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Hypoxia Directs Human Extravillous Trophoblast Differentiation in a Hypoxia-Inducible Factor—Dependent Manner



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Villous cytotrophoblasts are epithelial stem cells of the early human placenta, able to differentiate either into syncytiotrophoblasts in floating chorionic villi or extravillous trophoblasts (EVTs) at the anchoring villi. The signaling pathways regulating differentiation into these two lineages are incompletely understood. The bulk of placental growth and development in the first trimester occurs under low oxygen tension. One major mechanism by which oxygen regulates cellular function is through the hypoxia-inducible factor (HIF), a transcription factor complex stabilized under low oxygen tension to mediate cellular responses, including cell fate decisions. HIF is known to play a role in trophoblast differentiation in rodents; however, its role in human trophoblast differentiation is poorly understood. Using RNA profiling of sorted populations of primary first-trimester trophoblasts, we evaluated the first stage of EVT differentiation, the transition from epidermal growth factor receptor⁺ villous cytotrophoblasts into human leukocyte antigen-G⁺ proximal column EVT (pcEVT) and identified hypoxia as a major pcEVT-associated pathway. Using primary cytotrophoblasts, we determined that culture in low oxygen directs differentiation preferentially toward human leukocyte antigen-G⁺ pcEVT, and that an intact HIF complex is required for this process. Finally, using global RNA profiling, we identified integrin-linked kinase and associated cytoskeletal remodeling and adhesion to be among HIF-dependent pcEVT-associated signaling pathways. Taken together, we propose that oxygen regulates EVT differentiation through HIF-dependent modulation of various cell adhesion and morphology-related pathways. (*Am J Pathol* 2017, 187: 767–780; <http://dx.doi.org/10.1016/j.ajpath.2016.11.018>)

Proper formation and function of the placenta is required for normal fetal growth and development in utero.^{1,2} Early development of the human placenta occurs under low oxygen conditions, during which time the placenta anchors itself to the uterus and establishes the maternal-fetal interface.^{3,4} Cytotrophoblasts (CTBs), the proliferative epithelial stem cells of the placenta, either fuse and form syncytiotrophoblasts (STBs) in the floating chorionic villi, which comprise the gas/nutrient exchange portion of the placenta, or differentiate into extravillous trophoblasts (EVTs), which subsequently invade the uterus and remodel maternal spiral arterioles to gain access to maternal blood.^{2,3} Differentiation of CTBs into EVTs occurs in the anchoring villi, and involves a multistep process.⁵ In the first step, villous CTBs,

epidermal growth factor receptor (EGFR)⁺ cells adjacent to the villous mesenchyme, differentiate into human leukocyte antigen (HLA)-G⁺ proximal column EVT (pcEVTs), which remain proliferative. pcEVTs subsequently lose their proliferative potential in the distal column, and then mature into cells that invade the decidua and myometrium as either

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interstitial EVT_s or endovascular EVT_s.⁵ The latter remodel the maternal spiral arterioles, replacing endothelial cells, and thus taking control of maternal blood flow into the intervillous space.^{5,6}

The above process of EVT differentiation takes place under low oxygen tension,⁴ although the maturation process is thought to occur in an increasing oxygen gradient, particularly for the endovascular EVT, which eventually accesses oxygen-rich spiral arterioles.^{6,7} How oxygen regulates these cells in the early placenta has been the subject of numerous studies.^{7–12} Many hypoxia-induced signaling pathways act through the hypoxia-inducible factor (HIF) complex, a heterodimer formed from an α and a β subunit.¹³ Multiple α subunits exist (including HIF1 α and HIF2 α), each of which is stabilized in low oxygen, and subsequently translocate to the nucleus, where they bind to the constitutively expressed HIF1 β subunit [alias aryl hydrocarbon receptor nuclear translocator (ARNT)] to form the transcriptionally active complex.¹³ The HIF complex is critical for embryonic development in mice, where loss of either HIF1 β /ARNT or both of the main α subunits (HIF1 α /HIF2 α) leads to embryonic lethality at mid-gestation.^{14,15} The placental phenotypes associated with these transgenic animals include not just abnormal vascularization, but also abnormalities of the trophoblast.^{15,16} In fact, mouse trophoblast stem cells derived from either HIF1 α ^{-/-}/HIF2 α ^{-/-} or ARNT^{-/-} embryos cannot form trophoblast giant cells, cells analogous to human EVT_s, instead differentiating exclusively into multinucleated STBs.^{15,16} Similarly, HIF plays an important role in the development of the rat placenta, where hypoxia has been shown to promote both differentiation into trophoblast giant cells *in vitro* and trophoblast invasion of the uterus *in vivo*.^{17,18}

In the human placenta, the role of hypoxia has been more controversial.¹⁹ However, this is at least in part because of mixed terminology, specifically, the use of the term cytotrophoblast for both villous CTB (vCTB) and at least some subtypes of EVT.⁶ For this reason, many reviews on this subject summarize conclusions from these studies as hypoxia inducing proliferation of CTB and inhibiting EVT differentiation.^{6,20–22} Moreover, the role of HIF in inducing the EVT lineage in the human placenta, as it does in rodents, also remains unclear. We therefore set out to further evaluate the initial transition of vCTB into pcEVT at the molecular level, including the effect of oxygen tension and HIF.

