Comparison of syncytiotrophoblast generated from human embryonic stem cells and from term placentas

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Human embryonic stem cells (ESCs) readily commit to the trophoblast lineage after exposure to bone morphogenetic protein-4 (BMP-4) and two small compounds, an activin A signaling inhibitor and a FGF2 signaling inhibitor (BMP4/A83-01/PD173074; BAP treatment). During differentiation, areas emerge within the colonies with the biochemical and morphological features of syncytiotrophoblast (STB). Relatively pure fractions of mononucleated cytotrophoblast (CTB) and larger syncytial sheets displaying the expected markers of STB can be obtained by differential filtration of dispersed colonies through nylon strainers. RNA-seq analysis of these fractions has allowed them to be compared with cytotrophoblasts isolated from term placentas before and after such cells had formed syncytia. Although it is clear from extensive gene marker analysis that both ESC- and placenta-derived syncytial cells are trophoblast, each with the potential to transport a wide range of solutes and synthesize placental hormones, their transcriptome profiles are sufficiently dissimilar to suggest that the two cell types have distinct pedigrees and represent functionally different kinds of STB. We propose that the STB generated from human ESCs represents the primitive syncytium encountered in early pregnancy soon after the human trophoblast invades into the uterine wall.

BMP4 | human pluripotent stem cells | trophoblast | RNA-seq | syncytiotrophoblast

Syncytiotrophoblast (STB) is encountered during at least two stages of human placental development (1–3). The first coincides with early implantation when a multinucleated syncytium forms, presumably by cell fusion events, ahead of proliferating, mononucleated, cytotrophoblast (CTB) cells originating from polar trophectoderm (3, 4). This invasive syncytium emerges either during or soon after the trophoblast passes through the breached uterine epithelium and into the decidualized stromal layer beneath and appears to be responsible for hollowing out regions within the stroma to form lacunae (5), which become filled with fluid and cells from maternal blood and uterine glands and presumably provide a source of nutrients for the conceptus (3, 6). By about 12 d of gestation, soon after the blastocyst has sunk below the endometrial surface, strands of cytotrophoblast begin to form and penetrate through the primitive syncytium to form primary chorionic villi, which are subsequently invaded by extraembryonic mesoderm to form secondary and tertiary villi (villous trees) (2–4). The cytotrophoblast cells associated with the villi continue to divide and provide a progenitor cell population for the villous STB, which is the cell layer that covers the outer surface of the villi and forms the definitive interface involved in exchange of gases, nutrients, and excretory materials between the fetal placenta and maternal blood. Villous STB is also the major site for production of placental hormones. Cytotrophoblast cells at the tips of the anchoring villi proliferate and colonize the endometrium, thus expanding the placental bed and simultaneously remodeling maternal spiral arteries. The extent to which extravillous trophoblast becomes multinucleated and whether such cells are the source of islands of giant cells within the endometrium (7) is controversial; an alternative theory is that the latter are remnants of the original primitive syncytium formed during initial trophoblast invasion (2, 3). The extent to which the phenotypes of these different forms of STB resemble each other and whether the underlying processes that lead to cell fusion have features in common remain unclear.

Several main cell systems have been used to study STB formation. One has been to culture placental explants, where some of the features of STB formation and turnover can be preserved temporarily, provided the viability of the explant is maintained (8, 9). A second has been to use certain lines of choriocarcinoma cells, for example BeWo, which can be induced to undergo fusion by the addition of agents such as forskolin or dibutyryl cAMP to the culture medium (10, 11). A third approach has been to purify cytotrophoblast populations from whole placentas, usually from term collections, which, when cultured appropriately, fuse and form a multinucleated syncytium exhibiting many of the physiological features expected of STB within 2–3 d (12). An additional, fourth approach for generating what appears to be STB, is from human pluripotent stem cells, which, in absence of fibroblast growth factor-2 (FGF2) and in a process primed by the presence of the growth factor bone morphogenetic protein-4 (BMP-4), form areas of syncytium within the colonies (13–15). An up-regulation of genes expressed in placental syncytium accompanies this process of progressive differentiation (13, 16). When BMP4 is

Significance

Syncytiotrophoblast (STB) is responsible for nutrient and gas exchange in the human placenta. STB also forms when human embryonic stem cells (ESCs) differentiate to trophoblast. Here we compare ESC-derived STB with cytotrophoblasts isolated from term placentas before and after such cells had fused to form STB. Although both types of STB expressed all common trophoblast marker genes, there were dissimilarities indicative of altered function and ontology. We propose that STB derived from ESCs represents syncytial tissue encountered at the initiation of placental development. These cells may provide the first in vitro model for studying origins of diseases of placentation ranging from implantation failure and early pregnancy loss to intrauterine growth retardation and preeclampsia.

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See Commentary on page 5144.

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used in combination with two small compounds, the activin A signaling inhibitor A83-01, and the FGF2 signaling inhibitor PD173074 (BMP4/A83-01/PD173074; BAP treatment), the process becomes more efficient and synchronous, such that by 48 h, almost all of the cells in the colonies have become transiently positive for the transcription factor CDX2 and completely positive for the trophoblast marker KRT7 (14, 17). By day 6 of treatment, areas of syncytium emerge, and the culture medium begins to accumulate significant quantities of the placental hormones, chorionic gonadotropin (CG), placental growth factor (PGF), and progesterone (14). However, the extent to which these syncytial areas resemble STB associated with a functional human placenta remains unclear. Here our main goal was to characterize the syncytium formed when embryonic stem cells (ESCs) are driven along the trophoblast lineage and compare it with that generated when cytotrophoblast cells from placentas fuse.

Results

Isolation of Syncytial Areas from Colonies of BAP-Treated H1 ESC Colonies. H1 (WA01) ESCs were routinely maintained on mTeSR1 medium, which contains 100 ng/mL FGF2, and then passaged onto DME/F12/KOSR medium that had been conditioned by mouse embryonic fibroblasts (MEFs) at low FGF2 concentrations (4 ng/mL) for 24 h. At this stage, the conditioned medium was replaced with chemically defined DME/F12/KOSR medium that contained BMP4, A83-01, and PD173074 (BAP treatment) for up to 8 d (Fig. 1*A*). Under these conditions, the cells released detectable human CG (hCG), as measured by an ELISA, by day 5, with daily production rising significantly ($P < 0.01$) every day until day 8, at which stage the concentration of hormone in the medium had risen ~10-fold (Fig. 1*C*). Discrete zones of CG-alpha (CGA)-positive cells became visible within the colonies at day 4, and these increased in number and size over subsequent days until, at day 8, they occupied between 5% and 10% of the surface areas of the colonies (17). On this day, many of these areas were greater than 100 μm in diameter and contained many nuclei (Fig. 1*B*). The expansion of this CG-beta (CGB)-positive population over time correlated well with the release of hCG into the medium (Fig. 1*C*). We hypothesized that these CGB- and CGA-positive areas at day 8 were likely to be composed primarily of STB and STB precursor cells and that their expansion from day4 onward represented a progression of STB development and maturation (Fig. 1*B* and *SI Appendix*, Fig. S2; also see Fig. 3).

To isolate areas of presumed STB, day 8 colonies were dissociated, and different size fractions separated by passing the cell suspensions successively through nylon cell strainers with mesh sizes of 70 μm and 40 μm, respectively, thereby generating three cell size fractions (>70 μm, 40–70 μm, and <40 μm). Enzymatic cell dispersion with 0.25% trypsin–EDTA required an extended incubation time (up to 14 min) to achieve complete cell dissociation (*SI Appendix*, Table S3). Although it was possible to use trypsin to provide the three cell size fractions, the RNA extracted from these cells was extensively degraded and unsuitable for library preparation. Therefore, this approach was abandoned. By contrast, nonenzymatic treatment with "Gentle Cell Dissociation Reagent" followed by repeated pipetting dispersed the colonies effectively and allowed three cell fractions to be isolated with comparable efficiency to the enzymatic method (*SI Appendix*, Table S3 and Fig. S1) within 7 min. Moreover, intact RNA was readily recovered from each of the cell size fractions.

