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Comparison of extravillous trophoblast cells derived from human embryonic stem cells and from first trimester human placentas

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Abstract

Introduction—Preeclampsia and other placental pathologies are characterized by a lack of spiral artery remodeling associated with insufficient invasion by extravillous trophoblast cells (EVT). Because trophoblast invasion occurs in early pregnancy when access to human placental tissue is limited, there is a need for model systems for the study of trophoblast differentiation and invasion. Human embryonic stem cells (hESC) treated with BMP4- differentiate to trophoblast, and express HLA-G, a marker of EVT. The goals of the present study were to further characterize the HLA-G⁺ cells derived from BMP4-treated hESC, and determine their suitability as a model.

Methods—HESC were treated with BMP4 under 4% or 20% oxygen and tested in Matrigel invasion chambers. Both BMP4-treated hESC and primary human placental cells were separated into HLA-G⁺ and HLA-G⁻/TACSTD2⁺ populations with immunomagnetic beads and expression profiles analyzed by microarray.

Results—There was a 10-fold increase in invasion when hESC were BMP4-treated. There was also an independent, stimulatory effect of oxygen on this process. Invasive cells expressed trophoblast marker KRT7, and the majority were also HLA-G⁺. Gene expression profiles revealed that HLA-G⁺, BMP4-treated hESC were similar to, but distinct from, HLA-G⁺ cells isolated from first trimester placentas. Whereas HLA-G⁺ and HLA-G⁻ cells from first trimester placentas had highly divergent gene expression profiles, HLA-G⁺ and HLA-G⁻ cells from BMP4-treated hESC had somewhat similar profiles, and both expressed genes characteristic of early trophoblast development.

Conclusions—We conclude that hESC treated with BMP4 provide a model for studying transition to the EVT lineage.

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Keywords

Extravillous trophoblast; Human embryonic stem cell; BMP4; Trophoblast; Pre-eclampsia

1. Introduction

In humans, the outer layer of the blastocyst, the trophoblast, mediates initial attachment and subsequent invasion of embryo into maternal endometrium and then gives rise to the more advanced and specialized trophoblast cell types of the mature placenta: the cytotrophoblast, syncytiotrophoblast, and extravillous trophoblast (EVT) [1,2]. As cytotrophoblast grow through the syncytiotrophoblast, they form columns of cells and subsequently chorionic villi. The tips of chorionic villi harbor EVT, which invade the deciduum. While some EVT remain in the interstitium, others penetrate uterine spiral arteries, replacing the endothelial lining and musculoelastic tissues and creating low-resistance vessels [3]. Beginning at 10–12 weeks of gestation, this modification of spiral arteries ensures uninterrupted flow of maternal blood to the placental bed [4]. As EVT approach maternal blood vessels, they are exposed to increasingly higher O_2 tensions ($\sim 14\% O_2$), causing a gradual shift from proliferation to invasion [5]. By mid gestation, generation of EVT ends. If, by this stage, EVT has not adequately remodeled spiral arteries, placental insufficiency and pregnancy disorders such as preeclampsia may result [6]. It is evident in term placental sections from preeclampsia that interstitial EVT invasion does not extend as deeply as in normal pregnancies, and fewer EVT are found within spiral arteries [7–9]. The development of novel diagnostics or therapies for preeclampsia and other placental insufficiencies is hampered by the lack of suitable models to investigate EVT emergence and invasion during early placentation [10]. While human term placentas are easily obtained, they do not provide invasive EVT. In contrast, first trimester placental tissue will form EVT *in vitro*, but is not widely available, and cannot be maintained long term in culture [11,12]. Immortalized cell lines with EVT properties such as HTR8 (HTR-8/SVneo) [13], TEV-1 [14], Swan-71 [15], TCL1 [16] and SGHPL-4 [17] are widely used, but these cells are already committed to the EVT lineage and preclude investigation of their origins [18,19].

