Cytotrophoblast stem cell lines derived from human embryonic stem cells and their capacity to mimic invasive implantation events

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BACKGROUND: An effective embryonic–maternal interaction is crucial for successful human pregnancy. Failure of this process is a major cause of infertility and can lead to placental dysfunction resulting in recurrent miscarriage, fetal retardation and pre-eclampsia. Research is severely constrained by ethical and practical considerations; therefore, we aimed to generate cytotrophoblast stem (CTBS) cell lines from human embryonic stem cells (HESCs). METHOD: β-HCG was used as a marker of viable trophoblast cells. In defined culture, embryoid bodies were generated from HESCs and selected for trophoblast enrichment by rounds of cellular aggregation and disaggregation. Distinct CTBS cell lines were isolated and characterized. Spheroid cytotrophoblast bodies were generated and their interaction with luteal-phase endometrial stroma was analysed by real-time image analysis. RESULTS: Three CTBS cell lines were derived, which were maintained in the absence of residual HESCs, fibroblast feeder cells or extracellular matrix. CTBS cells displayed typical cytotrophoblast and syncytiotrophoblast characteristics and exhibited further differentiation to invasive endovascular cell phenotype. One cell line was generated with constitutive expression of enhanced green fluorescent protein (eGFP). Spheroid trophoblast bodies mimicked closely the early invasive stages of implantation when incubated with human endometrial stromal preparations *in vitro*. CONCLUSION: These human CTBS cell lines are a significant new model for investigating human placentation and may have considerable potential in cell therapy applications.

Key words: cytotrophoblast/embryonic stem cells/implantation

Introduction

The continual proliferation of trophoblast stem cells (TS) by the blastocyst and post-implantation embryo is of critical importance for the maintenance of pregnancy (Paria *et al.*, 2002; Red-Horse *et al.*, 2004). Initially, the rapidly dividing trophoblast cells generate syncytiotrophoblast by cell fusion. This terminally differentiated and nondividing syncytium penetrates the endometrial epithelium to invade underlying endometrial stroma where it forms a contiguous layer at the interface with maternal tissue and provides a primary barrier to protect the embryo from immune rejection. Syncytiotrophoblast also synthesizes and secretes HCG, a peptide hormone that prevents regression of the corpus luteum, thereby maintaining progesterone levels for uterine function and pregnancy. Extensive proliferation and differentiation of cytotrophoblast following initial implantation is responsible for the subsequent formation of the primate haemochorial placenta. In particular, differentiation of cytotrophoblast to invasive extravillous trophoblast and endovascular phenotypes enables the invasion and colonization of uterine spiral arterioles which are remodelled to allow much

greater maternal blood flow for the continuation of fetal development and growth. Endovascular cells also express membrane and soluble HLA-G, the nonclassical histocompatibility antigen believed to be involved in immune tolerance of the fetus (Le Bouteiller *et al.*, 2003). Aberrant development of trophoblast (including defective HLA-G function) is associated with serious complications, including recurrent miscarriage, pre-eclampsia and restricted fetal growth (Paria *et al.*, 2002; Laird *et al.*, 2003; Red-Horse *et al.*, 2004). Recently, elevated HLA-G secretion from the preimplantation blastocyst has been correlated with successful pregnancy outcome after assisted reproduction techniques (Noci *et al.*, 2005; Yie *et al.*, 2005).

The process of trophoblast differentiation is poorly understood in women because investigations are severely constrained by ethical and practical considerations.

Various cell lines have been generated from normal, malignant or transformed (immortalized) trophoblast and placental tissues, and these have provided useful *in vitro* models to investigate placental development and function (King *et al.*, 2000). However, many of these cell lines originate from late stage or term placental tissues or have lost functional capacity after transformation and therefore fail to recapitulate the physiology of trophoblast during the peri-implantation stages of development. Hence, we sought to generate human cytotrophoblast stem (CTBS) cell lines as a renewable source of progenitor trophoblast cells to serve in model systems of implantation.

In the mouse, TS cells isolated from pre- and post-implantation embryos can be maintained indefinitely in culture and have the capacity to differentiate along the trophoblast lineage (Tanaka *et al.*, 1998). However, so far the derivation of human TS cells from preimplantation blastocysts has not been achieved, highlighting the differences in early embryo and placental development between these species (Henderson *et al.*, 2002). Therefore, we investigated the use of human embryonic stem cells (HESCs) as a route to obtaining TS cell population. Although HESCs differentiate spontaneously to trophoblast-like cells in culture (Thomson *et al.*, 1998; Draper *et al.*, 2002), when supplemented with bone morphogenetic protein 4 (Xu *et al.*, 2002) or when Oct-4 is down-regulated (Matin *et al.*, 2004), these cells are terminally differentiated and display a limited proliferative capacity. Trophoblast differentiation can develop further when HESCs are aggregated to form embryoid bodies (EBs), but residual HESCs and other cell types in the culture resulting from spontaneous differentiation can confound the findings from these preparations (Gerami-Naini *et al.*, 2004). We surmised that the proportion of trophoblast cells that developed in human EBs *in vitro* would vary and sought to identify viable EBs containing trophoblast cells by measuring the secretion of the β-subunit of HCG (β-HCG) as secreted by syncytiotrophoblast. Such EBs might potentially contain a CTBS cell population for enrichment and purification.

Materials and methods

Materials

Human embryonic stem cell medium (HES medium) was prepared as described previously (Matin *et al.*, 2004). Unless stated otherwise, media and reagents were from Invitrogen (Paisley, UK) and antibodies from Sigma-Aldrich (Poole, UK).

HESC maintenance, differentiation and trophoblast enrichment

HESCs (H7 and H14, University of Wisconsin) of normal karyotype were maintained and passaged by standard protocols (Henderson *et al.*, 2002; Matin *et al.*, 2004). Batches of EBs were usually prepared from three T25 flasks of HESCs (dissociated with 1 mg/ml collagenase IV) and placed in HES medium (Thomson *et al.*, 1998) without basic fibroblast growth factor (FGF) in Petri dishes in 5% CO_2 in air. HESCs did not adhere to the Petri dish surface and instead aggregated to form EBs. On day 5, well-formed spherical EBs of approximately the same diameter (approximately 200 μm) were transferred singly to wells of a 96-well culture plate and cultured for a further 3 days before aliquots of medium were subjected to enzyme-linked immunosorbent assay (ELISA) as described previously (Acevedo *et al.*, 1997; Nishimura *et al.*, 1989).

To select for CTBS cells, EBs exhibiting high levels of β-HCG secretion were subjected to several rounds of selective enrichment by growth in conditioned 'TS' medium as described by Tanaka *et al.* (1998) for differentiation of murine TS cells from extraembryonic ectoderm. TS medium comprised Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 20% fetal calf serum, 1 mm sodium pyruvate, 100 μm β-mercaptoethanol, 2 mM L-glutamine and supplemented with fibroblast growth factor 4 (FGF4) and heparin. The medium was conditioned by incubation with confluent T25 flasks of mouse embryonic fibroblast (density of 5×10^6 per flask) for 72 h, filtered and stored at –20°C until used. This conditioned medium was diluted with fresh nonconditioned medium at a ratio of 70 : 30. Those EBs maintaining a high secretion of β-HCG were pooled, disaggregated and allowed to form new EBs, and this enrichment protocol was repeated consecutively for three rounds until all EBs displayed consistently high β-HCG secretion. EBs were disaggregated (0.25% trypsin-EDTA) and then single cells allowed to proliferate in adherent culture in TS medium without feeders. Medium was changed daily, and cells were passaged every 5 days by splitting 1 : 4 from T25 flasks. Density of cells was $1–2 \times 10^6$ per flask.