Analysis of Isolated Fractions. As anticipated, the control H1 ESC colonies could be dissociated almost completely into single cells <10 μm in diameter that had a large nucleus-to-cytoplasm ratio and a paucity of other organelles (Fig. 2 *A* and *E*). Although the <40-μm fraction from the BAP-treated cells was somewhat heterogeneous, it was also composed largely of eosin-positive mononucleated cells, although some larger clumps were also present (Fig. 2*B*). In thin sections, the mononucleated cells from the <40-μm fraction showed a more intense cytoplasmic incorporation of the uranyl acetate/lead stain (Fig. 2*F*) than the ESCs (Fig. 2*E*) and more heterochromatin within their nuclei. Whereas the >70-μm fraction was made up primarily of large sheets of

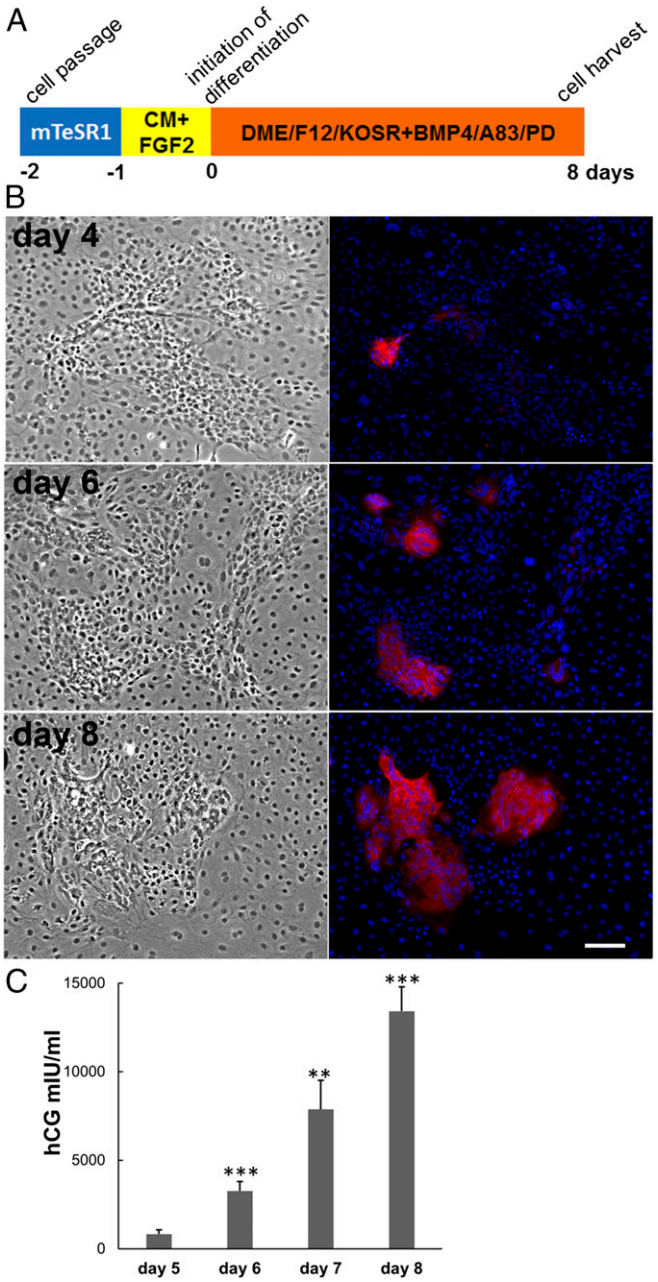


Fig. 1. Differentiation of H1 cells to STB. (*A*) Human ESCs (H1) were maintained on mTeSR1. Following the day of passaging, the medium was changed to MEF-conditioned medium supplemented with FGF2 (4 ng/μL) (CM+FGF2). After an additional day, the medium was replaced with DME/F12 and 20% KOSR supplemented with BMP4 (10 ng/mL), A83-01 (1 μM), and PD173074 (0.1 μM) (DME/F12/KOSR+BMP4/A83/PD) for 8 d. (*B*) Images of H1ESC BAP treated for 4 d, 6 d, and 8 d were captured under bright field (*Left*) and immunostained for CGB (red signals) and nuclear material (DAPI; blue signals, *Right*). (Scale bar, 100 μm.) (*C*) Daily production of hCG. Production of hCG began around day 5 of BAP treatment and peaked around day 8 before a subsequent decline (not shown). Error bars indicate means ± SEM for three experiments. Asterisks indicate significant differences from day 5 production (***$P < 0.001$, **$P < 0.01$).

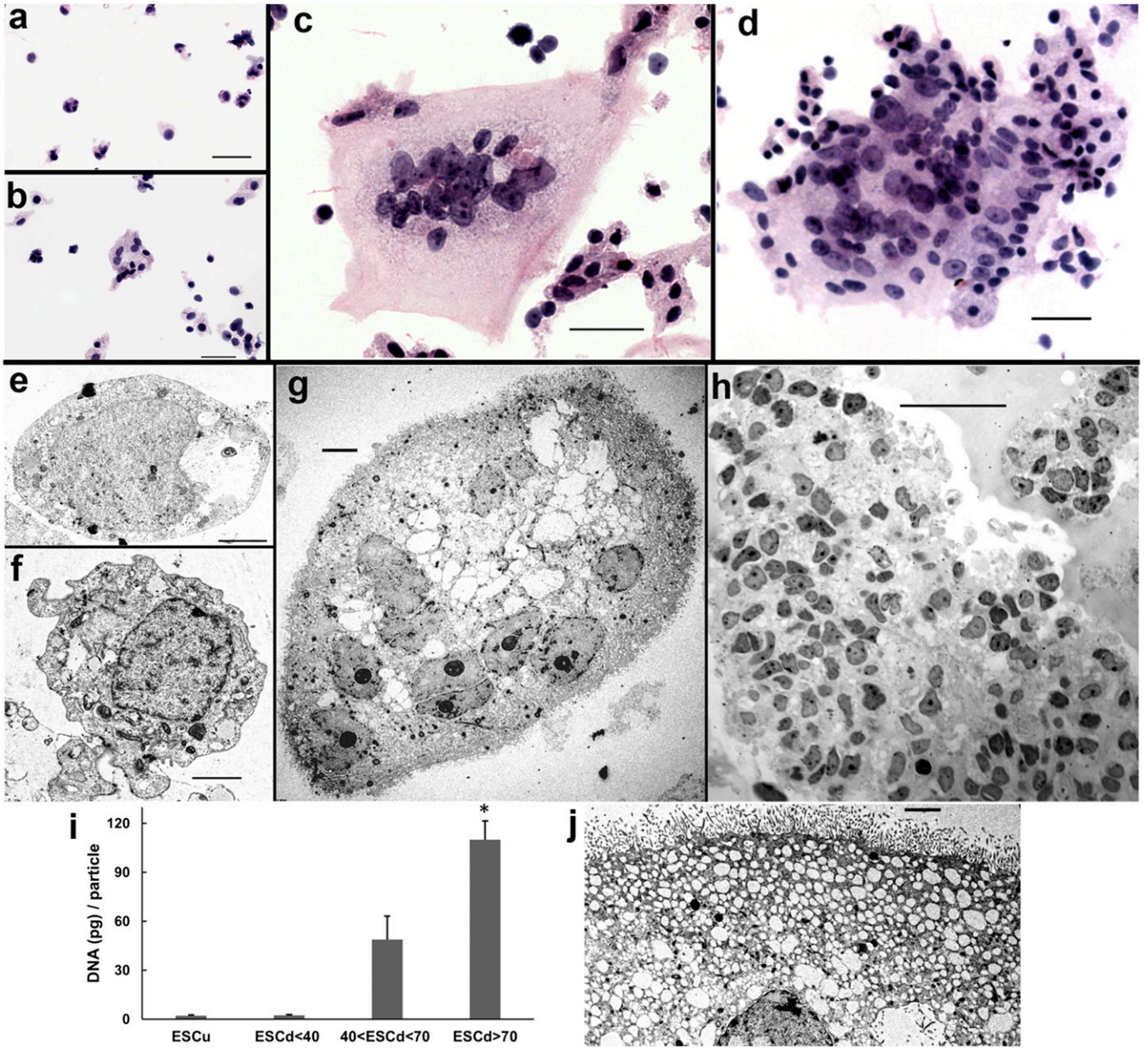


Fig. 2. STB generation from hESCs in vitro. (*A–D*) Hematoxylin and eosin-stained cells prepared by cytospin. (*A*) Control hESCs (ESCu); (*B*) <40-μm fraction; (*C*) >40 μm to <70 μm fraction; and (*D*) >70-μm fraction. (Scale bars in *A–D*, 20 μm.) (*E–G* and *J*) Transmission electron microscopy images of cells represented in *A–D*. (Scale bars in *E* and *F*, 2 μm and in *G*, 5 μm.) (*H*) Semithin section image of ESCd >70 STB stained with Toluidine Blue. (Scale bar, 20 μm.) (*I*) Comparison of DNA content per particle in four different preparations of ESCu, ESCd <40, ESCd >40 to <70, and ESCd >70. Error bars indicate means ± SE ($n = 3$). *$P < 0.05$, significant difference from ESCu. (*J*) Enlarged view (Scale bar, 2 μm.) of STB area showing microvilli on the cell surface.

cellular material, many of which appeared to have aggregated together during the cytospin procedure (Fig. 2*D*), the 40-μm to 70-μm fraction also contained similar, but generally less extensive, sheets of cells (Fig. 2*C*). The intertwined sheets from both cell size fractions contained many nuclei. Semithin and thin sections of cells in the >70-μm (Fig. 2 *H* and *J*) and 40-μm to 70-μm fractions (Fig. 2*G*) indicated that they were composed largely but not entirely of syncytium as defined by nuclei in a common cytoplasm. It was also evident that some surfaces of the syncytial areas were densely covered with microvilli (Fig. 2*J*). Finally, whereas the average DNA per cell in the H1 undifferentiated embryonic stem cells (ESCu, Fig. 2*I*) was about 3 pg, consistent with the presence of a single diploid nucleus, average values for the <40-μm fraction were slightly higher. By contrast, the DNA content per particle in the 40-μm to 70-μm and >70-μm fractions was 48.8 ± 14.5 pg and 110 ± 11.7 pg, respectively. These data, along with the information presented in *SI Appendix*, Table S3 suggest that the number of nuclei per cellular particle in the two larger cell fractions was ~16 and 37, respectively, and that the 40-μm to 70-μm and >70-μm fractions together comprised about 0.167% of the cells and ~5% of the total cellular DNA recovered. Yields of multicellular sheets from the same fractions from two different induced pluripotent stem cell (iPSC) lines derived from umbilical cord mesenchyme (MRucAi and MRuc3i) treated under similar BAP conditions were not greatly different (0.089 and 0.126%, respectively) from those achieved with H1 ESCs, although the experiments with iPSCs have only been performed once.