Human embryonic stem cells (hESC), as demonstrated first by Xu et al. [20] and later confirmed by others [12,21–26], differentiate efficiently into TB by the inclusion of BMP4, and mitigation of FGF2 signaling [27,28]. This differentiation is marked by down-regulation of *POU5F1* and other pluripotency genes and up-regulation of markers consistent with EVT and syncytiotrophoblast [20–22]. We previously demonstrated that some areas of hESC colonies treated with BMP4 for 6–9 days in the absence of FGF2 are syncytiotrophoblast-like and characterized by continuous cytoplasm and expression of GATA2, KRT7, KRT8 and hCGβ [22]. Additionally, regions of HLA-G⁺, putative EVT cells have been noted [22]. The goal of the present study was to characterize the putative EVT population further by comparing it with the analogous HLA-G⁺ population from first trimester primary placental samples and evaluate its potential as an *in vitro* model for investigating EVT invasion.

2. Materials/methods

2.1. Matrigel invasion assays

Human ES H1 (NIH code WA01) and human ES H9 (WA09) (WiCell Research Institute, Madison, WI) were maintained and passaged as per published methodologies [29]. Briefly, the hESC were cultured on irradiated mouse embryonic fibroblast (MEF) cells in 80% DMEM/F12 supplemented with 20% KnockOut serum replacement, 1 mM L-glutamine/0.1 mM 2-mercaptoethanol/1% nonessential amino acids (Life Technologies) and 4 ng/ml FGF2

(in house). H1 or H9 hESC were dispersed by using 1 mg/mL of collagenase Type IV (Life Technologies). For invasion assays, a small aliquot of the cell suspension was further dispersed into individual cells by using TrypLe (Life Technologies) and counted on a hemocytometer. Equivalent numbers of ESC from the collagenase suspension were transferred onto Matrigel invasion chambers with 8 μ m pores (Becton–Dickinson) in a 6-well plate at a density of $7.2\text{--}7.5 \times 10^4$ cells/chamber. Cells were cultured in irradiated MEF-conditioned medium supplemented with either 4 ng/ml FGF2 or 10 ng/ml BMP4 (R&D Systems) under a humidified atmosphere of 4% O₂/5% CO₂/91% N₂ or under a standard gas atmosphere of humidified air/5% CO₂ at 37 °C. After 5 days, cells and Matrigel on the upper side of the chambers were removed using a cotton swab, and membranes rinsed in PBS to remove any uninvaded cells. Invaded cells on the underside of the membrane were then fixed in 4% paraformaldehyde for 10 min, stained with DAPI, and five representative fields photographed. The manual counting feature of ImageJ was used to count the number of cells per field. Counts from duplicate chambers were averaged for each treatment group for each experiment. Invasion was analyzed statistically by two-way repeated measures ANOVA with oxygen concentration and growth factor (FGF2 or BMP4) as factors and number of invaded cells per frame as the outcome measure (SigmaPlot, Systat Software, Inc, San Jose, CA). Data were log-transformed to correct for non-normal distribution.

2.2. Immunocytochemistry

H1 hESC were maintained on Matrigel-coated plates in chemically defined mTeSR medium (STEMCELL Technologies Inc.). Cells were passaged to 6-well plates containing Matrigel-coated glass coverslips and cultured for 24 h in mTeSR (day–2). Cells were acclimated to MEF-conditioned medium containing 4 ng/ml FGF2 for a day (day–1) before treatment was begun in MEF-conditioned medium with either 4 ng/ml FGF2 or 10 ng/ml BMP4 (day 0). After 2, 4, 6 or 8 days of culture, cells were fixed in 4% paraformaldehyde for 10 min and stored in DPBS at 4 °C. Staining was carried out as previously described [22] with antibodies listed in Supplemental Table 1.

2.3. Magnetic bead separation

Placentas were collected from women undergoing surgery at 8–12 weeks of gestation by LMP (last menstrual period) due to fetal demise at University of Missouri Women's and Children's Hospital and isolated as previously described [30]. Briefly, villous tissue was minced with scissors and then incubated in dissociation solution (4.2 mM MgSO₄, 0.25% trypsin, 100 kU/ml DNase I in DPBS) for 30 min at 37 °C. Cells were passed through a 100 μ m cell filter, washed in Medium 199 and layered on a 40%/25% Percoll gradient. The trophoblast layer was collected at the interface then washed and resuspended in PBS/0.5% BSA/2 mM EDTA (PBS/BSA/EDTA).