Materials and Methods

Isolation and Culture of Primary Cytotrophoblast from Human Placental Samples

All fetal and placental tissues were collected under a protocol approved by the Human Research Protections Program Committee of the University of California San Diego Institutional Review Board; all patients gave informed consent for collection and use of these tissues.

First-trimester trophoblasts were isolated from 6- to 9-week (for microarray-based gene expression profiling experiments) or 8- to 10-week (for culture) gestational age placentas. Areas rich in chorionic villi were minced, washed in Hanks' balanced salt solution, and subjected to three sequential digestions: digestion 1, 300 μ g/mL of DNase I (10104159001; Roche, Pleasanton, CA) and 0.125% trypsin (Gibco, Carlsbad, CA); digestions 2 and 3, 0.25% trypsin and 300 μ g/mL of DNase I. The pelleted cells from the second and third digests were pooled and resuspended in Hanks' balanced salt solution (Thermo Fisher Scientific, Carlsbad, CA), and separated on a Percoll (Sigma-Aldrich, St. Louis, MO) gradient. For optimization of cell plating, we used laminin (50 μ g/mL; Santa Cruz Biotechnologies, Santa Cruz, CA), collagen IV (8 μ g/cm²; Sigma-Aldrich), and fibronectin (20 μ g/mL; Sigma-Aldrich). For the remaining experiments, fibronectin was used for plating. Cells were cultured in media containing Dulbecco's modified Eagle's medium/F12 (Gibco), 10% fetal bovine serum (Sigma-Aldrich), containing penicillin-streptomycin (Thermo Fisher Scientific), and gentamicin (Gibco) for 4 days. For hypoxic culture, cells were incubated at 2% oxygen in an XVIVO system (Biospherix, Parish, NY). For all experiments in hypoxia, medium was changed and cells were lysed (for either RNA or protein analysis) or fixed (for immunofluorescence and flow cytometry) in the XVIVO work chamber.

Constructs for Down-Regulation of ARNT

The vector consisting of a pLKO.1 lentiviral backbone containing a negative control (scramble) shRNA sequence was obtained from Addgene (Cambridge, MA). A set of five Mission shRNA Lentiviral constructs (also built using the pLKO.1 backbone) targeting the human *ARNT* gene were purchased (Sigma-Aldrich). HEK293FT cells were transfected with the shRNA constructs, Delta 8.2, and VSV-G using Lipofectamine 2000 (Sigma-Aldrich), according to the manufacturer's instructions. Lentiviral supernatants were concentrated with PEG-it virus precipitation solution (System Biosciences, Mountain View, CA). The concentrated viral particles were then incubated with target cells in the presence of 10 mg/mL polybrene (Sigma-Aldrich). Packaging and transduction efficiency were tested using a green fluorescent protein-expressing lentivirus in parallel experiments. Of the five ARNT-specific shRNA constructs, three were selected for further experiments on the basis of high-efficiency knockdown of ARNT protein levels in HEK293T cells; subsequent experiments were performed with this subset of three shRNAs pooled together. Isolated primary CTBs were transiently transduced at the time of plating. Transduction efficiency was determined by green fluorescent protein expression. Knockdown efficiency was determined by Western blot.

Immunofluorescence Staining of Tissues and Cells

Placental tissue was fixed overnight in formalin, paraffin embedded, and cut into sections (4 μ m thick). Sections were deparaffinized and rehydrated, and antigen retrieval was performed in citrate buffer. Sections were blocked in 10% fetal bovine serum and 0.5% fish gelatin and stained with rabbit anti-EGFR antibody (Santa Cruz Biotechnologies) and mouse anti-HLAG antibody (clone 4H84; Abcam, Cambridge, MA), followed by Alexa 488- or Alexa 596-conjugated secondary antibodies (Invitrogen, Carlsbad, CA). Stained tissue was imaged using a Leica DM IRE2 inverted fluorescence microscope (Leica Microsystems Inc, Buffalo Grove, IL).

Cultured cells were fixed in 4% paraformaldehyde in phosphate-buffered saline for 10 minutes, then permeabilized with 0.5% Triton X-100 for 2 minutes. Cells were stained with mouse anti-HLAG antibody (clone 4H84; Abcam), rabbit anti-Ki-67 antibody (Abcam), or Alexa 488-conjugated phalloidin (Invitrogen), counterstained with DAPI (Invitrogen), then visualized using a Leica DM IRE2 inverted fluorescence microscope. For determination of fusion index, nuclei and cells were counted in five randomly selected 10 \times objective fields per condition. Cell fusion was quantitated as a fusion index of (N-S)/T, where N is the number of nuclei in the syncytia, S is the number of syncytia, and T is the total number of nuclei counted.