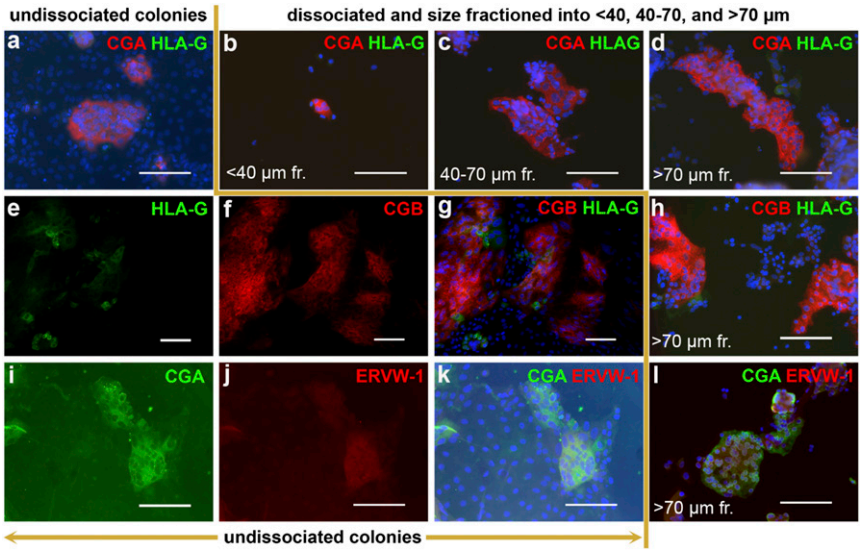


Fig. 3. Immunostained colonies at day 8 of differentiation (*A*, *E–G*, and *I–K*) and identical staining of three size-fractionated groups of dissociated cells deposited on glass slides by the cytospin procedure (*B–D*, *H*, and *L*). In all cases, hESCs were BAP treated for 8 d. All specimens were counterstained with DAPI (*A–D*, *G*, *H*, *K*, and *L*). (Scale bars, 100 μm.)

Additional Visible Phenotypic Features of the STB Fraction. When stained in situ at day 8, the presumed sycytial areas within the BAP-treated colonies were positive for CGA (Fig. 3 *A* and *I*), CGB (Fig. 3 *F* and *G*), ERVW-1 (Fig. 3 *J* and *K*), and negative for HLA-G (Fig. 3 *E* and *G*). The staining for CGA often appeared to be perinuclear, consistent with a Golgi localization (Fig. 3*I* and *SI Appendix*, Fig. S2*M*), but the same presumed syncytial regions positive for CGA were also positive for ERVW-1, with staining for the latter more uniform than for CGA (Fig. 3*J*). Similarly, the sheets of cells present in the 40-μm to 70-μm (Fig. 3*C*) and the >70-μm (Fig. 3*D*) fractions isolated from such cultures at day 8 were all CGA-positive and HLA-G negative. Some, but not all, expressed CGB (Fig. 2*H*). For the remainder of this paper, we concentrate mainly on the >70-μm fraction, which appears to be largely syncytial and less contaminated with mononucleated cells than the 40-μm to 70-μm fraction.

The second subunit of hCG, CGB, was rarely detected in day 4 cultures when CGA could first be identified but was expressed by day 6 (*SI Appendix*, Fig. S2*N*). However, at day 6 its expression was only partially coincident with that of CGA in subzones of the developing syncytial patches as viewed both in the intact colonies (*SI Appendix*, Fig. S2 *M*, *N*, and *P*) and in the isolated >70-μm fraction (*SI Appendix*, Fig. S2 *Q–T*). Another antigen, γ-aminobutyric acid A receptor, Pi (GABRP) that has previously been used as a trophoblast marker (18, 19), had a particularly interesting expression pattern. Its zones of expression within BAP-treated colonies at day 4 included only small areas that were CGA-positive (*SI Appendix*, Fig. S2 *A–D*). However, by day 6 there was more complete but not perfect coincidence of GABRP and CGA immunofluorescence (*SI Appendix*, Fig. S2 *E–H*). Gene expression data for the gene encoding gamma aminobutyric acid A receptor pi (*GABRP*), *CGA*, and *CGB5* (one of six *CGB* genes), measured by microarray mirrored that obtained by immunohistochemistry (*SI Appendix*, Fig. S2 *U–W*). *GABRP* expression changed from very low at 24 h to high at 72 h and 120 h (*SI Appendix*, Fig. S2*U*). Whereas *CGA* expression appeared to lag behind that of *GABRP* slightly (*SI Appendix*, Fig. S2*V*), *CGB5* transcripts were not detectable at all at 72 h, but were up-regulated by 120 h (*SI Appendix*, Fig. S2*W*).

Transcriptome Profile of the Size-Fractionated, Differentiated Cells and Comparison with Initiating H1 hESCs. Three separate experiments were performed to generate RNA from size-fractionated, ESC-derived cells. RNA-sequencing (RNA-seq) analysis was performed on each RNA preparation. In addition, RNA was isolated and analyzed in parallel from three different cultures of H1 ESCs that had been maintained in the same basal medium as had been used in the BAP treatments, except it lacked BMP4, A83-01, and PD173074 and had been supplemented with FGF2 (4 ng/mL). Previous studies have shown that culture in atmospheric oxygen (20%) conditions for an extended period can lead to a limited amount of spontaneous differentiation in H1 cells (20). Such changes may have occurred in one of the three ESC H1 samples (ESCu) samples shown in Fig. 4*A*, which appeared as an outlier relative to the other two samples (first lane in Fig. 4*A*). Based on the most highly regulated genes among all of the samples, the analysis was able to distinguish the ESCs from the mononuclear <40-μm fraction and the >70-μm STB fractions isolated after 8-d BAP-driven differentiation of the H1 cells. The <40-μm fraction and the >70-μm STB fractions also clustered separately, albeit closely, from each other and from the samples obtained from term placentas. The close clustering of the the <40-μm and >70-μm fractions by principal component analysis (Fig. 5*B*) probably reflects the fact that a continuum exists in the phenotypes created during the differentiation process initiated by BAP rather than gross cross-contamination. Importantly, the expression of many trophoblast marker genes, including ones considered to be characteristic of STB, were highly enriched in the >70-μm relative to the <40-μm fraction (Fig. 4*B*; see below), whereas a few more were down-regulated.

The RNA-seq transcriptome analysis provides further evidence that BAP-differentiated hESC is composed only of trophoblast. Of 63 trophoblast gene markers examined (18, 21), only one, *CDH1* (encoding E-cadherin), was not up-regulated in the ESC >70-fraction compared with the ESCu (Fig. 5*A*). It should be emphasized that *CDH1* was expressed robustly in all three types of cell [mean fragments per kilobase million (FPKM) values, 324, 323, and 290 for ESCu, differentiated ESCs <40 μm (ESCd <40), and differentiated ESCs >70 μm (ESCd >70), respectively]. Among, the highest up-regulated genes in the ESCd >70 fraction were those encoding hCG subunits (*CGA* and various *CGB* family members) (see also Fig. 6), *PGF*, enzymes involved in progesterone and estrogen biosynthesis (*HSD3B1*, *CYP11A1*, and *CYP19A1*), endogenous retroviral envelope proteins (*ERVW-1* and *ERVFRD-1*), and various transporters, e.g., *SLC40A1*, *SLC13A4*, and *SLC38A3*,