For the contrasting study with cells derived from hESC, colonies were grown for 5 days as described above in presence of BMP4 and absence of FGF2. Cells were fully dispersed with Accutase (Millipore) and transferred to a conical tube containing PBS/BSA/EDTA. Cells expressing HLA-G, the most definitive EVT marker, were isolated from both the placental cells and the BMP4-treated hESC by using an antibody that had been validated for flow cytometric use, i.e. in intact, unfixed cells [31]. The remaining HLA-G negative cells from each source were selected with a monoclonal antibody also validated for flow cytometry [32] that specifically recognizes cytotrophoblast and syncytiotrophoblast in human placental sections [33,34]. The antigen was named TROP2 (for trophoblast 2) but the official gene name has now been changed to TACSTD2. Briefly, placental cells or BMP4-treated hESC were incubated with an anti-HLA-G antibody conjugated to phycoerythrin (PE) and an anti-TACSTD2 antibody conjugated to biotin for 10 min at 4°C. Cells were washed in PBS/BSA/

EDTA ($\times 3$) then incubated in a 1:5 dilution of anti-PE antibody conjugated to magnetic beads (Miltenyi Biotec, Auburn CA) at 4 °C for 10 min to tag HLA-G⁺ cells. Cells were then separated into HLA-G⁺ and – fractions by using MS columns and a MimiMACS Separator held by a Multistand (Miltenyi) to capture magnetic beads. HLA-G⁺ cells that passed through the columns were incubated in a 1:5 dilution of streptavidin-conjugated magnetic beads (R&D Systems) at 4 °C for 10 min to tag HLA-G⁺/TACSTD2⁺ cells. A second MS column was used to collect the bead-bound (HLA-G⁺/TACSTD2⁺) cells. The abbreviations Placental/HLA-G⁺; Placental/TACSTD2; BMP-hESC/HLA-G⁺ and BMP-hESC/TACSTD2 are employed to refer to the separated cell populations for the remainder of the manuscript.

2.4. Microarray

Thirteen microarrays were run in total: $n = 3$ each from (1) Placental/HLA-G⁺; (2) Placental/TACSTD2; (3) BMP-hESC/HLA-G⁺; and (4) BMP-hESC/TACSTD2 groups and one sample of undifferentiated hESC was used as a control. Microarray analysis was performed by GenUs BioSystems (Northbrook, IL). RNA was purified using the Ribopure (Ambion) RNA isolation kit according to manufacturer's instructions. RNA quality was assessed with an Agilent Bioanalyzer. First and second strand cDNA was prepared from total RNA samples. The cRNA target was prepared from the DNA template and its quality verified on the Bioanalyzer. The cRNA was fragmented to uniform size and hybridized to Agilent Human GE 4 $\times$ 44K v2 arrays. Slides were washed and scanned on an Agilent G2565 Microarray Scanner. Data were analyzed with Agilent Feature Extraction and GeneSpring GX v7.3.1 software packages.

3. Results

3.1. Temporal and spatial pattern of trophoblast differentiation in BMP4-treated hESC

Expression of stem cell marker POU5F1 declined gradually over time in hESC treated with BMP4. On day 2 of treatment, colonies remained largely positive for POU5F1, although there was some loss at the periphery (Fig. 1A). By day 4, POU5F1 signals were absent from small colonies (Fig. 1) but expression persisted in a small central “core” in larger colonies (not shown).

In contrast, expression of trophoblast proteins HLA-G and CGA increased gradually over time. On day 2 of BMP4 treatment, a few cells in scattered locations throughout the colonies showed punctuate staining for HLA-G, a canonical EVT marker at the cell surface (Fig. 2). HLA-G expression became progressively more widespread throughout the colony on days 4–8 (Fig. 2B,C,D). On days 6 and 8 of BMP4, some HLA-G⁺ cells were clustered and HLA-G staining was continuous across multiple cells within clusters (Fig. 2D). Immunostaining for chorionic gonadotropin subunit CGA appeared later than that for HLA-G, with a few positive cells on day 6 and many more by day 8 (Fig. 2G,H). CGA⁺ cells appeared first near the periphery of colonies. Many CGA⁺ cells were clustered and appeared to be syncytialized. Neither HLA-G nor CGA was detected in cells maintained under pluripotency conditions (+FGF2) (Supplemental Fig. 2).

