin protein levels were markedly higher in 16d EBs compared to undifferentiated KCL-002 hES cells (Fig. 2c). This expression pattern was very different from that observed during EB development of the other hES cell line derived in our laboratory under identical conditions, KCL-003, which showed no increases in mesenchymal differentiation as determined by vimentin staining (Fig. 2c).

Figure 1. Analysis of hES cell pluripotency marker genes. (a) qPCR analysis of Nanog and Sox-2 mRNA expression in KCL-001, -002 and -003 hES cell colonies, compared to fibroblast negative controls (Co). Data normalized to GAPDH and presented relative to expression from Co cells. Data represent mean $\pm$ SEM ($*P < 0.05$). (b–e) Immunostaining of KCL-002 (b and d) and KCL-003 (c and e) colonies grown on feeder cells showed hES cell-specific immunopositivity for antigens TRA 1-60 and Oct-3/4, associated with undifferentiated hES cells. Bars = 100 μ m.

In vivo analysis of KCL-002 pluripotency

We further performed teratoma assays to provide *in vivo* confirmation of pluripotency of KCL-002 cells. Injection of KCL-002 cells into the testis of SCID mice resulted in formation of large teratomas within 12 weeks. Histological analysis showed that the teratomas contained derivatives from all three germ layers as identified morphologically, namely, urinary epithelium and retinal pigmented epithelium characteristic of ectoderm, endodermal derivatives such as gut-like and simple squamous epithelia, and cartilage and bone which are mesodermal-derived tissues (Fig. 3a).

Adjacent sections of KCL-002-derived teratomas were further immunostained using antibodies directed against proteins indicative of each germ layer to provide molecular confirmation of differentiation. As shown in Fig. 3b, ectodermal tissues were identified by neurofilament staining, which was observed in small areas around the periphery of tumour structures, and intracellular cytokeratin immunopositivity was observed in localized areas in structures bearing epithelium-like

morphology. In contrast, vimentin antibody staining representing mesenchymal tissues was widespread throughout the teratoma sections. Additional staining for cartilage marker protein type II collagen further demonstrated presence of mesenchymal derivatives (Fig. 3b). Parallel immunostaining was also performed on teratomas derived from the control KCL-001 hES cell line (Fig. 3c). Semi-quantitative analysis integrated over several sections demonstrated that a greater proportion of KCL-002 teratomas expressed vimentin, in contrast to neurofilament or cytokeratin (Fig. 3b). In comparison, this relationship was not observed in KCL-001 teratomas (Fig. 3c). Taken together, these data demonstrate the pluripotent potential of KCL-002 cells and suggest that they may harbour a slight predisposition towards mesenchymal cell derivation.

Discussion

In this study, we have provided the initial characterization of an hES cell line derived in our laboratory, KCL-002. This cell line bears typical morphology of hES

Figure 2. Analysis of germ layer markers during development of EBs *in vitro* from KCL-002 hES cell line. (a) Expression of undifferentiated hES cell markers and germ layer marker genes during 16d EB differentiation time course of KCL-002 cells. cDNA was generated from RNA isolated at indicated time points and expression of pluripotent hES cell markers (pluri; Nanog, Sox-2), ectodermal markers [ecto; neural cell-adhesion molecule-1 (NCAM-1)], endodermal markers [endo; alpha-fetoprotein (AFP)] and mesodermal markers [meso; vascular endothelial growth factor receptor-2 (VEGFR-2) and α -actin] were measured by qPCR as described in the Materials and methods section (* $P < 0.05$). Data normalized to GAPDH and presented relative to day 0 expression. (b) Immunostaining for proteins indicative of ectodermal (ecto: neurofilament), endodermal/ectodermal (endo/ecto: cytokeratin) and mesoderm/mesenchyme (meso: vimentin) differentiation at d0 and in d8 EBs derived from KCL-002 cells, using specific antibodies as described in the Materials and methods section. Arrows point to relevant positive immunolabelling. Blue/purple staining – haematoxylin nuclear counterstain. Bars = 50 μ m. (c) Flow cytometric analysis of vimentin expression in dissociated d0 and d16 EBs derived from KCL-002 and KCL-003 cells, showing differences in developmental potential. FSC, forward scatter.