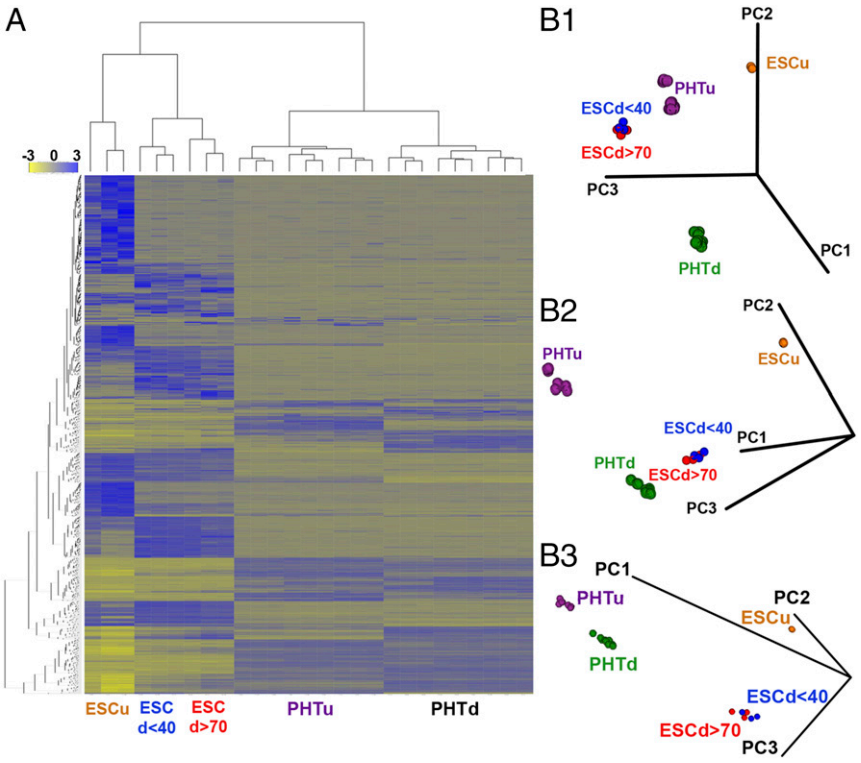


Fig. 4. Clustering and heatmap analysis (*A*) and principal component analysis (*B*, 1–3) of 1,284 genes that differed significantly in expression [false discovery rate (FDR) <0.01] in at least one cell fraction relative to at least one other. Expression was assessed from RNA-seq data performed on undifferentiated ESCs (ESCu; $n = 3$), two different size fractions from BAP-differentiated cells ($n = 3$ for each), 8-h cultures of cytotrophoblasts derived from term placentas (PHTu; three placentas; three preparations from each), and from 48-h cultures from batches of cells from the same placentas (PHTd). In *B*, three different views of the same image are displayed.

and a number of transcription factors implicated in trophoblast differentiation, e.g., *GCM1*, *GATA2*, *GATA3*, *OVOL1*, *DLX3*, *TFAP2A*, *TFAP2C*, and *PPARG*.

The same group of trophoblast marker genes, again with the exception of *CDH1*, were up-regulated in the <40-μm fraction (ESCd <40) relative to ESCu (*SI Appendix*, Fig. S3*A*). However, transcripts for some of these genes were more enriched in the ESC >70 fraction than in the largely mononucleated ESCd <40 fraction, e.g., *CGB*, *CGA*, *CYP11A1*, *PGF*, and *LGALS16*, whereas for some others, e.g., *VTCN1*, *GABRP*, and *ITGB6*, the situation was reversed (Fig. 5*B*).

There was no significant up-regulation of a majority of genes associated with mesoendoderm differentiation (*SI Appendix*, Table S4). There were three exceptions, *HAND1*, *KDR*, and *SNAI1*, of which the former two were uniquely expressed in the ESC-derived cell fractions. *HAND1* is a well-known trophoblast-associated gene (22, 23), whereas *KDR* encodes one of the two VEGF receptors and is expressed in human extravillous CTB (24). *SNAI1* is a transcriptional repressor of *CDH1* and may have a role in controlling trophoblast invasion (25). Ectodermal markers were also not expressed (*SI Appendix*, Table S5). Together these data are consistent with the conclusion that both <40-μm and >70-μm fractions are composed of trophoblast, and contain little, if any, mesoendoderm or ectodermal derivatives.

A comparison of the transcriptome profiles of the >70-μm and <40-μm fractions, as expected, revealed less dramatic differences than those observed between the >70-μm fraction and the undifferentiated H1 cells (Fig. 5*B*). There was significant ($P \leq 0.001$ and $q < 0.05$) up-regulation of expression of genes encoding CGB/LH family members, *CGA*, *CDH5* (VE-cadherin), *PGF*, *ERVW-1*, *ERVFRD-1*, *INSL4* (insulin-like 4), and *MUC15*, also several transporters, including *SLC38A3*, *SLC13A4*, and *SLC40A1*, and several transcription factors, e.g., *CEBPA*, *PPARD*, *GATA2*, *HEY1*, *DLX3*, *GCM1*, *TFAP2C*, *MSX2*, and *OVOL1*. Expression of 19 other marker genes was not significantly different between fractions (Fig. 5*B*, marked with gray bar). Among these were ones that encoded several additional transcription factors, e.g., *PPARG*, *ELF3*, *GATA3*, *MXD1*, and *TFAP2A*. A few marker genes were expressed significantly higher in the <40-μm fraction than in the >70-μm fraction (*VTCN1*, *LAMA1*, *GABRP*, and *ITGB6*).

A Comparison of the Transcriptome Profiles of ESC-Derived STB with STB Generated from Cytotrophoblast Isolated from Term Placenta. When cytotrophoblast isolated from term placentas (PHTu) underwent differentiation and formed STB (PHTd), there was the expected rise in expression of the genes, such as *CGA*, *CGB*, *PGF*, *ERVWR-1*, and *PSG* family members, which are anticipated markers of STB (*SI Appendix*, Fig. S3*B*). A few other trophoblast signature genes were down-regulated during the differentiation process, among them *OVOL1* and *ERVFRD-1*. The former is a key transcription factor regulating fusion of progenitor cytotrophoblast (26), whereas ERVFRD-1 (formerly called syncytin-2) has recently been proposed to be a more important facilitator of trophoblast fusion than ERVW-1 (syncytin-1) (27). Presumably once cell fusion had begun, these two genes became quickly down-regulated in PHTd.

We then made a comparison between the >70-μm size fraction STB generated from ESCs and PHTd formed from fusion of PHTu cells. The majority of trophoblast marker genes showed no more than a twofold difference in $\log_2$ expression values between the two types of STB (Fig. 5*C*). Some were expressed equivalently (gray bar in Fig. 5*C*). However, others were highly enriched in one cell type versus the other. For example, transcripts for *GABRP*, *SLC38A3*, *LAMA1*, and *VTCN1*, the transcription factor *HEY1*