3.2. Matrigel invasion assay

To test whether BMP4-treated hESC contain a population of mobile, potentially invasive trophoblast, hESC were plated on Matrigel invasion chambers and either treated with BMP4 or maintained in an undifferentiated state (+FGF2). Additionally, the effect of oxygen on invasion was tested at concentrations that we have previously found to alter differentiation of BMP4-treated hESC, ambient (20%) O₂ and low (4%) O₂ [22] (Fig. 3). BMP4 had a significant, independent effect on invasion in H9 ($p = 0.038$) and H1 ($p = 0.016$), whereas

the effect of oxygen reached statistical significance only in H1 ($p = 0.025$, H9 $p = 0.07$). In BMP4-treated hESC maintained under 20% O₂, 40–100 times more invasive cells were observed as compared to hESC maintained on FGF2 and 4% O₂ (Fig. 3). In BMP4-treated cultures, cells on the underside of chamber membranes on day 5 were almost uniformly positive for trophoblast marker KRT7, and roughly 70% of cells were HLA-G⁺, although there was some variation from experiment to experiment (Fig. 3). In contrast, the small number of cells penetrating the Matrigel layer under FGF2 failed to stain for either KRT7 or HLA-G (Supplemental Fig. 2). Additionally, some invaded BMP4-treated hESC express CDH5, which is found in trophoblast from anchoring, but not floating villi [35]. ITGA1, which is found on EVT within the deciduum, but not within the anchoring villi [36], was not expressed (Fig. 3).

A time course was conducted to determine when the invasive process began and when it was maximal. A small number of invaded cells were apparent on the underside of chamber membranes by day three and the number of these cells markedly increased through day five (Supplemental Fig. 3).

3.3. Comparison of gene expression in HLA-G⁺, BMP4-treated hESC and HLA-G⁺ placental cells

Primary first trimester human placental cells were separated with magnetic beads into HLA-G⁺ (EVT) and HLA-G⁻/TACSTD2⁺ (cytotrophoblast) populations (Supplemental Fig. 4). Microarray analysis identified 4579 genes that were differentially expressed ($p < 0.05$, >2-fold) between these cell types (Supplemental Table 2). DAVID analysis was used to identify meaningful functional clusters among the differentially expressed genes. For those up-regulated in Placental/HLA-G⁺ EVT relative to Placental/TACSTD2⁺, the most overrepresented categories were related to cell invasion (Table 1). Among genes up-regulated in cytotrophoblast relative to EVT, the most overrepresented categories were related to immune function, including cytokine production, immune cell activation and antigen presentation (Table 1).

The same separation procedure was used to select HLA-G⁺ and HLA-G⁻/TACSTD2⁺ cells from hESC cultured with BMP4 for five days. For genes differentially expressed between placental/HLA-G⁺ and placental/TACSTD2 cells, expression profiles were more similar between placental/HLA-G⁺ and BMP-hESC/HLA-G⁺ cells than between placental/HLA-G⁺ and placental/TACSTD2 cells (Fig. 4A). Thus, HLA-G⁺ cells derived from hESC have some characteristic features of EVT. However, HLA-G⁺ cells from placentas and BMP4-treated hESC clustered separately and were clearly distinct (Fig. 4A,B).

Expression of well-established lineage markers supported similarities between BMP-hESC/HLA-G⁺ and placental/HLA-G⁺ cells but also emphasized their distinctiveness (Fig. 5). GATA6, a marker of primitive endoderm, also widely expressed in endocrine tissues [37], was robustly expressed in placental/HLA-G⁺ cells (Fig. 5). Otherwise, placental/HLA-G⁺, placental/TACSTD2 cells and BMP-hESC/HLA-G⁺ cells all expressed differentiated trophoblast markers at levels far exceeding the weak expression of mesendoderm markers in these cells (Fig. 5). However, of the mature trophoblast marker genes, only KRT7 and -8 were expressed in BMP-hESC/HLA-G⁺ cells at levels comparable to primary placental cells (Fig. 5C).