cells, and expresses characteristic pluripotency markers that are used to define pluripotent cells – Nanog, Sox-2, Oct-3/4 and TRA 1-60. Through a combination of *in vitro* studies utilizing EB differentiation cultures, *in vivo* teratoma assays, and gene and protein expression experiments, data presented indicate that KCL-002 cells are pluripotent and can give rise to all three embryonic germ layers. The data also suggest that despite formation of ectodermal and endodermal derivatives, this particular hES cell line might harbour basal differentiation bias both *in vitro* and *in vivo*, towards mesodermal or mesenchymal lineage commitment, as marked by VEGFR-2, α -actin and vimentin expression. That different hES cell lines exhibit

varying basal differentiation potentials is now well-established, based originally on *in vitro* differentiation experiments from many laboratories using serum-containing media, as we have also performed in this study (23,24,30). Heterogeneity amongst different hES cell lines and differentiation potentials have also been more accurately defined recently using serum-free, defined conditions where there is a greater degree of control over the differentiating cell pool (25,26,31,32). It remains to be determined whether KCL-002 cells exhibit a similar bias towards mesodermal/mesenchymal differentiation when subjected to directed differentiation approaches using specific factors that can drive pluripotent cells towards

Figure 3. *In vivo* analysis of KCL-001 and -002 pluripotency. (a) Histological analysis showing germ layer derivatives present in teratomas resulting from transplantation of KCL-002 hES cells into SCID mice. Ectodermal (ecto) derivatives were identified by urinary epithelium (UE) and retinal pigmented epithelium (RPE); endodermal (endo) derivatives were identified by gut-like epithelium (GE) and simple squamous epithelium (SE), and mesodermal (meso) derivatives were identified by cartilage (Ca) and bone (Bo) tissues. Sections stained with H+E. (b) Immunostaining of KCL-002 teratoma sections for ectodermal marker neurofilament (A, B), endodermal/ectodermal marker cytokeratin (C, D), mesenchyme markers vimentin (E, F) and type II collagen (G), using specific antibodies as described in the Materials and methods section. (H) Quantification of areas of teratomas expressing neurofilament (NF), cytokeratin (C) and vimentin (Vim) using point-counting as described in the Materials and methods section. (c) Immunostaining of teratomas from control line KCL-001 for the same ectodermal (A), endo/ectodermal (B) and mesenchyme markers (C), and corresponding quantification of immunopositive areas as described in the Materials and methods section and for (b). Bars = 100 μ m.

different germ layers. Nevertheless, we have recently reported that KCL-002 cells are very useful for directed differentiation of mesodermally derived haematopoietic stem cell derivatives, such as monocytes and macrophages (33). Further studies are also required to determine the extent of passage-dependent effects on differentiation capabilities of KCL-002.

Implications of observed heterogeneity in basal developmental potentials amongst different hES cell lines could be significant for studies involving developmental specification and expansion of germ layer derivatives, cell lineage commitment, differentiation of specific cell types and efficient generation of cells for regenerative medicine purposes. It is thus important to screen and characterize hES cell lines for their intrinsic differentiation potentials to understand the molecular mechanisms driving commitment and differentiation, and to enable more efficient differentiation to the lineage of choice. Our data suggest that KCL-002 cells would represent one such hES cell line that could be useful for studies involving mesodermal and mesenchymal cell derivation.

Acknowledgements

This study was supported by the Nuffield Foundation – Oliver Bird Rheumatism Programme and the Medical Research Council. The authors acknowledge technical expertise of Carl Hobbs for histology guidance and Dr Lawrence Moon for assistance with the qPCR studies and statistics advice, and Prof Paul Sharpe for critical reading of the manuscript. Cosimo De Bari is a fellow of the UK Medical Research Council.

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