Cell lines were cryopreserved using the freezing medium of fetal calf serum supplemented with 10% dimethylsulphoxide (DMSO). Briefly, the medium was removed from the flask and cells were washed with 5 ml PBS before the addition of trypsin-EDTA solution for 5 min. Complete medium was added to neutralize the enzyme, and cells were recovered by aspiration, washed by centrifugation and resuspended in ice-cold freezing medium in cryovials which were placed in a polystyrene container overnight at –70°C before transfer to liquid nitrogen. Vials of cells were thawed at 37°C in a water bath, and the cells were washed by centrifugation thrice before transfer to fresh TS medium in a T25 flask.

ELISA determination of β-HCG concentration in cell cultures

Concentrations of β-HCG were determined using a sandwich enzyme immunoassay kit (DRG Diagnostics, Marburg, Germany). The standards and samples were incubated with 100 μl antiHCG enzyme conjugate for 30 min at room temperature followed by a five-times washing procedure. A second incubation with 100 μl substrate solution for 10 min was terminated with the addition of 50 μl stop solution. Absorbance was read at 450 ± 10 nm with a microtitre plate reader. The concentration of β-HCG in the samples was determined from the standard curve as m IU/ml.

Analysis of CTBS cell lines

Trophoblast phenotype of the cell lines were analysed by immunolocalization following previously described methods for the pan trophoblast

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marker cytokeratin 7 (Kam *et al.*, 1999; Haigh *et al.*, 1999), stage-specific embryonic antigen 1 (SSEA1; Henderson *et al.* 2002) and human placental lactogen (HPL; King *et al.* 2000). Additionally, RT–PCR was performed with primer sequences for marker genes of HESCs and trophoblast. Total RNA (2 μg) was reverse transcribed using 1 μg oligo-dT primer with Moloney murine leukaemia virus (MMLV) Reverse-Transcriptase (Promega, Cowley, UK) in a 40 μl reaction volume containing 1.25 mM dNTPs at 37°C. PCR was performed using 1 μl of cDNA in 50 μl reaction volume containing 15 pmol of each primer, 0.2 mM dNTPs and 1 unit *Taq* polymerase (Promega). Primer sequences used and conditions of these reactions were as follows:

β-actin-F: 5´-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3´;
β-actin-R: 5´-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3´ (60°C annealing, ×23 cycles).
Oct4-F: 5´-CGACCATCTGCCGCTTTGAG-3´;
Oct4-R: 5´-CCCCCTGTCCCCCATTCCTA-3´ (60°C annealing, ×23 cycles).
Sox2-F: 5´-CCCCCGGCGGCAATAGCA-3´;
Sox2-R: 5´-TCGGCGCCGGGGAGATACAT-3´ (60°C annealing, ×38 cycles).
FGF4-F: 5´-CTACAACGCCTACGAGTCCTACA-3´;
FGF4-R: 5´-GTTGCACCAGAAAAGTCAGAGTTG–3´ (56°C annealing, ×40 cycles).
Nanog-F: 5´-GCCTCAGCACCTACCTACCC-3´;
Nanog-R: 5´-GGTTGCATGTTCATGGAGTAG-3´ (60 annealing and ×30 cycles).
Eomes-F: 5´-TCACCCCAACAGAGCGAAGAGG-3´;
Eomes-R: 5´-AGAGATTTGATGGAAGGGGGTGTC-3´ (57°C annealing, ×35 cycles).
Cdx2-F: 5´-CCTCCGCTGGGCTTCATTCC-3´;
Cdx2-R: 5´-TGGGGGTTCTGCAGTCTTTGGTC-3´ (60°C annealing, ×35 cycles);
HLA-G-F: 5´-GCGGCTACTACAACCAGAGC-3´;
HLA-G-R: 5´-GCACATGGCACGTGTATCTC-3´ (55°C annealing, ×26 cycles).
CD9-F: 5´-TTGGACTATGGCTCCGATTC-3´;
CD9-R: 5´-GATGGCATCAGGACAGGACT-3´ (55°C annealing, ×26 cycles).
CK7-F: 5´-ACAGAGCTGCAGTCCCAGAT-3´;
CK7-R: 5´-GTAGGTGGCGATCTCGATGT-3´ (55°C annealing, ×26 cycles).

Fluorescence-activated cell sorter

Trophoblast cells were prepared for cell sorting by dissociating CTBS cells into single cells with trypsin-EDTA solution. To block nonspecific binding sites, the cells were resuspended at 5×10^6/ml in fluorescence-activated cell sorter (FACS) buffer with 40% normal goat serum on ice for 20 min. About 90 μl of the cell suspension was aliquoted into FACS tube, and 10 μl of G233 (marker for HLA-G) and W6/32 HLA class I control was added. G233 supernatant with NaN_3 (mouse IgG_{2a}) was kindly given by Dr Ashley King, University of Cambridge. The cells were incubated on ice for 30 min and washed twice by centrifugation before being labelled with anti-mouse polyvalent immunoglobulin fluorescein isothiocyanate (FITC) conjugate for 30 min on ice. The cells were washed again and resuspended in 300 μl buffer.

Constitutive expression of enhanced green fluorescent protein in HESCs

A pCAG-GFP expression vector was constructed by excision of enhanced green fluorescent protein (eGFP) from pEGFP-1 (Clontech, Cowley, UK) with XhoI and NotI and insertion into the pCAG vector (Niwa *et al*., 2002). H7 cells were seeded at the equivalent of 2×10^5

per well of a 6-well plate on Matrigel (Becton Dickinson, Cowley, UK). The next day they were transfected using ExGen 500 (Fermentas, Hanover, Germany) according to the manufacturer's instructions. The DNA/NaCl ExGen mixture was then added directly to the normal growth medium in the well. The plate was centrifuged at 280 *g* for 5 min and placed back in the incubator. The medium was exchanged the next day with HES growth medium containing puromycin (at 1 μg/ml). Viable colonies were picked after 2–3 weeks.

Endometrial–CTBS spheroid co-culture

The invasive implantation potential of the CTBS cell lines was examined using spheroid culture. This technique has been shown to maintain extravillous cytotrophoblast phenotype of first trimester placental tissue (Korff *et al*., 2004). Aggregates of CTBS cells were generated in a similar manner to EBs from confluent monolayers (three T25 flasks) following brief trypsinization and incubation in nonadherent (Petri dish) culture for 5–10 days, where they formed spheroid trophoblast bodies (TBs). These TBs were explanted to Matrigel culture in TS medium or to endometrial stromal co-culture. For the latter, luteal-phase endometrial biopsies were obtained from women undergoing hysterectomy under full ethical approval and patient consent. Endometrial epithelial and stromal cells were isolated as described previously (Laird *et al*., 1993). Preparations were embedded in Matrigel on membrane inserts and primed with progesterone for 24 h before the start of co-culture with CTBS. In monolayer co-culture, TBs were cultured on a confluent layer of stromal or epithelial cells in culture wells. The co-cultures were maintained in 500 μl of either conditioned TS medium or serum-free HES medium up to 6 days.

Time-lapse microscopy

CTBS cultures or TB–endometrial co-cultures were continuously monitored under an inverted microscope in a humidified physiological chamber at 37°C in 5% CO_2 in air for up to 3 days. Preliminary experiments indicated no difference in the viability of cells maintained under these conditions compared with a standard incubator. Regions of interest (ROI) were identified and programmed for analysis using Simple PCI software (Digital Pixel, Brighton, UK) with control over xyz scan, transmitted light and image capture. Routinely 20 ROIs were identified with image capture every 15 min.