In contrast, three transcription factors characteristic of early mouse trophoblast development (CDX2, HAND1 and EOMES) were more highly expressed in the HLA-G⁺, BMP4-treated hESC than in either primary placental cell type, while other transcription factors characteristic of early trophoblast development were expressed at similar levels in BMP-hESC/HLA-G⁺ and placental cells (Fig. 5B).

BMP-hESC/HLA-G⁺ also displayed continued expression of stemness genes that are normally down-regulated during differentiation. For example, POU5F1 expression was only about one-fifth of that in undifferentiated hESC, whereas in placental cells its expression was barely above background (GEO GSE42112). Among genes characteristic of undifferentiated hESC, expression profiles in BMP-hESC/HLA-G⁺ cells clustered more closely to undifferentiated hESC than to placental cells (Fig. 4).

Gene expression profiles in BMP-hESC/HLA-G⁺ and BMP-hESC/TACSTD2 were highly similar, in contrast to placental/HLA-G⁺ and placental/TACSTD2 cell profiles, which were clearly distinct from each other (Supplemental Fig. 4). Of the few genes up-regulated in BMP-hESC/HLA-G⁺ relative to BMP-hESC/TACSTD2 cells, only one, the uncharacterized *C8ORF47*, was also up-regulated in placental/HLA-G⁺ vs. placental/TACSTD2 cells. In contrast, there were 21 genes significantly down-regulated in HLA-G⁺ vs. HLA-G⁻ cells in both the BMP4-treated hESC and placentas (Supplemental Table 3). We additionally identified 1215 genes that were differentially expressed across all six pairs of HLA-G⁺ vs HLA-G⁻/TACSTD2⁺ cells, regardless of source ($p < 0.05$, fold change > 1.5). These generally reflected large, consistent differences in primary placental cells coupled with small changes in the BMP4-hESC. One of the most consistent was *LHX2*, which encodes a transcription factor that regulates *CGA* transcription in pituitary and thyroid ([38,39], but which has not been studied in trophoblast.

4. Discussion

The genes that we found to be up- or down-regulated specifically in primary placental EVT largely overlapped with those noted in a previous study. Apps et al. [40] compared global gene expression profiles in EGFR⁺ and HLA-G⁺ cells from first trimester placentas. Among the twenty genes we found most up-regulated in placental/HLA-G⁺ cells relative to placental/TACSTD2 cells, several had also been noted in the App et al. study (*KCNK12*, *AQPEP*, *ADAM19*, *JAM2*, *LAIR2*, *MCAM*, *ESAM*). Other genes Apps et al. cited as being most enriched in EVT (*SLC02A1*, *PAPPA*, *SERPINE2*, *TIMP2*) were also highly up-regulated in placental HLA-G⁺ cells in our study (2–18 fold), just not to the extent noted in theirs. There was also concordance between our TACSTD2⁺ cytotrophoblast cells and the EGFR⁺ cytotrophoblast cells analyzed by Apps et al. For example, *SLC40A1*, *SLC22A11*, *ITGB5*, *MPP1*, *CNTNAP*, *NRP1*, *PAGE4* and *COLEC12* were significantly up-regulated relative to HLA-G⁺ cells in both studies. On the other hand, the twenty genes most enriched in our analyses of TACSTD2⁺ cells did not appear on the Apps et al. list of most enriched genes in EGFR⁺ cells. The explanation for these conflicting data probably relates in part to stage differences in starting material and also to differences in cell-sorting methods. Whereas both studies utilized HLA-G to select EVT, different antigens (EGFR versus TACSTD2) were used for cytotrophoblast selection, likely leading to collection of different populations of cytotrophoblast. TACSTD2 (TROP2) was first identified through generation of monoclonal antibodies to BeWo cells that react specifically with syncytiotrophoblast and cytotrophoblast in human placental sections [33], but the antigen is not unique to trophoblast. It is also expressed, like EGFR, in tumors and in epithelial cells from multiple normal tissues [41].