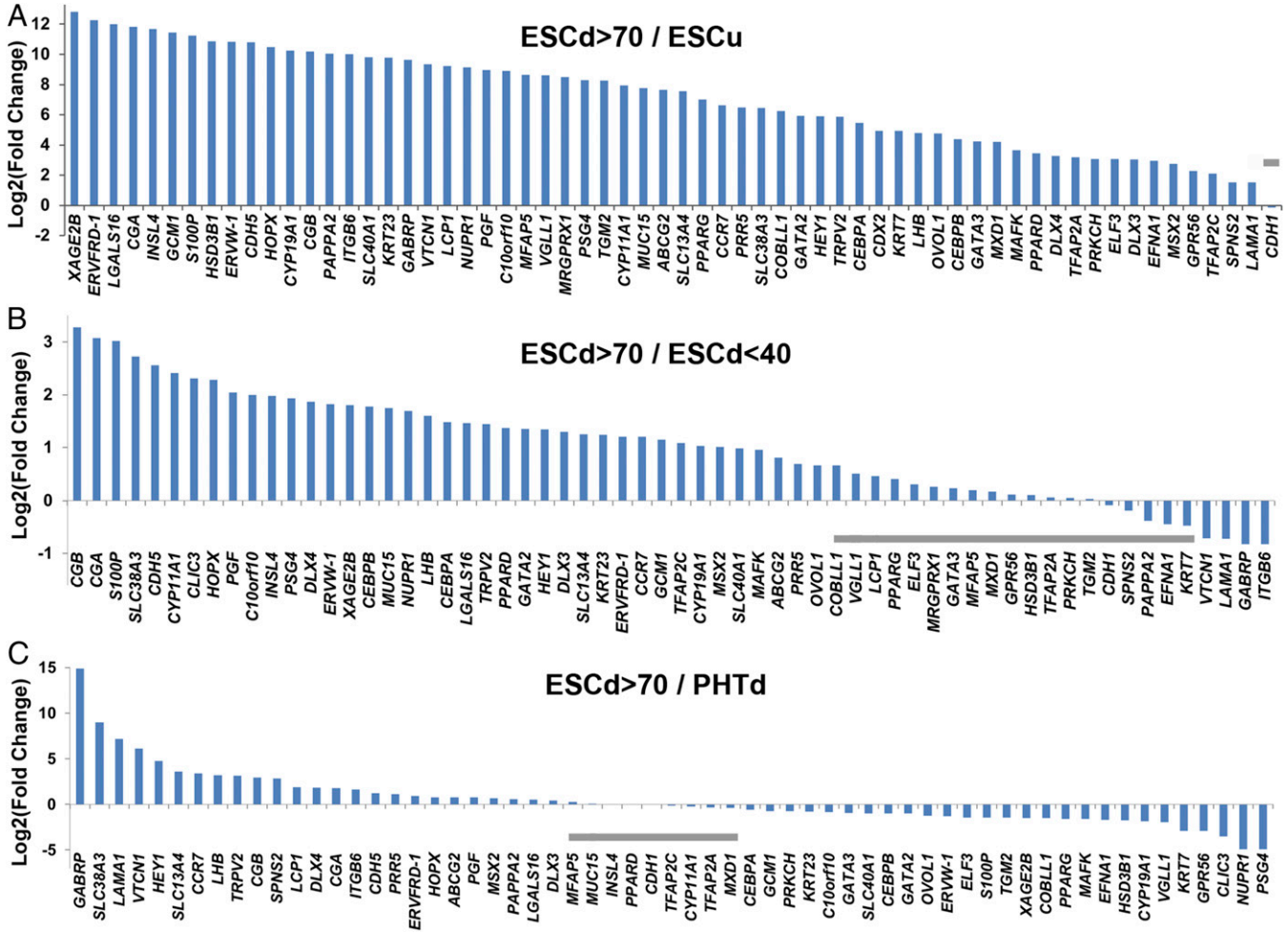


Fig. 5. Fold-change differences in relative expressions of known trophoblast gene markers in comparisons of different cell fractions: (*A*) >70-μm size fraction (ESCd >70) from BAP-differentiated ESCs relative to control ESCs that had been cultured in parallel (ESCu); (*B*) >70-μm size fraction (ESCd >70) from BAP-differentiated ESCs relative to the <40-μm fraction (ESCd <40); (*C*) >70-μm size fraction (ESCd >70) from BAP-differentiated ESCs relative to STB generated from cultured cytotrophoblast isolated from term placentas (PHTd, *C*). Sixty-two transcripts of selected trophoblast markers are compared in *A* and *B*, whereas only 61 genes are shown in *C*. The missing gene in *C* is *MRGPRX1* whose expression in PHTd was undetectable. Gray horizontal bars indicate where differences in gene expression are not significant ($q > 0.05$).

and the chemokine *CCR2* were barely detectable in the PHTd fraction, although significantly expressed in ESCd >70 cells. The genes encoding LHB family members, (*LHB*, *CGB*, *CGB5*, *CGB8*, and *CGB7*), as well as *CGA*, although significantly up-regulated in PHTd relative to PHTu (*SI Appendix*, Fig. S3*B*), were much more strongly expressed in the >70-μm size fraction derived from ESCs (ESCd >70) (Fig. 6). By contrast, a cohort of other genes, including *KRT7*, *GPR56*, *CLIC3*, *NUPR1*, and *PSG4*, were expressed significantly higher in placental STB (PHTd) than in the the >70-μm size fraction (ESCd >70) derived from ESCs (Fig. 5*C*). Other highly expressed genes with biased expression toward PHTd relative to ESCd >70 cells and that have been linked to placental trophoblast function but are not shown in Fig. 5*C*, include *CSH1* (placental lactogen), *B2M* (beta-2 microglobulin), *ENG* (endoglin), *ALPP* (placental alkaline phosphatase), *PPARG*, *SPINT1* (Kunitz type 1 serine peptidase inhibitor), *PSG* family members (*PSG1–9 PSG11*; pregnancy-specific β1 glycoproteins), and *LGALS* family members (*LGALS1*, *3*, *8*, *9*, *13*, and *14*; L-galectins). Overall, this comparison indicated many similarities between the two cell types but also some major differences. Additional distinctions are explored in the following sections.

A Comparison of the Expression of Transporter Genes in ESCd >70 and STB Generated from PHTd. A major function of placental STB is in transport of solutes to and from the conceptus, especially involving the SLC and ABC transporter families. There are 492 genes in the human *SLC* superfamily. Of these, RNA-seq revealed that 254 were measurably expressed (cutoff FKPM value >1) in the ESCd >70 cells and 231 in the PHTd cells. The expression of the top 25 SLC genes in terms of transcriptome abundance are shown in *SI Appendix*, Fig. S4*A*. Although the majority of these genes showed quite similar levels of expression in the two types of STB, there were a few striking differences. In particular, *SLC28A2*, *SLC13A4*, *SLC7A2*, *SLC35F2*, and *SLC38A3* were much more strongly expressed in ESCd >70 than in PHTd, whereas the reverse was true for *SLC40A1*, *SLC44A2*, *SLC43A2*, and *SLC38A9*. The inferred roles of the protein products of these genes is shown in *SI Appendix*, Table S6.

Of the 81 known ATP-binding cassette (ABC) transporter genes, 25 were significantly expressed above background in both the ESCd >70 and PHTd (*SI Appendix*, Fig. S4*B*). Again there were some differences between the two kinds of STB. For example, transcripts for *ABCG2*, *ABCA7*, *ABCD3*, and *ABCB4* were higher in ESCd >70 than the PHTd. Overall, however, the profiles were quite similar. A brief summary of the likely functions

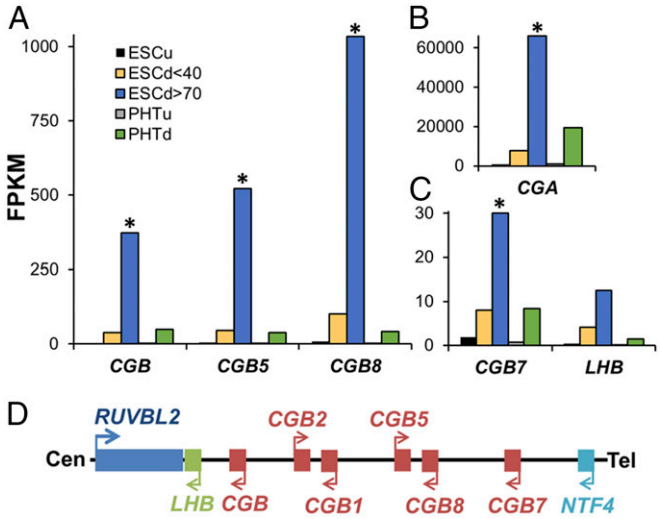


Fig. 6. RNA-seq–based gene expression values (FPKM) for *CGB*, *CGB5*, and *CGB8* (*A*), *CGA* (*B*), and *CGB7* and *LHB* (*C*) in different cell fractions. Asterisks indicate statistically significant differences ($q < 0.0005$) for expression in ESCd >70 ($n = 3$) relative to expression values in any other cell fraction. (*D*) Schematic diagram of the ~58-kb region *CGB/LHB* gene loci at chromosome 19q13.3 based on the Ensembl database (51).

of the protein products of the differentially expressed *ABC* genes are listed with the *SLC* in *SI Appendix*, Table S6.

Potential for Steroid Metabolism in ESCd >70 and STB Generated from PHTd. Placental STB is actively involved in steroid metabolism, and this potential is also evident in the ESCd >70 and PHTd. For example ESCd >70 and PHTd cells expressed similar members of the hydroxysteroid dehydrogenase family (HSD) gene family (*SI Appendix*, Fig. S5*A*). Five transcripts (those for *HSD3B1*, *HSD17B4*, *HSD11B2*, *HSD17B12*, and *HSD17B1*) predominated in both STB types. Similarly the dominant presence of transcripts for *CYP11A1* and *CYP19A1*, which encode P450 side chain cleavage enzyme and aromatase, respectively, confirms the potential of both types of syncytial cell to synthesize sex steroids from cholesterol (*SI Appendix*, Fig. S5*B*).

Expression of Genes Encoding Extracellular Matrix Components Distinguish ESCd >70 from STB Generated from PHTd. Despite the fact that ESCd >70 and PHTd cells express a host of gene markers consistent with a trophoblast identity and lack gene signatures for the three main germ-line lineages, they are clearly distinct sorts of cell. One particular distinguishing feature is in the expression of genes encoding extracellular matrix components, perhaps best illustrated by the extensive family of collagen genes (*SI Appendix*, Fig. S6*A*). PHTd expressed only a few of those genes, e.g., *COL4A1*, *COL4A2*, and *COL17A1*, and then relatively weakly, whereas expression of at least nine collagen genes, including *COL1A1*, *COL1A2*, and *COL3A1*, was uniquely associated with ESCd >70 STB. Laminin genes were also differentially expressed (*SI Appendix*, Fig. S6 *B* and *C*), as were genes encoding various proteoglycans, such as *HSPG2* (perlecan), *DCN* (decorin), *LUM* (lumican), *SDC4* (syndecan), and extracellular glycoproteins, including *FBLN1* (fibulin 1), *FN1* (fibronectin 1), *MATN2* (matrilin-2), *AGRN* (agrin), and *EFEMP1* (fibulin 3). Some of these genes were sufficiently active in one cell type relative to the other, that the presence of their transcripts was virtually diagnostic, e.g., *MATN2*, *HSPG2*, *LUM*, and *MDK* for ESCd >70, and *FN1* for PHTd. Overall, the data clearly demonstrate differences between ESCd >70 and PHTd cells in their potential to produce extracellular matrix components.