Results

Derivation of CTBS cell lines

Trophoblast-containing EBs were first prepared by aggregation of single HESCs in ES medium without basic FGF in nonadherent Petri dish culture (5 days) and then transferred singly to wells of a 96-well culture plate (3 days). β-HCG was detected in most wells (4 × 96 well plates), but only 3.8% of the wells had concentration of hormone greater than 500 m IU/ml (Figure 1A). The EBs in these wells were of equivalent size and morphology, indicating that any increase in β-HCG was most likely due to the proportion of trophoblast cells rather than a greater overall number of cells. Control cultures of EBs in HES medium without basic FGF exhibited only basal β-HCG levels (<50 m IU/ml) indicating poor trophoblast differentiation. Thereafter, EBs selected initially (i.e. displaying β-HCG levels above 500 m IU/ml) were pooled, disaggregated and re-aggregated and subjected to culture in TS medium. After three rounds of culture, all EBs secreted high levels of β-HCG and were subjected to adherent culture conditions. Four cell lines were generated initially, but two lines
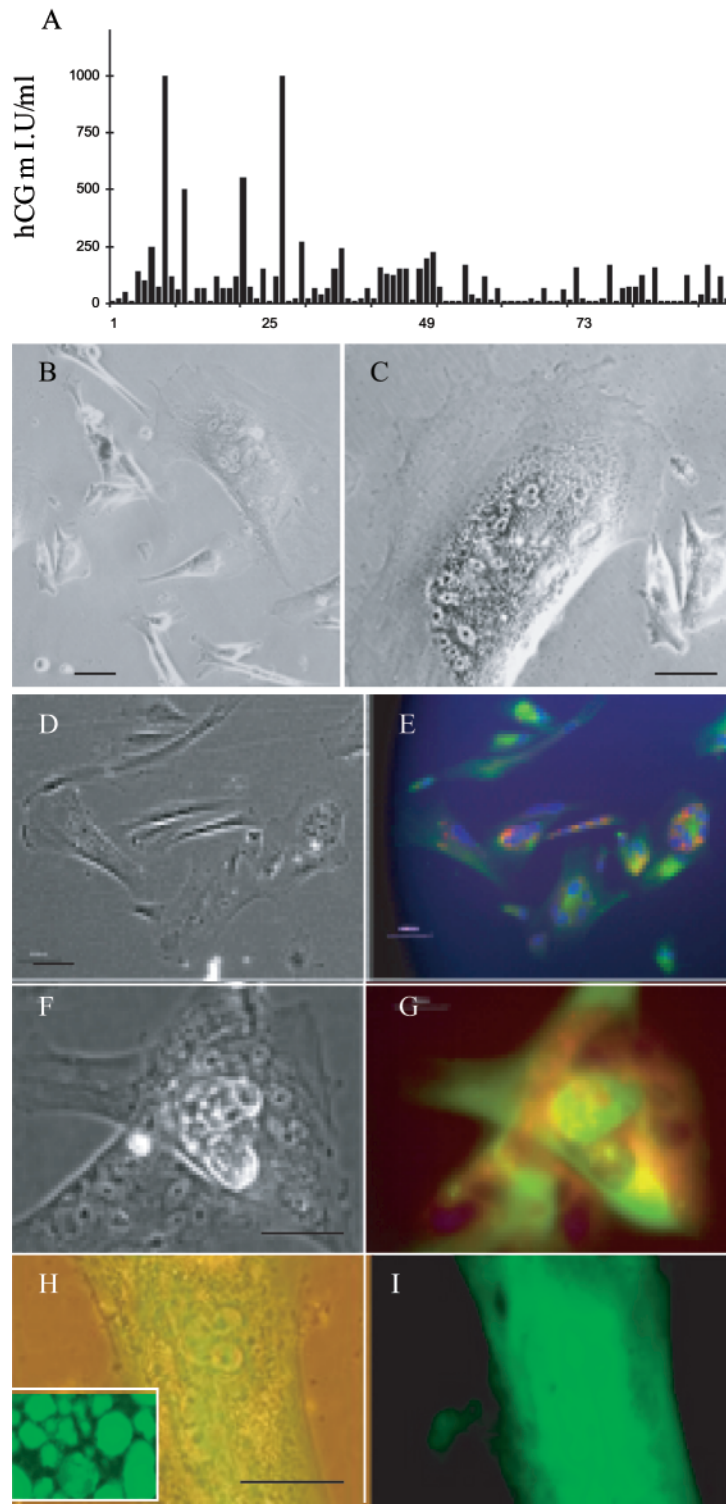
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Figure 1. Derivation and initial characterization of human cytotrophoblast stem (CTBS) cell lines; bars = 20 µm throughout. (**A**) Histogram of β-HCG concentration in culture medium in 96-wells containing single embryoid bodies. (**B**) Adherent epithelial CTBS cells in 'trophoblast stem (TS) cell' culture without feeder cells. (**C**) Adherent multinucleated syncytiotrophoblast in same culture as in panel B. (**D**) and (**E**) adherent CTBS cells under phase contrast and UV light displaying cytokeratin (green) and β-HCG (red/orange) immunolocalization (nucleus, blue). (**F**) and (**G**) single CTBS cells fusing to adherent multinucleated syncytium under phase contrast and UV. Single cells mainly cytokeratin$^+$ and syncytium mainly β-HCG$^+$. (**H**) Adherent green fluorescent protein (GFP)-syncytial trophoblast (phase contrast and UV) and (**I**) UV alone. Inset: low power of GFP-trophoblast vesicles.

failed to proliferate after passages 8 and 12. The remaining two lines have retained persistent proliferative capacity for more than 30 passages (CTBS1 from H7 HESC and CTBS2 from H14 HESC) and form epithelial-like cell colonies with single and multinucleated cells (Figure 1B). An additional CTBS cell line (CTBS–GFP1) was generated by the same

methods but from H7 HESCs with constitutive expression of eGFP (Figure 1H and I). Continuous proliferation of each cell line was related to the persistence of a mononuclear cytotrophoblast population (relative to syncytium formation) as determined by immunostaining for cytokeratin and β-HCG (Figure 1D–G). Cell proliferation was maintained by regular cell passage every 5 days, because this inhibited terminal differentiation.

When CTBS cells (CTBS1 and 2) and TBs were returned to mouse embryonic fibroblast feeders with HES medium they failed to revert to or generate either HESC colonies or EBs with pluripotent developmental capacity other than trophoblast.

Characterization of CTBS cell lines

CTBS cells displayed dual immunolocalization of CK7 and HCG and also expressed HPL and SSEA1. Most (>80%) CTBS cells were single mononuclear cells which did not form colonies. However with time in culture before passage, cells fused to form syncytium (see below). They did not localize antibody markers to undifferentiated HESCs, namely Tra-1-60, SSEA3 or SSEA4. The mRNA expression for Oct-4, Sox2, FGF4 and Nanog in the derived cell lines was absent, but trophoblast-related mRNAs for Cdx2 (caudal-related homeobox), HLA-G and Cd9 were up-regulated or for Ck7 was maintained (Figure 2A). Expression of eomesodermin, a marker of murine post-implantation trophoblast, was absent in the CTBS cells but present in HESCs.