We cannot rule out the possibility that placental cells isolated from spontaneous fetal demises are abnormal in some way, particularly as placental abnormalities can contribute to fetal losses. Attempts were made to minimize this possibility by collecting tissue only within 2 weeks of previous fetal heart rate detection and within 48 h of diagnosis of demise and by utilizing only placental tissue with normal gross morphology, particularly appropriately branched fine villi. The high concordance in gene expression in HLA-G⁺ cells in this study

and that of cells from elective terminations from Apps et al. suggests that our cells were generally normal.

Among the genes identified in both studies as being highly up-regulated in HLA-G⁺ cells, several have not previously been identified as enriched in, and therefore potential markers of, EVT. Although *AQPEP*, *MCAM*, *PAPPA* [42], *SERPINE2* and *ADAM19* [42–46] have been noted in EVT and implicated in invasion, *KCNK12*, *JAM2*, *SLC0A2* and *ESAM* have not. These are opportune targets for further investigation. Similarly, of the 20 genes most down-regulated in both placental/HLA-G⁺ and BMP-hESC/HLA-G⁺ cells, only *ERN1* [47], *MXD1* [48], *CYP3A7* [49], *SLC22A11* [50] and *CD70* [51] have been previously identified in placenta. Also intriguing was the gene, *C8orf47*, which was up-regulated in both placental/HLA-G⁺ and BMP-hESC/HLA-G⁺. This gene encodes two variants, both completely uncharacterized.

Unexpectedly, there were relatively few gene expression changes that distinguished BMP-hESC/HLA-G⁺ from BMP-hESC/TACSTD2 cells. At day 5, colonies are comprised of a uniform population of cells with a “cobblestone” (i.e. epithelial) phenotype, except in the central region of larger colonies. No syncytial regions can be observed at d5 but a substantial fraction of cells are HLA-G⁺. The high degree of similarity between HLA-G⁺ and HLA-G[−] cells on this day suggests that HLA-G does not mark a distinct cell type but rather may be expressed stochastically in cells that are poised to differentiate along more than one trophoblast lineage.

Indeed, even among the cells that passed through the Matrigel-coated invasion membrane, HLA-G expression was not universal but KRT7 expression was. These cells may therefore represent EVT at the earliest stages of differentiation and not homologs of EVT isolated from 8 to 12 week placenta. This conclusion is supported by the continued low levels of *POU5F1* mRNA in HLA-G⁺ cells that yet expressed multiple genes characteristic of early trophoblast differentiation and the continuing presence of Ki67, indicating cells that have not terminally differentiated. Thus, BMP4-treated hESC provide a powerful tool for the investigation of factors controlling early trophoblast lineage decisions.

Multiple subtypes of EVT, including endovascular EVT in spiral arteries, interstitial EVT in decidua and EVT of anchoring villi have been identified immunohistologically in human placenta [52]. Whether these represent different differentiated cell types and the extent to which they can interconvert remains unknown. It is also not clear whether EVT and syncytiotrophoblast can emerge from the same or separate populations of cytotrophoblast. Cytotrophoblast cells isolated from late gestation spontaneously differentiate only to syncytiotrophoblast [53], whereas cultures of first trimester cytotrophoblast can form both syncytiotrophoblast and EVT depending on culture conditions [30], suggesting that there is a bipotential cytotrophoblast population at this stage. However, others reported that explants from the tips of first trimester anchoring villi lose the ability to generate syncytiotrophoblast after several days in culture but continue to produce EVT for weeks, and proposed that a cytotrophoblast population capable of giving rise only to EVT is found at the anchoring villus tips [1,54]. That such cells have only been identified in explants and not in dispersed cytotrophoblast cultures indicates that the anchoring villus niche may be necessary for its extended survival in culture. The HLA-G⁺ BMP-hESC that we have identified may represent this population, as they have both stemness characteristics and EVT characteristics, and express markers consistent with cells of the anchoring villus. Specifically, they express HLAG [55] and CDH5 [35], which are found in anchoring but not floating villi, but do not express ITGA1, which is found in the interstitial and endovascular EVT only [35,36]. Further tracking will be necessary to determine the differentiation potential of HLA-G⁺ BMP-hESC and to identify key features of a potential EVT stem cell

niche. These questions are important to our understanding of placental pathologies such as preeclampsia, in which inadequate trophoblast remodeling of spiral arteries may result from deficiencies in a specific subtype of trophoblast or EVT.