Relative Expression of Genes for Pregnancy-Specific Glycoproteins and L Galectins in ESCd >70 and STB Generated from PHTd. The pregnancy-specific glycoprotein (PSG) family of genes is expressed abundantly in the human placenta and can be detected in maternal serum, with concentrations rising as placental size increases (28). Interestingly, the PSG genes were expressed almost exclusively by PHTd, with only *PSG4* transcripts detected above background in ESCd >70 (*SI Appendix*, Fig. S6*D*). Again, these data suggest that ESCd >70 collected at day 8 after BAP exposure represent STB from early stages of pregnancy. They further confirm the distinctiveness of ESCd >70 and PHTd.

Certain members of a family of genes whose protein products encode β-galactoside binding lectins are known to be highly expressed in placentas. The concentration of at least one of these antigens (LGALS13) rises in maternal serum as pregnancy progresses (29, 30). Transcripts for seven members of this large gene family were detectable in ESCd >70 and PHTd cells (*SI Appendix*, Fig. S6*D*), and, as with PSG members, expression was much higher in the latter than in the former.

Metalloproteinases and Their Inhibitors. Transcripts for 28 ADAM family genes were detected in either the ESCd >70 or PHTd cells, with the top 16 shown in *SI Appendix*, Fig. S7. A few, including those for *ADAMTS20*, *ADAMTS2*, *ADAMTS18*, and *ADAMTS3* were uniquely associated with ESCd >70 cells. However, perhaps the most dramatic difference between the two cell types was in the relative expression of *MMP2* and *TIMP1*. The former, in particular, was very highly expressed and up-regulated more than 70-fold in ESCd >70 relative to PHTd cells. *TIMP1* transcripts were also ~9-fold more abundant in ESCd >70 cells.

Quantitative PCR Confirmation of Expression of Selected Genes. The expression patterns of two genes only expressed in ESCd >40 and ESCd >70 cells (*GABRP* and *VTCN1*), one gene expressed strongly in PHTd cells (*PSG4*), and a fourth (*KRT7*) expressed more generally in trophoblast were confirmed by quantitative PCR (qPCR) (*SI Appendix*, Fig. S8). The *GAPDH* gene used for normalization showed some variation across cell types, as did other housekeeping genes (*SI Appendix*, Table S4), but this variability was not sufficient to alter interpretation of the qPCR data.

Discussion

In this paper, we describe a characterization of the syncytial areas that emerge when human pluripotent stem cells differentiate along the trophoblast lineage. These structures materialize within the colonies as regions that stain positively for CGA and CGB, as well as for a number of other marker genes for placental STB, approximately a week after the stem cells are exposed to the growth factor BMP4 (13, 31, 32). Differentiation is enhanced under conditions that minimize FGF2 signaling, which is usually achieved by excluding FGF2 from the medium and, more recently, by including inhibitors that block pathways needed to maintain stem cell pluripotency (14, 15, 33). The areas are positive for CGA, CGB, and ERVW-1 (Syncytin-1) and have been assumed to be syncytial because, as noted by others studying placental STB formation (34), they lack the characteristic, membrane-associated desmosome staining that demarks surrounding mononucleated cells and demonstrate strands of microfilaments that extend across what appears to be a continuous cytoplasm containing several nuclei that are strongly positive for the transcription factor GATA2 (32). Here, we show that sheets of this putative syncytium can be effectively isolated within a few minutes of dispersing the colonies with a gentle cell dissociation reagent. Then, different size fractions of cell can be captured by filtration through nylon strainers. Our subsequent analyses focused mainly on two cell fractions, one that was largely mononucleated and <40 μm in diameter, whereas a second became trapped on a 70-μm mesh screen. The intermediate size fraction (40–70 μm) contained a mixture of cell types, provided an

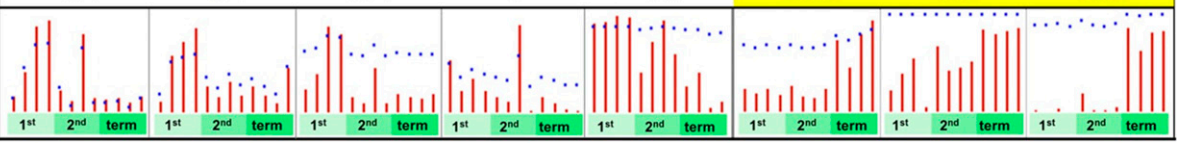
FRKM	GABRP	NTRK2	WFDC2	VTCN1	COL4A5	PSG4	CSH1	ALPP
ESCu	1.3	<0.1	38	0.4	26	<0.1	0	<0.1
ESCd<40	1994	142	224	444	242	4	0.7	0.2
ESCd>70	1074	99	181	259	85	14	0.3	0.4
PHTu	<0.1	<0.1	0.3	0.6	0.7	14	73	96
PHTd	<0.1	<0.1	0.2	4	1.3	450	56	88
Expressions in normal placentae at 1st, 2nd and term								

Fig. 7. Comparative expression of a select group of genes (*Top*) determined by RNA-seq in different cell fractions with their expression in first and second trimesters and term placental tissue as assessed by microarray. The data for the placental tissue (*Bottom*) were obtained from GEO profile (www.ncbi.nlm.nih.gov/geoprofiles; GSE9984) (52). Red bars represent transformed hybridization signal counts and blue dots are percentile rank within the sample at first trimester (four datasets at *Left*; 45–59 d of gestation); second trimester (four datasets in *Center*; 109–115 d); and term (four datasets at *Right*; C-section) placentas (data ID: 76726592). Data identifications are as follows: *GABRP*, 76726592; *NTRK2*, 76743177; *WFDC2*, 76725440; *VTCN1*, 76741153; *COL4A5*, 76734514; *PSG4*, 76721077; *CSH1*, 76728022; and *ALPP*, 76726212. Yellow highlights illustrate genes expressed differentially between ESCd >70 μm and PHTd and at different stages of pregnancy.

intermediate transcriptome profile, and was, therefore, of less immediate interest. Subsequent RNA-seq analysis performed on the <40-μm and >70-μm size fractions allowed the component cell types to be compared with each other and with the progenitor ESCs from which they were derived.

The morphological assessments made on the >70-μm cell fraction at day 8 of differentiation confirmed that the structures were largely but not entirely syncytial and were rich in endoplasmic reticulum and vesicles presumed to contain secretory product (Fig. 2*G*). Areas of cell surface richly decorated with microvilli could be observed and likely represent the apical surface of the syncytium. Cells with single nuclei, possibly in the process of fusing with their neighbors, also appeared to be present in the sheets (Figs. 2 *C* and *D* and 3 *D*, *H*, and *I*). Our immunohistochemical data show that the syncytial areas both in situ and when dissociated lacked appreciable staining for HLA-G, but were positive for ERVW-1. The appearance of *CGA* preceded *CGB*, and its initial synthesis may, as originally predicted by others (35), be initiated before syncytium formation even begins, whereas CGB may be a feature of a mature syncytium. Of particular interest to us was the localization of GABRP, a protein whose presence seems to foreshadow syncytium formation (*SI Appendix*, Fig. S2) and whose transcripts are more abundant in the <40-μm mononucleated cells than in the >70-μm syncytial fraction (Fig. 5*B*). At day 4 of differentiation, GABRP is present in discrete areas of the colonies that contain a few CGA-positive cells, although by days 6 and 8, there is a higher degree of colocalization of the two antigens (*SI Appendix*, Fig. S2 *A–L*). The possible involvement of GABRP, normally recognized as the pi subunit of the GABA receptor, in formation of syncytium is probably worthy of future attention.

The paper further confirms the ability of BAP treatment of hESCs to generate trophoblast and no other lineages. All transcripts for an arbitrarily selected but relatively large set of trophoblast marker genes, with the exception of *CDH1*, were up-regulated relative to the initiating H1 ESCs (Fig. 5*A*). Also, there was no evidence for the expression of genes indicative of the presence of ectoderm, endoderm, or mesoderm derivatives in either the >70-μm or <40-μm cell fractions (*SI Appendix*, Tables S4 and S5). A few genes, *HAND1* being a good example, that have been implicated in emergence of mesoderm, were expressed in the BAP-treated H1 cells, but such genes are also trophoblast associated and provide ambiguous diagnostic information in distinguishing the one lineage from the other. Nonetheless, the combined data presented in Fig. 5 and *SI Appendix*, Fig. S3 and Tables S4 and S5 provide unequivocal evidence that the hESCs had been driven efficiently and unidirectionally to trophoblast by BAP exposure and that the >70-μm fraction expressed many of the hallmark genes anticipated for syncytiotrophoblast.