To further assess the subtype of trophoblast cells, the comparative cell surface expression of histocompatibility HLA class I (pan HLA antibody W6/32) and HLA-G (antibody G233) antigens was determined by FACS and immunolocalization.
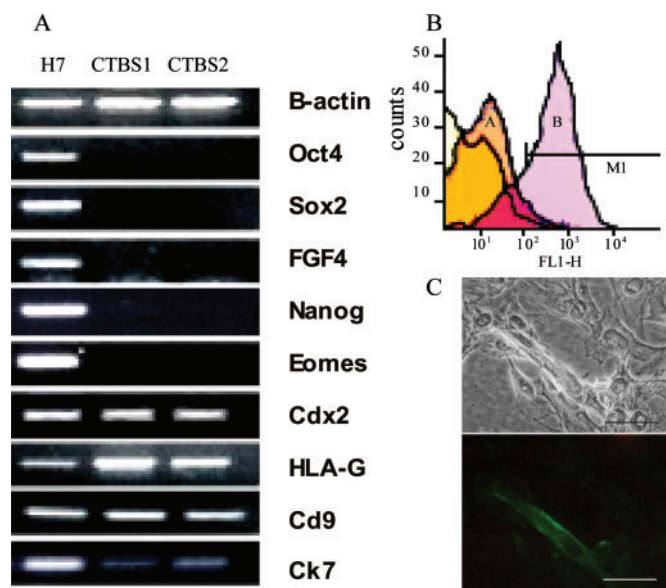

Figure 2. RT–PCR and fluorescence-activated cell sorter (FACS) analysis of trophoblast stem (TS) cells. (**A**) Gene expression (RT–PCR) for undifferentiated human embryonic stem cells (HESCs) (H7) and cytotrophoblast stem (CTBS) 1 and 2 cell lines. (**B**) FACS analysis profile for CTBS2 (similar data for CTBS1 not shown) in early culture. A proportion of cells express nonclassical HLA-G (peak A), whereas the majority express all forms of HLA class I. (**C**) Phase contrast and immunofluorescent labelling of cells used for FACS analysis indicating HLA-G staining; bar = 20 μm.

The majority of cells (approximately 90%) expressed HLA class I histocompatibility antigens (Loke and King, 1995), consistent with extravillous trophoblast (Figure 2B). The expression of HLA-G was relatively weak in most cells, but a small proportion (approximately 10%) of cells displayed strong immunoreactivity (Figure 2B and C). Some cells expressed vimentin, a marker of interstitial cytotrophoblast (King et al., 2000).

CTBS cell differentiation to endovascular cell phenotype

Following extended culture for one week or more in T25 flasks, the proportion of HLA-G+ cells increased considerably (>90%). These cells exhibited distinct endothelial cell morphology similar to cultures of differentiating cytotrophoblast from first trimester human placental tissue (Nagamatsu et al., 2004) and also resembling endothelial morphological differentiation from primate embryonic stem cells (Levenberg et al., 2002; Kaufman et al., 2004). Significantly, however, the cells co-expressed HLA-G and the platelet endothelial cell adhesion molecule-1 (PECAM-1), both markers of invasive endovascular (endothelial-like) cytotrophoblast (Red-Horse et al., 2004) (Figure 3). Vascular endothelial (VE)-cadherin and E-cadherin immunolocalization were weak or absent on endovascular cells but strong on a relatively small proportion (<5%) of multinucleated cells also present at this stage and most likely equivalent to the syncytial giant cells found in stroma of the developing placenta. As determined by RT–PCR, adherent endovascular trophoblast in culture exhibited PECAM-1 mRNA expression but neither vascular endothelial growth factor receptor 1 (flt-1) nor VE cadherin exhibited the expression in comparison with EBs, again distinguishing these cells from a true endothelial phenotype (Figure 3I).

Functional analysis of CTB cells

To determine their functional capacity, CTBS cells were monitored by continuous time-lapse recording for cell–cell fusion and the formation of nonproliferative, syncytiotrophoblast (Paria et al., 2002). Adherent cells displayed progressive migration across the culture dish promoted by pseudopodia-like extension of cells. When cells occasionally converged they fused to form multicellular syncytiotrophoblast cells (Figure 1G) that were β-HCG- and Ck7-positive but HLA class I negative. This cell fusion was captured unequivocally by digital time-lapse microscopy. Single cells fusing with each other, single cells fusing with multinucleated cells and multinucleated cells fusing with each other (Figure 4) were all observed.

The invasive implantation potential of the CTBS cell lines was investigated by generating TBs. When cultured in extracellular matrix (Matrigel) drops, these spheroid aggregates developed characteristic outgrowths, which expressed β-HCG and cytokeratin (Figure 5Ai and ii). On further culture with primary human endometrial tissue (luteal phase), prepared using well-characterized protocols (Laird et al., 1993), TBs attached to both epithelial and stromal cells. qSignificantly, as shown by time-lapse microscopy (Figure 5B), TBs with stromal cell cultures displayed a characteristic circular migratory movement and exhibited polar outgrowths from which endovascular cells
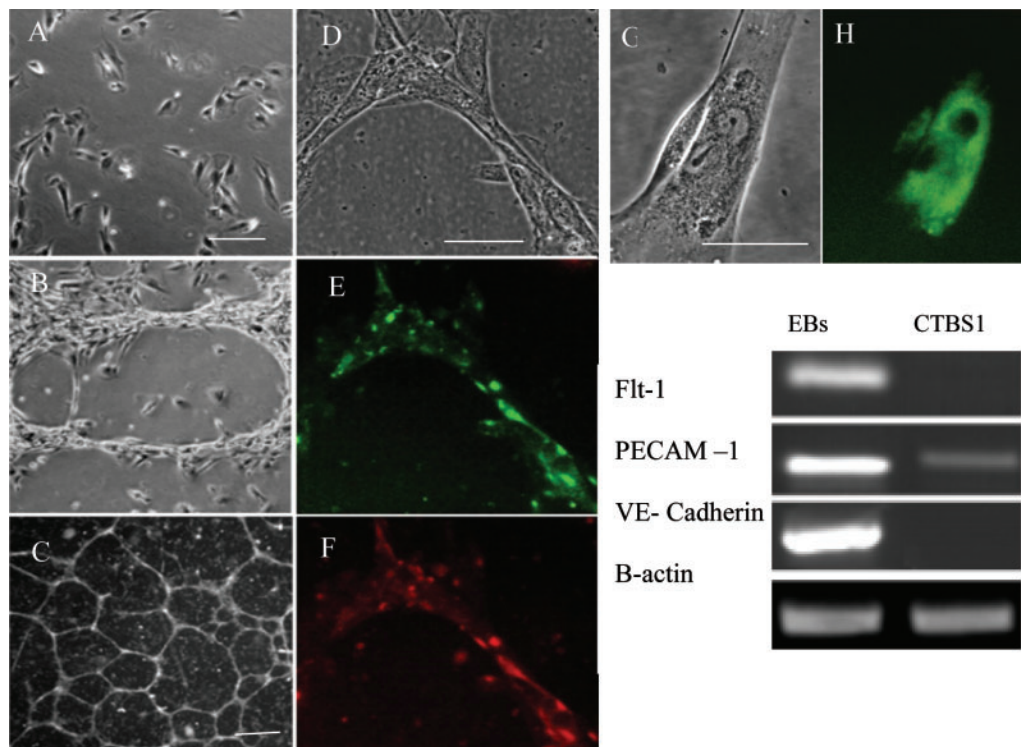
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Figure 3. Differentiation of cytotrophoblast stem (CTBS) cell line to endovascular cells in 'trophoblast stem (TS) cells' conditioned medium. (**A**) Phase-contrast micrograph of single adherent cytotrophoblast of CTBS1 cell line; bar = 20 μm. (**B**) The cells in (A) after 1–2 weeks in culture. Long aggregates display typical endothelial-like morphology; bar = 15 μm. (**C**) Dark field, low-power micrograph of culture flask; bar = 100 μm. (**D**) Phase contrast of endovascular cell aggregate displaying co-expression of (**E**) HLA-G and (**F**) platelet endothelial cell adhesion molecule-1 (PECAM-1); bar = 20 μm. (**G**) Phase contrast of multinucleated 'giant cells' near endovascular cell. (**H**) Endothelial E-cadherin immunolocalization was much greater in giant cells than in endovascular cells; bar = 20 μm. (**I**) Gene expression (RT–PCR) for endothelial markers in EBs before selection and after endovascular trophoblasts differentiation from CTBS 1 line.