In rodents, a combination of histochemistry on pre-and post-implantation conceptuses, *in vitro* trophoblast stem cell studies and *in vivo* genetic manipulations have enabled characterization of several key transcription factors, such as HAND1, that regulate trophoblast differentiation and provide for emergence of a functional placenta [56]. HAND1 is also expressed in human placental cells and choriocarcinoma cells [57] but presently there are no direct means to elucidate its role in early cell fate decisions of human trophoblast. Our observation that HAND1 expression in BMP4-treated hESC is an order of magnitude higher than in 8–12 week placental cells encourages us to believe that the BMP4-hESC model will provide one additional tool to study the function of HAND1 and other transcription factors in the emergence of sub-lineages of human placental cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

EVT	extravillous trophoblast
hESC	human embryonic stem cells
BMP4	bone morphogenetic protein 4
FGF2	fibroblast growth factor 2

Fig. 1. H1 hESC were immunostained for POU5F1 (Oct4) (Green B,D) on days 2 (A–B) and 4 (C–D) of BMP4 treatment. No POU5F1 signals were detected on days 6 or 8 (not shown). Nuclei are counterstained with DAPI (Blue A,B). Scale Bar (D): 200 μ m. Negative controls are shown in Supplemental Fig. 2.

Fig. 2.

Acquisition of trophoblast lineage markers by hESC over 8 days of culture in BMP4. H1 hESC were immunostained for HLA-G (Green, A,B,C,D), and CGA (Green, E,F,G,H) on d2, 4, 6 and 8 of treatment with BMP4. Nuclei were counterstained with DAPI (blue) Drawings indicate position of image within an hESC colony relative to the colony edge and central core of less differentiated cells. Scale bar (H): 50 μ m. Negative controls are shown in Supplemental Fig. 1.

Fig. 3. H1 and H9 hESC were plated on matrigel invasion chambers and treated with BMP4 for 5 days. (A) Approximately 70% of cells that passed through the matrigel and adhered to the underside of the chamber membrane were positive for extravillous trophoblast marker HLA-G (green). (B) Nearly 100% of these invaded cells were positive for trophoblast marker KRT7 (green). (C) Proliferation marker Ki67 (red). (D) CDH5 (VE-cadherin), green. (E) ITGA1 (integrin alpha 1), green. Nuclei are counterstained with DAPI (blue). Scale bar (E): 50 μ m. Positive and negative controls are shown in Supplemental Fig. 2. The number of invaded cells on the membrane was averaged across five randomly selected fields of view in three trials each in H1 (F) and H9 (G) hESC. $*p < 0.05$, Holm-Sidak pairwise comparisons.

Fig. 4.

Microarray analysis of gene expression profiles in trophoblast derived from first trimester placental tissues and hESC. (A) Clustering of primary and hESC-derived trophoblast expression profiles for genes that are differentially expressed between primary (placental) HLA-G⁺ and HLA-G⁺/TACSTD2⁻ cells (4579 probes >2-fold, paired *T*-test < 0.05 in placental samples). (B) Clustering of primary and hESC-derived trophoblast expression profiles for genes differentially expressed between undifferentiated hESC and all six trophoblast samples from BMP4-hESC or all six trophoblast samples from first trimester placentas (>6 fold in 6/6 comparisons, 3212 probes). The condition trees were generated using values normalized to the 75th percentile intensity of each array. Red/Orange = up-regulated relative to median. Blue/Grey = down-regulated relative to median of 10 samples.

Fig. 5.

Expression of lineage marker genes by microarray analysis. Expression was normalized to the 75th percentile (=1.0) of fluorescence intensity on the array. This normalized value is shown on the *y*-axis. Error bars represent standard errors. (A) Endoderm – expressed genes. (B) Genes expressed in mature trophoblast (log scale). (C) Transcription factor genes expressed in early trophoblast development.

