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 placentation 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 (BFGF) in Petri dishes in 5% CO2 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 × 10^5 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 × 10^4 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 mIU/ml.

Analysis of CTBS cell lines

Trophoblast phenotype of the cell lines were analysed by immunolocalization following previously described methods for the pan trophoblast...
marker cyto-keratin 7 (Kam et al., 1999; Haigh et al., 1999), stage-spe-
cific embryonic antigen 1 (SSEA1; Henderson et al. 2002) and human pla-
cental lactogen (HPL; King et al. 2000). Additionally, RT–PCR was per-
formed with primer sequences for marker genes of HESCs and tropho-
blast. Total RNA (2 μg) was reverse transcribed using 1 μg oligo-dT primer with Moloney murine leukemia virus (MMLV) Re-
verse-Transcriptase (Promega, Cowley, UK) in a 40 μl reaction vol-
ume 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′-ATCTGGGACACACTTCTACATGAGTCG-3′;
β-actin-R: 5′-CGTCATATCTCCTGTTGCTGATCCACATCTGC-3′;
60°C annealing, ×23 cycles).
Oct4-F: 5′-CGACCAGCTGGCGCTTTAG-3′;
Oct4-R: 5′-CCCCCTGTCCCCCATTCTCA-3′ (60°C annealing, ×23 cycles).
Sox2-F: 5′-CCCCCGGCAGAATAC-3′;
Sox2-R: 5′-TCGCGGCAGGGAGGAT-3′ (60°C annealing, ×38 cycles).
FGF4-F: 5′-CTACAACGCTCATGCTCTTACA-3′;
FGF4-R: 5′-GTTGACCAGAAGTGCACTTGG-3′ (56°C annealing, ×40 cycles).
Nanog-F: 5′-GCTTCAGACCTACTTACCAC-3′;
Nanog-R: 5′-GGTTGTCTCTGGATGAGTAG-3′ (60 annealing and ×30 cycles).
Eomes-F: 5′-TCACCCCCAAGACAGCGAAGAG-3′;
Eomes-R: 5′-AGAGATTGTGAAGGAGGAGGGTGTC-3′ (57°C annealing, ×35 cycles).
Cdx2-F: 5′-CCTCCGCTGGCTTACCCTC-3′;
Cdx2-R: 5′-TGCCGTTCTCAGCTTGGTG-3′ (60°C annealing, ×35 cycles).
HLA-G-F: 5′-GGGCTACTACAACAGACAG-3′;
HLA-G-R: 5′-GCCATGGCACGATCTTCTC-3′ (55°C annealing, ×26 cycles).
CD9-F: 5′-TGGAGACTATGGCTCGTATT-3′;
CD9-R: 5′-GATGCGATCGCAGCACAGT-3′ (55°C annealing, ×26 cycles).
CK7-F: 5′-ACAGAGCTGCAGTCCCGAT-3′;
CK7-R: 5′-GTAGGTGGCGATCCTGAT-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 nonspe-
cific binding sites, the cells were resuspended at 5 × 10^5/ml in fluo-
rescence-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 tubes and 10 μl of G233 (marker for HLA-G) and W6/32
supernatant with NaN 3 (mouse
Reverse-Transcriptase (Promega, Cowley, UK) in a 40 μl reaction vol-
ume 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:

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Sox2-F: 5′-CCCCCGGCAGAATAC-3′;
Sox2-R: 5′-TCGCGGCAGGGAGGAT-3′ (60°C annealing, ×38 cycles).
FGF4-F: 5′-CTACAACGCTCATGCTCTTACA-3′;
FGF4-R: 5′-GTTGACCAGAAGTGCACTTGG-3′ (56°C annealing, ×40 cycles).
Nanog-F: 5′-GCTTCAGACCTACTTACCAC-3′;
Nanog-R: 5′-GGTTGTCTCTGGATGAGTAG-3′ (60 annealing and ×30 cycles).
Eomes-F: 5′-TCACCCCCAAGACAGCGAAGAG-3′;
Eomes-R: 5′-AGAGATTGTGAAGGAGGAGGGTGTC-3′ (57°C annealing, ×35 cycles).
Cdx2-F: 5′-CCTCCGCTGGCTTACCCTC-3′;
Cdx2-R: 5′-TGCCGTTCTCAGCTTGGTG-3′ (60°C annealing, ×35 cycles).
HLA-G-F: 5′-GGGCTACTACAACAGACAG-3′;
HLA-G-R: 5′-GCCATGGCACGATCTTCTC-3′ (55°C annealing, ×26 cycles).
CD9-F: 5′-TGGAGACTATGGCTCGTATT-3′;
CD9-R: 5′-GATGCGATCGCAGCACAGT-3′ (55°C annealing, ×26 cycles).
CK7-F: 5′-ACAGAGCTGCAGTCCCGAT-3′;
CK7-R: 5′-GTAGGTGGCGATCCTGAT-3′ (55°C annealing, ×26 cycles).

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 exam-
going 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 condi-
tioned 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% CO2 in air for up to 3 days. Preliminary exper-
iments 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 nonad-
herent 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 dif-
f erentiation. Thereafter, EBs selected initially (i.e. displaying
β-HCG levels above 500 m IU/ml) were subjected to adherent culture
conditions. Four cell lines were generated initially, but two lines

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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. 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 (Puria et al., 2002). Adherent cells displayed progressive migration across the culture dish promoted by pseudopodial-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...
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), 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.
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 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 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

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

**Acknowledgements**

We are grateful to Liz Tuckerman for technical assistance with endometrial cultures. R.H. thanks the Malaysian Government for a studentship. J.D. are supported by the BBSRC. Centre for Stem Cell Biology is a MRC Resource Centre for Human Embryonic Stem Cells.

**Competing interests statement**

There are no competing interests.

**References**


Submitted on November 7, 2005; resubmitted on December 6, 2005; accepted on December 20, 2005