streamed. After about 24–36 h in co-culture, these trophoblast outgrowths were the site of erosion of the extracellular matrix of the stroma. This was identified by the rapid retraction of the trophoblast vesicle owing to the dissolution of underlying extracellular matrix of the stromal cells (Figure 5B2–5). The erosion site was characterized by extravillous (HLA–G$^+$) trophoblast that expressed matrix metalloproteinase (MMP)-2 (gelatinase A; Figure 5B4i and ii); single GFP-trophoblast cells with endometrial stroma in culture displayed a similar response.

Discussion

As far as we are aware, these are the first human CTBS cell lines to be derived from HESCs and then maintained independently without feeder cells. Although trophoblast cells can be derived directly from HESCs (Xu *et al.*, 2000; Matin *et al.*, 2004), they rapidly lose proliferative capacity after forming syncytium, and any self-renewing progenitor CTBS population is lost. In the mouse, trophoblast cells proliferate in response to FGF4 via their FGFR2 receptor (Tanaka *et al.*, 1998), and a stem cell population can be generated. Therefore, we used TS medium containing FGF4 and heparin to maintain CTBS cells derived from HESCs, which could be subsequently enriched, and selected for, to create three cell lines. These CTBS cell lines differ from many of the immortalized placental lines (Pavan *et al.*, 2003; Feng *et al.*, 2005) in that they have

multipotent capacity to differentiate to various trophoblast phenotypes including syncytiotrophoblast and extravillous cytotrophoblast. This differentiation process is initiated in the first trimester of human pregnancy by mechanisms that are largely unknown, although recent evidence supports the contention that early cytotrophoblast has a bipotential capacity. In the mouse, withdrawal of FGF4 from TS medium induced trophoblast differentiation, but for our human stem cell lines, FGF4-supplemented medium was required for cell proliferation and subsequently allowed differentiation to occur. This suggests a difference between mouse and human TS cells. The addition of FGF4 and heparin to early human villous explants inhibits syncytiotrophoblast regeneration in favour of clumps of FGF2R-positive cytotrophoblast which can be differentiated to extravillous cytotrophoblast (Baczyk *et al.*, 2005). The CTBS cell lines exhibited a similar bipotential differentiation in culture, such as cell–cell fusion to syncytium, an invasive implantation into endometrial stroma and terminal differentiation to endovascular cells that expressed HLA-G. Moreover, following the constitutive expression of eGFP in HESCs, it was possible to derive a GFP–CTBS cell line to provide unambiguous identification in co-culture with other cell types.

Clonally derived HESCs maintain full pluripotency and proliferation (Amit *et al.*, 2000) suggesting that CTBS cells develop from a homogeneous HESC population rather than multiple (i.e. ES and TS) precursors. This finding differs from
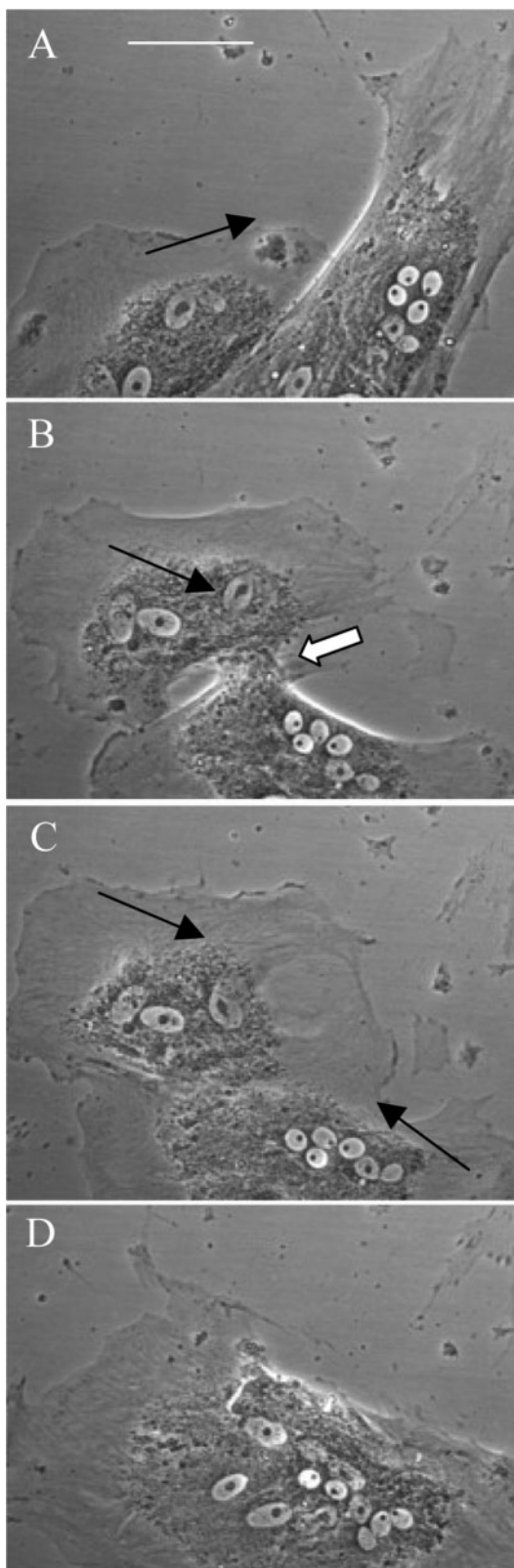
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Figure 4. Separate image frames from time-lapse movie in a sequence over approximately 16 h of cell culture of cytotrophoblast stem (CTBS) cells (CTBS 2 line) showing cell fusion event; bar = 10 μm. Black arrows indicate direction of movement of cells. (**A**) Multinucleated cell (on left) comes into proximity with stationary cell on right. (**B**) Cell–cell membrane fusion creates cytoplasmic bridge (white arrow). (**C**) Both cells retract membranes and granular cytoplasm intermingles. (**D**) Nuclei aggregate in centre of larger syncytium.

the mouse, where trophoblast cells may be derived from the early embryo and extraembryonic ectoderm (Beddington and Robertson, 1989) but not from murine ESCs without conditional gene expression (Tanaka *et al*., 1998; Niwa *et al*., 2000; Velkey and O'Shea, 2003). Our cell lines continued proliferative capacity without HESCs or feeder cells, strongly indicating the presence of a subpopulation of CTBS. This view was reinforced by complete failure of the cell line to revert to HESCs or pluripotency (other than trophoblast lineage) under permissive conditions, thereby confirming the absence of any residual HESCs in the lines and also their restricted developmental capacity as shown also for mouse TS cells (Tanaka *et al*., 1998). The latter can be derived from a cell 'niche' that spans the entire extraembryonic ectoderm and chorionic ectoderm (Uy *et al*., 2002), but it remains unclear whether a similar wide range of trophoblast development supports human TS cells. The direct developmental progression *in vitro* would possibly be the differentiation of HESCs to the immediate trophectodermal stem cell phenotype of the preimplantation blastocyst. However, the specific routes of derivation of our CTBS lines still require verification as initial EB formation may induce various trophoblast cell types from which the cell lines were ultimately selected. For practical purposes we adopted a relatively simple procedure for selecting viable EBs based on an appropriate secretory marker (β-HCG), followed by rounds of enrichment by cell disaggregation and EB regeneration. The HCG receptor is expressed on invasive cytotrophoblast as well as syncytiotrophoblast, and similar observations have been reported for EB differentiation to trophoblast (Gerami-Naini *et al*., 2004). The method was effective but did not allow prospective analysis of the differentiation process. Nevertheless, subsequent antibody and gene profile analyses of the cell lines using trophoblast-specific markers were consistent with the derivation of CTBS cells.