Table 1

Genes that were up-regulated in HLA-G⁺ cells and HLA-G⁻/TACSTD2⁺ cells from first trimester human placentas or in HLA-G⁺ and HLA-G⁻/TACSTD2⁺ cells from BMP4-treated hESC were analyzed using the functional annotation tool in DAVID. Enrichment scores are based on Fisher Exact Scores for all functional categories grouped within the annotation cluster. A higher enrichment score indicates the likelihood that the functional cluster is more represented among the differentially expressed genes than would be expected by chance alone.

Annotation cluster	Enrichment score	Number genes	Selected genes
Functional clusters upregulated in primary HLAG⁺ vs. HLAG⁻/TACSTD2⁺ cells			
Extracellular matrix	9.01	122	COL4A1, FN1, LAMA1, MMP2, TGFB2
Cytoskeletal protein binding	6.35	81	ACTA1, CFL2, DMD, MYL3, STMN1
Cell cycle	6.1	113	AURKB, CDKN1C, CHEK1, MCM2, SMAD3
Extracellular region	5.92	353	ADAM12, BMP4, CSH1, ITGA5, PAPP
Cell junction	5.61	86	AQP3, CLDN19, GJA5, JAM2, LI MA1
Glycoprotein/signal peptide	5.11	487	ADAM19, EPHB3, GPR98, PSG1, SLC6A19
Contractile fiber part	4.9	29	ACTN1, CRYAB, PDLIM3, MYL4, TPM1
Cell adhesion	4.58	98	DSCAM, CDH1, ESAM, ITGB1, LAMA4
Immunoglobulin domain	3.52	69	CEACAM1, HAPLN1, ISGF3, PSG2, SEMA4B
Angiogenesis	2.99	41	KLF5, ANGPT1, CDH5, EPAS1, NOS2
Functional clusters upregulated in primary HLAG⁻/TACSTD2⁺ vs. HLAG⁺ Cells			
Inflammatory response/host defense	31.6	207	CCL2, CXCL1, C1QC, IL1A, TNF
Transmembrane	18.37	972	AGPAT3, ATP7A, CD36, CLEC2B, SLC38A6
Lysosome	13.25	76	ATP6V0A1, CTSB, GLA, LI PA, VAMP4
Immune cell activation/differentiation	12.1	114	CXCR4, MICA, CSF1, SOD1, TLR1
Regulation of cytokine production	11.02	80	CD40, IL1A, IL1B, IL10, PTGS1
Pleckstrin homology	7.13	73	AGAP2, ARHGAP9, DOCKIO, PHLDA1 RALGPS1,
Antigen processing and presentation	6.98	118	CD28, FCER1G, ICAM1, KIR3DL1, HLA-A
Immunoglobulin domain	6.52	115	CD300A, FCAR, ICAM1, SIGLEC1 SLAMF6
Regulation of protein kinase activity	5.47	171	JUN, MAPK1, PRKCD, RPS6KA3, STAT5
Apoptosis	5.12	149	BCL2, CASP1, FAS, NAIP, SRC
Functional clusters in genes differentially expressed between HLAG⁺ and HLAG⁻/TACSTD2⁺ BMP₄-hESC			
<i>Upregulated in HLAG⁺</i>			
Extracellular region	1.6	9	AREG, CCL2, DLK1, SPINK1, TTR
<i>Upregulated in HLAG⁻/TACSTD2⁺</i>			
Circulatory system	1.3	5	CORIN, PMCH, KEL, MYLK2, GCGR
Intracellular signaling	1.09	10	DOK3, RREB1, RAPGEF6, KSR1, RHOV
Regulation of protein localization	1.09	6	GATA2, SORBS1, P2RX2, NLRP12, NLRP2
Chromatin organization	1.07	10	SHPRH, HIST1H2AI, H2AFJ, HDAC11, HIST3H2BB
SH3 domain	1.02	5	FYB, SORBS1, C10RF113, SH3TC1, NEBL
Tissue morphogenesis	1.02	5	FGF7, TBX3, MYLK2, KGFLP2, PTCH1
Hormone activity	1.01	4	PPY, ERVFRDE1, PMCH, GAST
Contractile fiber	0.99	4	TNNT3, ITGB1BP2, MYLK2, TRIM63