Our experiments also provided us with the opportunity to compare transcriptional profiles of STB generated from hESC trophoblast cells with those derived from term placentas. Although term villous cytotrophoblasts are not abundant compared with earlier in pregnancy (36), they appear to be committed to form STB. The cells can be isolated and, within 48 h, begin to fuse and give rise to areas of multinucleated cells (12). RNA-seq analysis performed on cultured cytotrophoblast before they had differentiated and on the same cells cultured under conditions that promoted cell fusion revealed expression of all of the same trophoblast markers up-regulated in the hESCs treated with BAP (Fig. 5 and *SI Appendix*, Fig. S3). Additionally, there was little evidence for the presence of markers characteristic of lineages other than trophoblast. On the other hand, it was clear that there were major dissimilarities between the STB from the term placentas (PHTd) and the BAP-differentiated hESCs (>70-μm fraction), despite the basic trophoblastic phenotype both presented. These differences were evident in the cluster analyses performed on the RNA-seq data (Fig. 4 *A* and *B*) and even in the relative expressions of trophoblast markers (Fig. 5*C*). For example, several trophoblast marker genes, including the aforementioned *GABRP*, were highly expressed in the BAP-differentiated hESCs but barely at all in placental STB, whereas the situation was reversed for others, such as *CSH1*, and *PSG*, and *LGALS* family members (Fig. 5*C* and *SI Appendix*, Fig. S6*D*). There were also major discrepancies in the expression of *KRT* genes (*SI Appendix*, Fig. S6*E*), which are frequently used diagnostically to identify trophoblast in sections of pregnant endometrium (37, 38). Moreover, whereas the ESCd >70 μm and PHTd would both appear to be engaged in synthesis of CG, the expression of *CGB* genes and its partner, *CGA*, was manyfold higher in the former than in the latter (Fig. 6). Fig. 7 illustrates the correlation between the relative expression values for eight trophoblast marker genes in the ESCd >70-μm fraction and PHTd cells and their expression in placental villous samples obtained at different stages of pregnancy. In particular, *GABRP*, *NTRK2*, *WFDC2*, *VTCN1*, and even *COL4A5* appear to be features of the early placental samples, whereas *PSG4*, *CSH1*, and *ALPP* (placental alkaline phosphatase) are associated most strongly with term placentas. Together, these observations suggested that the >70-μm fraction represents STB from early stage pregnancies. However, the situation may be more complicated than that, as the clustering analyses in Fig. 4 indicate major disparities in gene expression, even for genes encoding proteins engaged in anticipated placental functions, such as transporters

(*SI Appendix*, Fig. S4) and steroid metabolism (*SI Appendix*, Fig. S5). Such data seem inconsistent with any perception that the two cell types have particularly close pedigrees, despite the fact that the culture systems used to generate the STB from hESCs and from term placental cytotrophoblast were dissimilar.

If, as seems reasonable, PHTd represents the STB that overlays placental villi, what then is the in vivo equivalent of the STB formed from BAP-treated ESC colonies? As pointed out in the introduction, there is an earlier form of STB, a syncytium that emerges during the initial breaching of the uterine epithelium (6), but it is unlikely under present restrictions for conducting research on human subjects that such specimens will ever become available. This syncytial tissue then invades maternal stromal tissue, degrades the matrix with which it makes contact, hollows out lacunae, and colonizes blood capillaries and uterine glands and thereby gains access to solutes and macromolecules necessary to support the growing conceptus. Additionally, it also provides a base through which growing cytotrophoblast columns migrate outward to establish the rudiments of the placental villous tree. This early syncytium is also the major form of trophoblast present when the mother must respond to trophoblast signals to maintain her pregnancy, particularly the hormone hCG, which is required for continued corpus luteum support and avoidance of endometrial shedding and menstruation, which would normally occur after about 2 wk if pregnancy recognition had not ensued (39). Is it possible that the hESC-derived STB represents this early syncytium? Its invasive features, particularly the high expression of MMP2, its potential for substantial hCG and extracellular matrix production, and its broad expression of transporter genes, including that for the iron-carrier SLC40A1, make this hypothesis a feasible one. Another alternative is that this STB is a homolog of villous STB from very early pregnancy. However, detailed transcript analysis of such cells has not yet been published; nor have there been experiments to determine whether purified cytotrophoblasts from the first trimester terminations can be induced to fuse in vivo in a manner similar to that possible with cells obtained at term. The amount of tissue available is undoubtedly a limiting factor in conducting such experiments.

Unfortunately we know little about implantation in human and related primates and virtually nothing about the characteristic of the invasive STB that paves the way for placenta formation (2). In particular, it is not completely clear how this syncytium forms, although it is believed to have its origins through proliferation and fusion of a population of cytotrophoblast cells derived from polar trophectoderm overlaying and in immediate contact with the epiblast. However, can an epiblast origin for this syncytium be definitely ruled out given the paucity of histological data? Such a beginning would reconcile the controversies that have raged about whether or not human pluripotent stem cells of the epiblast type can differentiate into trophoblast (40–42), a concept not readily accepted by some embryologists, but supported by an imposing array of experimental data (13–19, 31–33, 43–48), including the results presented in this paper where we have confirmed the STB nature of the >70-μm fraction but also demonstrated many features in gene expression in common with epiblast stem cells (Fig. 4).

Materials and Methods

Human ESC Culture and Differentiation. Human ESC (H1; WA01) originated from WiCell Research Institute and were cultured in six-well tissue culture plates (Thermo Scientific) on Matrigel (BD Bioscience)-coated plates. For maintenance, these cells were cultured in mTeSR1 medium (Stemcell Technologies). The culture medium was changed daily, and cells were passaged every 5–6 d by using Gentle Cell Dissociation Reagent (Stemcell Technologies). They were cultured under an atmosphere of 95% (vol/vol) air and 5% (vol/vol) CO_2 at 37 °C. For trophoblast differentiation, a procedure described in Amita et al. (14) was used. Briefly, the day after passaging onto Matrigel-coated dishes at 1.2×10^4 cells/cm^2, the culture medium was changed to DME/F12 medium (Thermo Scientific) with knock-out serum replacement (KOSR, Invitrogen) that had been conditioned by MEFs and supplemented with FGF2 (4 ng/mL). After 24 h, the conditioned medium was replaced with daily changes of DME/F12/KOSR medium lacking MEF conditioning and minus FGF2, but containing BMP4 (10 ng/mL), A83-01 (1 μM), and PD173074 (0.1 μM) (BAP treatment) for up to 8 d (14). Control cultures were maintained in conditioned medium containing 4 ng/mL FGF2.

Cell Separation on Strainers. Cell sorting by relative cell diameter was conducted after completely dissociating the colonies. Complete cell dissociations could be achieved either after 14 min in 0.25% Trypsin-EDTA (Life Technologies) or a six-min incubation with Gentle Cell Dissociation Reagent followed by complete physical dispersions by repeated pipetting. Large cells (>70 μm) were then collected by passing the suspension successively through a series of nylon cell strainers (70 μm and 40 μm, Fisher Scientific) to generate three cell size fractions (>70 μm, 40–70 μm, and <40 μm). The captured cells on the strainer were recovered by inverting the strainer and rinsing with culture medium. Similarly, middle size cells (40–70 μm) were obtained from the <70-μm fraction by collection on a 40-μm cell strainer after removal of <70-μm cells. The three size fractions were used in all subsequent analyses.

Portions of the size-fractioned cells were fixed in 4% (vol/vol) paraformaldehyde/PBS solution for 15 min and resuspended (5.0×10^2 and 1.0×10^5 cells/100 μL PBS). The suspensions were loaded into a Shandon single cytofunnel and centrifuged for 5 min at 1,000 × *g* in a Shandon CytoSpin 4 cytocentrifuge (Thermo Scientific).

Derivation Primary Human PHTu and PHTd. Placental tissue samples were collected by the Obstetrical Specimen Procurement Unit at Magee-Womens Hospital of the University of Pittsburgh Medical Center. The work was performed under an exempt protocol approved by the Institutional Review Board (IRB) at the University of Pittsburgh. Under the protocol, patients provided written consent for the use of deidentified discarded tissues for research upon admittance to the hospital. Primary villous PHTs were derived and cultured according to published procedures (12, 49, 50) from three term human placentas (one female and two males). Multiple primary cultures were established from each placenta at a density of 3.5×10^5 cells/cm^2 in DMEM supplemented with 10% (vol/vol) FBS and antibiotics under a 5% (vol/vol) CO_2/air atmosphere at 37 °C. Triplicate cultures from each placenta were harvested at 9 h (PHTu) before syncytium formation and subsequently at 48 h (PHTd) when syncytium formation had occurred. The sieving technique used for the hESC-derived cells was thus unnecessary for the term placental STB. Total RNA was extracted from each sample (3 × 3 at 9 h and 48 h, respectively) to provide a total of 18 samples for RNA-seq analysis.

Additional methods are described in *SI Appendix, SI Materials and Methods*.