Compared with HESCs, mRNA expression for Oct-4, Sox2, FGF4 and Nanog in CTBS cell lines was absent, but trophoblast-related mRNAs for Cdx2 (caudal-related homeobox; Strumpf *et al*., 2005), HLA-G and Cd9 were up-regulated, whereas CK7 expression was maintained. The expression together of the latter three genes is considered as a verification for extravillous cytotrophoblast (King *et al*., 2000) which invades the uterine decidua during placentation (Pijnenborg *et al*., 1980; Enders *et al*., 1997). Surprisingly, eomesodermin (eomes), a marker of mouse early post-implantation trophoblast (Russ *et al*., 2000), was expressed strongly in HESCs but was weak or absent in the CTBS cells. Several reports have highlighted differences between mouse and human ESCs (Henderson *et al*., 2002; Ginis *et al*., 2004) including eomesodermin expression in HESCs but not mouse ES cells (Ginis *et al*., 2004). Furthermore, the expression of some trophoblast markers in stock cultures of HESCs may relate to spontaneous differentiation to trophoblast lineage. We had previously shown that expression of trophectodermal markers in such cultures occurred predominantly in the SSEA(−) and SSEA1(+) subsets of cells, consistent with their expression in the differentiated derivatives of the HESCs rather than in the HESCs themselves (Henderson *et al*., 2002; Ginis *et al*., 2004).
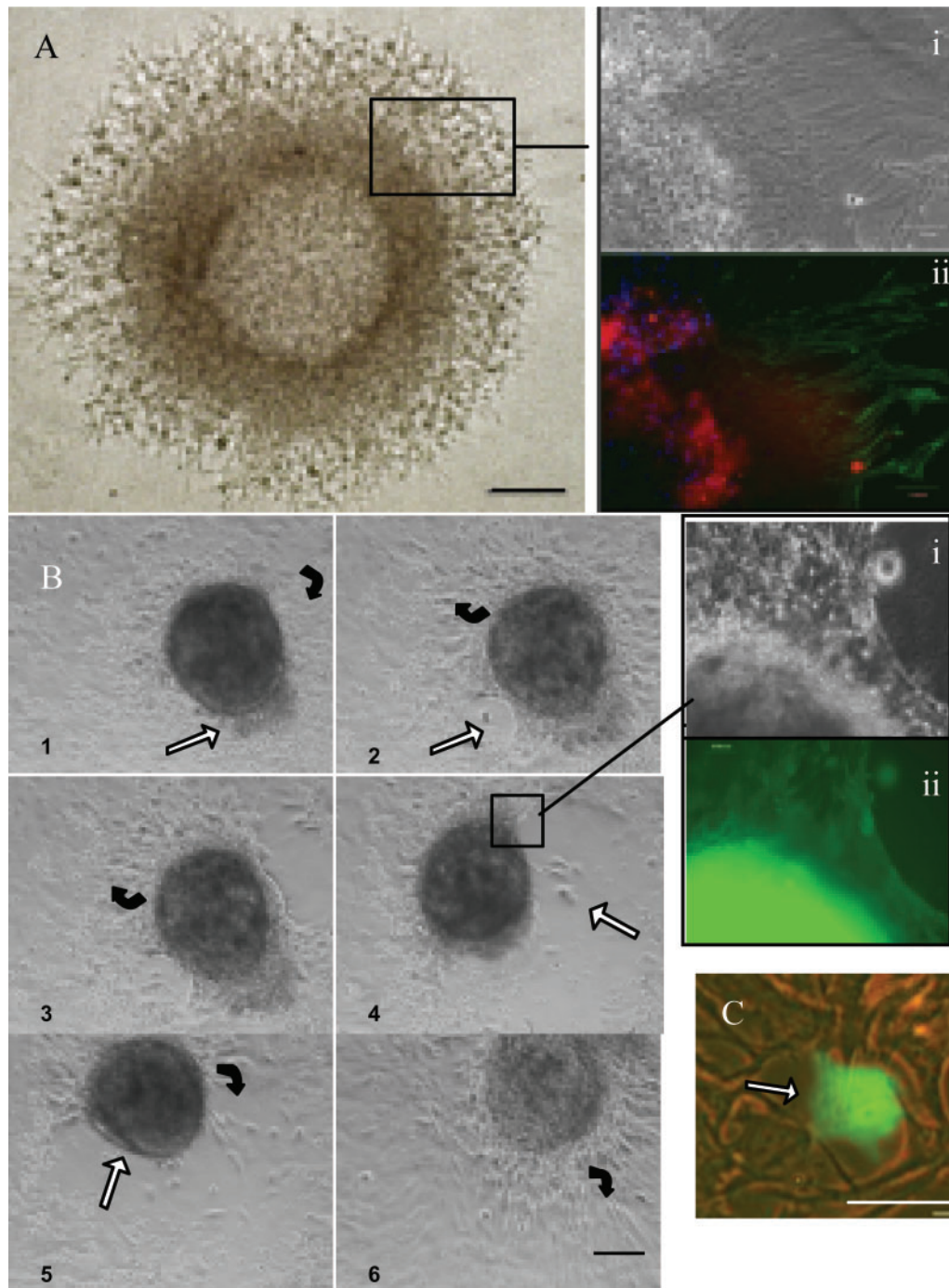
Figure 5. Cytotrophoblast stem (CTBS) spheroid trophoblast bodies (TBs) in extracellular matrix and endometrial co-culture. (**A**) TB (CTBS1 cell line) in Matrigel culture for 5 days with long microvilli protrusions of syncytium. Inset (i) and (ii) shows higher magnification phase contrast and immunostaining displaying cytokeratin 7 (green) and β-HCG (red) in syncytial bed; bar = 100 μm. (**B**) Separate image frames from sequence of time-lapse movie (over approximately 48 h) of TB in co-culture with endometrial stromal cells; bar = 150 μm. Black arrows throughout indicate direction of migration of vesicle. (1), white arrow indicates invasive cytotrophoblast outgrowth; (4 and 5), white arrow indicates stromal erosion site; (4) inset (i) and (ii), higher magnification of margin at erosion site showing phase contrast and matrix metalloproteinase (MMP)-2 immunolocalization. (**C**) CTBS-GFP cells in co-culture with endometrial stroma; bar = 20 μm.

The differentiation of several trophoblast subtypes in the cell cultures probably accounts for the physiological attributes of the CTBS cell to mimic implantation processes *in vitro*. Syncytiotrophoblast was readily generated, and the formation of syncytium by cell–cell fusion was determined precisely using time-lapse cultures under the microscope.

Two cell lines were initially lost under the prevailing culture conditions because they rapidly generated syncytium with no further proliferative capacity, and the proportion of mitotically active single trophoblast cells was insufficient to sustain the lines. Whether these lines had a greater propensity to form syncytium than those that retained proliferative capacity

is not clear. Regular passaging of adherent cells in culture inhibited syncytium formation, but we are presently investigating culture medium that may inhibit syncytiotrophoblast while retaining cell proliferation. Such conditions may allow the rescue of an early trophectodermal stem cell phenotype.

Following extended culture without passaging, CTBS cells formed the endothelial phenotype of invasive endovascular cells as displayed by cytotrophoblast from first trimester placental tissue (Nagamatsu *et al*., 2004). Previously, the exact origin of these cells has been in question, but their derivation from CTBS cell lines provides definitive evidence of their differentiation from cytotrophoblast. Significantly, although these cells expressed some markers of endothelial cells, they lacked expression of vascular endothelial growth factor receptor 1 (flt-1) and exhibited the nonclassical histocompatibility antigen, HLA-G on their surface. Thus, although their morphology closely resembled that of endothelial cells in culture, they were clearly a separate phenotype, as determined by biomarkers.

To gain an indication of the physiology of CTBS cells and their derivatives during implantation events, spheroidal trophoblast bodies, TBs (akin to EBs formed from HESCs) were generated to resemble the implanting embryo. Matrigel, an extracellular matrix preparation rich in laminin and collagen, was used to mimic the endometrial deciduas (Tarrade *et al*., 2002; Hemberger *et al*., 2004) along with human stromal cell preparations from late luteal phase endometrial biopsy tissue (Laird *et al*., 1993). In response to Matrigel culture, TBs exhibited extensive differentiation with projections of endothelial-like cells, which were cytokeratin 7-positive. Cells within the body of the TBs expressed HCG. A similar response to Matrigel has been observed with EBs where a proportion of the cells differentiate to trophoblast (Gerami-Naini *et al*., 2004). Significantly, TBs in co-culture with endometrial stromal cells exhibited a migration around the culture dish with cycles of erosion of extracellular matrix followed by reattachment. Because this co-culture system was essentially two dimensional, the migration of the TB most likely represents a regulated invasion process across the stromal culture rather than through it. Indeed, a proliferative column of cytotrophoblast was observed along with the faster migrating endovascular cells. A similar process of trophoblast invasion has been observed also for human blastocyst co-culture with stromal cells *in vitro* (Carver *et al*., 2003). During implantation, cytotrophoblast expresses various zinc-dependent proteolytic enzymes that cleave constituents of the extracellular matrix called matrix metalloproteinases (MMPs). A complex interaction with tissue inhibitors of MMPs (TIMPS) ensures a carefully controlled regulation of invasion. MMP-2 (gelatinase A) was identified as a key enzyme correlated with first trimester invasive capacity of human cytotrophoblast (Huppertz *et al*., 1998; Xu *et al*., 2000; Wang *et al*., 2001; Staun-Ram *et al*., 2004) and activity alters in cytotrophoblast (Campbell *et al*., 2004) and plasma of women that develop pre-eclampsia (Myers *et al*., 2005). Therefore, immunolocalization of MMP-2 specifically at the edge of erosion site between TBs and stroma was good preliminary evidence that the CTBS cells were secreting physiologically relevant factors. A much more detailed examination of the mechanisms regulating gene expression and enzyme activity in this model system is now in progress.

Overall, these cell lines therefore represent a significant advance for studies related to human implantation, for example cell cultures to mimic the early embryo invasion process, or investigations of placental dysfunctions such as pre-eclampsia.

In the future, somatic nuclear replacement techniques (therapeutic cloning) may enable HESCs and subsequently, trophoblast cell lines to be generated from patients with specific implantation and placental dysfunctions. Moreover, efficient generation of endovascular cytotrophoblast may have a wide utility for regenerative medicine. Their pseudo-vasculogenic and invasive characteristics might be utilized in a variety of cell therapies remote from the uterus but related to angiogenesis and vessel remodelling, especially as expression of HLA-G (Kovats *et al*., 1990) and indoleamine 2,3-deoxygenase (Mellor *et al*., 2002) render the cells naturally refractory to immune rejection.

Competing interests statement

There are no competing interests.

References

Acevedo HF, Hartsock RJ and Maroon JC (1997) Detection of membrane-associated human chorionic gonadotrophins and its subunits on human cultured cancer cells and the nervous system. Cancer Detect Prev 21,295–303.

Amit M, Carpenter M, Inokuma M, Chiu C-P, Harris C, Waknitz M, Itskovitz-Eldor J and Thomson J (2000) Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol 227,271–278.

Baczyk D, Dunk C, Huppertz B, Maxwell C, Reister F, Giannoulias D and Kingdom JC (2005) Bi-potential behavior of cytotrophoblasts in first trimester chorionic villi. Placenta June 9. (epub ahead of print)

Beddington R and Robertson E (1989) An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. Development 105,733–737.

Campbell S, Rowe J, Jackson CJ and Gallery ED (2004) Interaction of cocultured decidual endothelial cells and cytotrophoblasts in preeclampsia. Biol Reprod 71,244–252.

Draper J, Pigott C, Thomson J and Andrews PW (2002) Surface antigens of human embryonic stem cells: changes on differentiation in culture. J Anat 200,249–258.

Carver J, Martin K, Spyropoulou I, Barlow D, Sargent I and Mardon H (2003) An in vitro model for stromal invasion during implantation of the human blastocyst. Hum Reprod 18,283–290.

Enders A, Lantz K, Peterson P and Hendrickx A (1997) From blastocyst to placenta: the morphology of implantation in the baboon. Hum Reprod Update 3,561–573.

Feng HC, Choy MY, Deng W, Wong HL, Lau WM, Cheung AN, Ngan HY and Tsao SW (2005) Establishment and characterization of a human first-trimester extravillous trophoblast cell line (TEV-1). J Soc Gynecol Investig 12,21–32.

Gerami-Naini B, Dovzhenko O, Durning M, Wegner F, Thomson J and Golos. T (2004) Trophoblast differentiation in embryoid bodies derived from human embryonic stem cells. Endocrinology 145,1517–1524.

Ginis I, Luo Y, Miura T, Thies S, Brandenberger R, Gerecht-Nir S, Amit M, Hoke A, Carpenter M, Iskovitz-Eldor J *et al*. (2004) Differences between human and mouse embryonic stem cells. Dev Biol 269,360–280.

Haigh T, Chen C, Jones C and Aplin J (1999) Studies of mesenchymal cells from 1st trimester human placenta: expression of cytokeratin outside the trophoblast lineage. Placenta 20,615–625.

Hemberger M, Hughes M and Cross JC (2004) Trophoblast stem cells differentiate in vitro into invasive trophoblast giant cells. Dev Biol 271,362–371.

Henderson JK, Draper JS, Baillie HS, Fishel S, Thomson JA, Moore HD and Andrews PW (2002) Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. Stem Cells 20,329–337.

Huppertz B, Kertschanska S, Demir AY, Frank HG and Kaufmann P (1998) Immunohistochemistry of matrix metalloproteinases (MMP), their substrates, and their inhibitors (TIMP) during trophoblast invasion in the human placenta. Cell Tissue Res 291,133–148.

Kam E, Gardner L, Loke Y and King A (1999) The role of trophoblast in the physiological change in decidual spiral arteries. Hum Reprod 14,2131–2138.