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1. Gude NM, Roberts CT, Kalionis B, King RG (2004) Growth and function of the normal human placenta. *Thromb Res* 114(5–6):397–407.
2. James JL, Carter AM, Chamley LW (2012) Human placentation from nidation to 5 weeks of gestation. Part I: What do we know about formative placental development following implantation? *Placenta* 33(5):327–334.
3. Huppertz B (2008) The anatomy of the normal placenta. *J Clin Pathol* 61(12): 1296–1302.
4. Boyd JD, Hamilton WJ (1970) *The Human Placenta* (Heffer & Sons, Cambridge).
5. Hertig AT, Rock J, Adams EC (1956) A description of 34 human ova within the first 17 days of development. *Am J Anat* 98(3):435–493.
6. Huppertz B (2007) The feto-maternal interface: Setting the stage for potential immune interactions. *Semin Immunopathol* 29(2):83–94.
7. Loke YW, King A (1995) *Human Implantation: Cell Biology and Immunology* (Cambridge Univ Press, Cambridge).
8. Miller RK, et al. (2005) Human placental explants in culture: Approaches and assessments. *Placenta* 26(6):439–448.
9. Simán CM, Sibley CP, Jones CJ, Turner MA, Greenwood SL (2001) The functional regeneration of syncytiotrophoblast in cultured explants of term placenta. *Am J Physiol Regul Integr Comp Physiol* 280(4):R1116–R1122.
10. Wice B, Menton D, Geuze H, Schwartz AL (1990) Modulators of cyclic AMP metabolism induce syncytiotrophoblast formation in vitro. *Exp Cell Res* 186(2):306–316.
11. Orendi K, Gauster M, Moser G, Meiri H, Huppertz B (2010) The choriocarcinoma cell line BeWo: Syncytial fusion and expression of syncytium-specific proteins. *Reproduction* 140(5):759–766.

12. Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss JF, 3rd (1986) Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. *Endocrinology* 118(4):1567–1582.
13. Schulz LC, et al. (2008) Human embryonic stem cells as models for trophoblast differentiation. *Placenta* 29 Suppl A:S10–S16.
14. Amita M, et al. (2013) Complete and unidirectional conversion of human embryonic stem cells to trophoblast by BMP4. *Proc Natl Acad Sci USA* 110(13):E1212–E1221.
15. Sudheer S, Bhushan R, Fauler B, Lehrach H, Adjaye J (2012) FGF inhibition directs BMP4-mediated differentiation of human embryonic stem cells to syncytiotrophoblast. *Stem Cells Dev* 21(16):2987–3000.
16. Ezashi T, Telugu BP, Roberts RM (2012) Model systems for studying trophoblast differentiation from human pluripotent stem cells. *Cell Tissue Res* 349(3):809–824.
17. Yang Y, et al. (2015) Heightened potency of human pluripotent stem cell lines created by transient BMP4 exposure. *Proc Natl Acad Sci USA* 112(18):E2337–E2346.
18. Xie W, et al. (2013) Epigenomic analysis of multilineage differentiation of human embryonic stem cells. *Cell* 153(5):1134–1148.
19. Marchand M, et al. (2011) Transcriptomic signature of trophoblast differentiation in a human embryonic stem cell model. *Biol Reprod* 84(6):1258–1271.
20. Ezashi T, Das P, Roberts RM (2005) Low O2 tensions and the prevention of differentiation of hES cells. *Proc Natl Acad Sci USA* 102(13):4783–4788.
21. Bai Q, et al. (2012) Dissecting the first transcriptional divergence during human embryonic development. *Stem Cell Rev* 8(1):150–162.
22. Hu D, et al. (2013) The basic helix-loop-helix transcription factor Hand1 regulates mouse development as a homodimer. *Dev Biol* 382(2):470–481.
23. Knöfler M, et al. (2002) Human Hand1 basic helix-loop-helix (bHLH) protein: Extraembryonic expression pattern, interaction partners and identification of its transcriptional repressor domains. *Biochem J* 361(Pt 3):641–651.
24. Iacob D, et al. (2008) Decorin-mediated inhibition of proliferation and migration of the human trophoblast via different tyrosine kinase receptors. *Endocrinology* 149(12): 6187–6197.
25. Paznekas WA, Okajima K, Schertzer M, Wood S, Jabs EW (1999) Genomic organization, expression, and chromosome location of the human SNAIL gene (SNAI1) and a related processed pseudogene (SNAI1P). *Genomics* 62(1):42–49.
26. Renaud SJ, et al. (2015) OVO-like 1 regulates progenitor cell fate in human trophoblast development. *Proc Natl Acad Sci USA* 112(45):E6175–E6184.
27. Lokossou AG, Toudic C, Barbeau B (2014) Implication of human endogenous retrovirus envelope proteins in placental functions. *Viruses* 6(11):4609–4627.
28. Sørensen S, Momsen G, Ruge S, Pedersen JF (1995) Differential increase in the maternal serum concentrations of the placental proteins human chorionic gonadotrophin, pregnancy-specific beta 1-glycoprotein, human placental lactogen and pregnancy-associated plasma protein-A during the first half of normal pregnancy, elucidated by means of a mathematical model. *Hum Reprod* 10(2):453–458.
29. Huppertz B, Meiri H, Gizurarson S, Osol G, Sammar M (2013) Placental protein 13 (PP13): A new biological target shifting individualized risk assessment to personalized drug design combating pre-eclampsia. *Hum Reprod Update* 19(4):391–405.
30. Than NG, et al. (2014) Placental Protein 13 (PP13): A placental immunoregulatory galectin protecting pregnancy. *Front Immunol* 5:348.
31. Xu RH, et al. (2002) BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat Biotechnol* 20(12):1261–1264.
32. Das P, et al. (2007) Effects of fgf2 and oxygen in the bmp4-driven differentiation of trophoblast from human embryonic stem cells. *Stem Cell Res (Amst)* 1(1):61–74.
33. Sarkar P, et al. (2015) Activin/nodal signaling switches the terminal fate of human embryonic stem cell-derived trophoblasts. *J Biol Chem* 290(14):8834–8848.
34. Douglas GC, King BF (1990) Differentiation of human trophoblast cells in vitro as revealed by immunocytochemical staining of desmoplakin and nuclei. *J Cell Sci* 96(Pt 1): 131–141.
35. Hoshina M, Boothby M, Boime I (1982) Cytological localization of chorionic gonadotropin alpha and placental lactogen mRNAs during development of the human placenta. *J Cell Biol* 93(1):190–198.
36. Benirschke K, Kaufmann P, Baergen RN (2006) *Pathology of the Human Placenta* (Springer, New York), 5th Ed.
37. Gauster M, Blaschitz A, Siwetz M, Huppertz B (2013) Keratins in the human trophoblast. *Histol Histopathol* 28(7):817–825.
38. Uhlen M, et al. (2010) Towards a knowledge-based Human Protein Atlas. *Nat Biotechnol* 28(12):1248–1250.
39. Ticconi C, et al. (2007) Pregnancy-promoting actions of HCG in human myometrium and fetal membranes. *Placenta* 28 Suppl A:S137–S143.
40. Bernardo AS, et al. (2011) BRACHYURY and CDX2 mediate BMP-induced differentiation of human and mouse pluripotent stem cells into embryonic and extraembryonic lineages. *Cell Stem Cell* 9(2):144–155.
41. Roberts RM, et al. (2014) Differentiation of trophoblast cells from human embryonic stem cells: To be or not to be? *Reproduction* 147(5):D1–D12.
42. Lee CQ, et al. (2016) What is trophoblast? A combination of criteria define human first-trimester trophoblast. *Stem Cell Rep* 6(2):257–272.
43. Douglas GC, VandeVoort CA, Kumar P, Chang TC, Golos TG (2009) Trophoblast stem cells: Models for investigating trophectoderm differentiation and placental development. *Endocr Rev* 30(3):228–240.
44. Erb TM, et al. (2011) Paracrine and epigenetic control of trophectoderm differentiation from human embryonic stem cells: The role of bone morphogenic protein 4 and histone deacetylases. *Stem Cells Dev* 20(9):1601–1614.
45. Li Y, et al. (2013) BMP4-directed trophoblast differentiation of human embryonic stem cells is mediated through a ΔNp63+ cytotrophoblast stem cell state. *Development* 140(19):3965–3976.
46. Warmflash A, Sorre B, Etoc F, Siggia ED, Brivanlou AH (2014) A method to recapitulate early embryonic spatial patterning in human embryonic stem cells. *Nat Methods* 11(8):847–854.
47. Gupta R, Ezashi T, Roberts RM (2012) Squelching of ETS2 transactivation by POU5F1 silences the human chorionic gonadotropin CGA subunit gene in human choriocarcinoma and embryonic stem cells. *Mol Endocrinol* 26(5):859–872.
48. Telugu BP, et al. (2013) Comparison of extravillous trophoblast cells derived from human embryonic stem cells and from first trimester human placentas. *Placenta* 34(7): 536–543.
49. Nelson DM, Johnson RD, Smith SD, Anteby EY, Sadovsky Y (1999) Hypoxia limits differentiation and up-regulates expression and activity of prostaglandin H synthase 2 in cultured trophoblast from term human placenta. *Am J Obstet Gynecol* 180(4): 896–902.
50. Schaiff WT, et al. (2005) Peroxisome proliferator-activated receptor-gamma and retinoid X receptor signaling regulate fatty acid uptake by primary human placental trophoblasts. *J Clin Endocrinol Metab* 90(7):4267–4275.
51. Nagirnaja L, et al. (2010) Genomics and genetics of gonadotropin beta-subunit genes: Unique FSHB and duplicated LHB/CGB loci. *Mol Cell Endocrinol* 329(1–2):4–16.
52. Mikheev AM, et al. (2008) Profiling gene expression in human placentae of different gestational ages: An OPRU Network and UW SCOR Study. *Reprod Sci* 15(9):866–877.