Kaufman DS, Lewis RL, Hanson ET, Auerbach R, Plendl J and Thomson JA (2004) Functional endothelial cells derived from rhesus monkey embryonic stem cells. Blood 103,325–332.

King A, Thomas L and Bischof P (2000) Cell culture models of trophoblast II: trophoblast cell lines—a workshop report. Placenta 21 (Suppl. A), S113–S119.

Korff T, Krauss T and Augustin HG (2004) Three-dimensional spheroidal culture of cytotrophoblast cells mimics the phenotype and differentiation of cytotrophoblasts from normal and preeclamptic pregnancies. Exp Cell Res 297,415–423.

Kovats S, Main E, Librach C, Stubblebine M, Fisher S and Demars R (1990) A class I antigen, HLA-G expressed in human trophoblasts. Science 248,220–223.

Laird SM, Li TC and Bolton AE (1993) The production of placental protein 14 and interleukin 6 by human endometrial cells in culture. Hum Reprod 8,795–798.

Laird SM, Tuckerman EM, Cork BA, Linjawi S, Blakemore AI and Li T-C (2003) A review of immune cells and molecules in women with recurrent miscarriage. Hum Reprod Update 9,163–174.

Le Bouteiller P, Pizzato N, Barakonyi A and Solier C (2003) HLA-G, preeclampsia, immunity and vascular events. J Reprod Immunol 59,219–234.

Levenberg S, Golub JS, Amit M, Itskovitz-Eldor J and Langer R (2002) Endothelial cells derived from human embryonic stem cells. Proc Natl Acad Sci USA 99,4391–4396.

Loke Y and King A (1995) Trophoblast expression of major histocompatibility complex class I antigens. In *Human Implantation, Cell Biology and Immunology*. Cambridge University Press, Cambridge, pp. 82–101.

Matin MM, Walsh JR, Gokhale PJ, Draper JS, Bahrami AR, Morton I, Moore HD and Andrews PW (2004) Specific knockdown of Oct4 and β2-microglobulin expression by RNA interference in human embryonic stem cells and embryonal carcinoma. Stem Cells 22,659–668.

Mellor AL, Chandler P, Lee GK, Johnson T, Keskin DB, Lee J and Munn DH (2002) Indoleamine 2,3-dioxygenase, immunosuppression and pregnancy. J Reprod Immunol 57,143–150.

Myers JE, Merchant SJ, Macleod M, Mires GJ, Baker PN and Davidge ST (2005) MMP-2 levels are elevated in the plasma of women who subsequently develop preeclampsia. Hypertens Pregnancy 24,103–115.

Nagamatsu T, Fujii T, Ishikawa T, Kanai T, Hyodo H, Yamashita T, Osuga Y, Momoeda M, Kozuma S and Taketani Y (2004) A primary cell culture system for human cytotrophoblasts of proximal cytotrophoblast cell columns enabling in vitro acquisition of the extra-villous phenotype. Placenta 25,153–165.

Nishimura R, Kitajima T, Hasegawa K, Takeuchi K and Mochizuki M (1989) Molecular forms of human chorionic gonadotrophin in choriocarcinoma serum and urine. Jpn J Cancer Res 80,968–974.

Niwa T, Masui S, Chambers I, Smith A and Miyazaki J-A (2002) Phenotypic complementation establishes requirement for specific POU domain and generic transactivation function (of) ct-3/4 in embryonic stem cells. Mol Cell Biol 22,1526–1536.

Niwa H, Miayazaki J and Smith AG (2000) Quantitative expression of Oct3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 24,372–376.

Noci I, Fuzzi B, Rizzo R, Melchiorri L, Criscuoli L, Dabizzi S, Biagiotti R, Pellegrini S, Menicicci A and Baricordi O (2005) Embryonic soluble HLA-G as a marker of developmental potential in embryos. Hum Reprod 20,138–146.

Paria BC, Reese J, Das SK and Dey SK (2002) Deciphering the cross-talk of implantation: advances and challenges. Science 296,2185–2188.

Pavan L, Tarrade A, Hermouet A, Delouis C, Titeux M, Vidaud M, Therond P, Evain-Brion D and Fournier T (2003) Human invasive trophoblasts transformed with simian virus 40 provide a new tool to study the role of PPAR-gamma in cell invasion process. Carcinogenesis 24,1325–1336.

Pijnenborg R, Dixon G, Robertson WB and Brosens A (1980) Trophoblastic invasion of human deciduas from 8 to 18 weeks of pregnancy Placenta 1,3–19.

Red-Horse K, Zhou Y, Genbacev O, Prakobphol A, Foulk R, McMaster M and Fisher SJ (2004) Trophoblast differentiation during embryo implantation and formation of the maternal–fetal interface. J Clin Invest 114,744–754.

Russ AP, Wattler S, Colledge WH, Aparicio SA, Carlton MB, Pearce JJ, Barton SC, Surani MA, Ryan K, Nehls MC, Wilson V and Evans MJ (2000) Eomesodermin is required for mouse trophoblast development and mesoderm formation. Nature 404,95–99.

Staun-Ram E, Goldman S, Gabarin D and Shalev E (2004) Expression and importance of matrix metalloproteinase 2 and 9 (MMP-2 and -9) in human trophoblast invasion. Reprod Biol Endocrinol 2,59.

Strumpf D, Mao CA, Yamanaka Y, Ralston A, Chawengsaksophak K, Beck F and Rossant J (2005) Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. Development 132,2093–2102.

Tanaka S, Kunath T, Hadjantonakis A-K, Nagy A and Rossant J (1998) Promotion of trophoblast cell proliferation by FGF4. Science 282,2072–2075.

Tarrade A, Goffin F, Munaut C, Lai-Kuen R, Tricottet V, Foidart JM, Vidaud M, Frankenne F and Evain-Brion D (2002) Effect of matrigel on human extravillous trophoblasts differentiation: modulation of protease pattern gene expression. Biol Reprod 67,1628–1637.

Thomson J, Iskovitz-Eldor J, Shapiro S, Waknitz M, Swiergiel J, Marshall V and Jones J (1998) Embryonic stem cell lines derived from human blastocysts. Science 282,1145–1147.

Uy G, Downs K and Gardner R (2002) Inhibition of trophoblast stem cell potential in chorionic ectoderm coincides with occlusion of the ectoplacental cavity in the mouse. Development 129,3913–3924.

Velkey JM and O'Shea S (2003) Oct4 RNA interference induces trophectoderm differentiation in mouse embryonic stem cells. Genesis 37,18–24.

Wang M, Li Q, Shao L and Zhu C (2001) Expression of matrix metalloproteinase-2, -9, -14, and tissue inhibitors of metalloproteinase-1, -2, -3 in the endometrium and placenta of rhesus monkey (*Macaca mulatta*) during early pregnancy. Biol Reprod 65,31–40.

Xu P, Wang Y-L, Zhu S-J, Luo S-Y, Piao Y-S and Zhang L-Z (2000) Expression of matrix metalloproteinase-2, -9, -14, and tissue inhibitors of metalloproteinase-1 and matrix proteins in human placenta during the first trimester. Biol Reprod 62,988–994.

Xu R, Chen X, Li D, Addicks G, Glennon C, Zwaka T and Thomson J (2002) BMP4 initiates human embryonic stem cell differentiation to trophoblast. Nat Biotechnol 20,1261–1264.

Yie SM, Balakier H, Motamedi G and Librach CL (2005) Secretion of human leukocyte antigen-G by human embryos is associated with a higher in vitro fertilization pregnancy rate. Fertil Steril 83,30–36.