Flow Cytometry and Fluorescence Activated Cell Sorting

For flow cytometric analysis, cells were fixed with 4% paraformaldehyde. Cells were incubated with phosphatidylethanolamine-conjugated mouse anti-HLA-G (MEM-G/9; ExBio, Vestec, Czech Republic) and Allophycocyanin-conjugated mouse anti-EGFR (BioLegend, San Diego, CA) antibodies at room temperature for an hour, washed, and resuspended in an appropriate volume of fluorescence activated cell sorting buffer (1 \times phosphate-buffered saline, 10% fetal bovine serum). Flow cytometric analysis was performed using a BD FACS-Canto Flow Cytometer (BD Biosciences, San Jose, CA).

To obtain the populations of sorted CTBs or sorted EVT, freshly isolated first-trimester trophoblasts were stained with phosphatidylethanolamine-conjugated mouse anti-HLA-G (87G; Biolegend) and Allophycocyanin-conjugated mouse anti-EGFR (BioLegend) and sorted using a BD Influx instrument (BD Biosciences).

Automated ISH and IHC

For both *in situ* hybridization (ISH) and immunohistochemistry (IHC), placental tissue samples were fixed in neutral-buffered formalin, embedded in paraffin, and cut into sections (4 μ m thick). Both procedures were performed

on a Ventana Discovery Ultra automated stainer (Ventana Medical Systems, Tucson, AZ).

For ISH, slides were deparaffinized, and subjected to antigen retrieval and protease treatment as described by the manufacturer (ACD-Bio, Hayward, CA). ISH was performed using the RNAscope method with probes specific to human TEAD2, GCM1, HIF1A, and ASCL2, all from ACD-Bio. Probes consisted of the z-DNA method, where two adjacent 18- to 25-bp probes hybridize to the mRNA sequence.²³ The z-DNA probe has a 14-bp linker region and an adjacent 25-bp sequence; when two z-pairs hybridize next to each other, they contain a 50-bp backbone to which a subsequent probe and amplifier sequence hybridize. This allows for up to 8000 labels for each target mRNA.²³ After amplification steps, the probes were visualized with 3,3'-diaminobenzidine and slides were counterstained with hematoxylin. Each of the probes used in the study were tested in tissues where their expression level and pattern could be readily validated. Both negative (bacterial *dapB* mRNA) and positive control probes (cyclophilin PPIB mRNA) were used on selected tissues to ensure probe specificity. Each dot corresponds to a single RNA message (mRNA); larger dots may be multiple mRNAs closely localized.

For IHC, placental sections were stained with a rabbit monoclonal antibody against HIF1A (final dilution of 1:225; CellMarque, Rocklin, CA) or with a rabbit polyclonal antibody against integrin-linked kinase 1 (ILK1; final dilution of 1:75; Cell Signaling, Danvers, MA). Antigen retrieval was performed using Cell Conditioning 1 solution (Ventana Medical Systems) for 40 minutes at 95°C. The primary antibodies were incubated on the sections for 1 hour at 37°C. Primary antibodies were visualized using 3,3'-diaminobenzidine as a chromagen using the UltraMap system (Ventana Medical Systems), followed by hematoxylin as a counterstain. Slides were rinsed, dehydrated through alcohol and xylene, and coverslipped.

Both ISH and IHC slides were analyzed by conventional light microscopy on an Olympus BX43 microscope (Olympus, Waltham, MA).

Western Blot Analysis

After washing with ice-cold phosphate-buffered saline, cells were scraped in lysis buffer containing 1% Triton X-100 and 0.5% SDS, 150 mmol/L NaCl in 50 mmol/L Tris, pH 7.5, with 100 \times HALT protease inhibitor cocktail (Thermo Fisher Scientific), and 100 \times EDTA (Thermo Fisher Scientific). Samples were sonicated for 15 seconds at 20% amplitude, spun at 15,000 \times g for 10 minutes to pellet any insoluble material, and the supernatant was collected. The protein concentration of each sample was determined using a BCA protein assay reagent (Thermo Fisher Scientific). Samples were mixed with 10 \times SDS-PAGE sample buffer (20 mmol/L Tris, pH 8.0, 2% SDS, 2 mmol/L dithiothreitol, 1 mmol/L Na₃VO₄, 2 mmol/L ethylenediamine tetraacetic acid, and 20% glycerol) plus 5% 2-mercaptoethanol (Bio-Rad, Hercules, CA) and

boiled for 10 minutes. Total cellular protein (30 µg) was separated by 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1 hour at room temperature in 20 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, and 0.05% Tween 20 (Thermo Fisher Scientific) containing 5% nonfat dried milk (Bio-Rad). The membranes were then incubated with the primary antibody overnight at 4°C. Primary antibodies included mouse anti-ARNT (BD Biosciences), rabbit anti-HIF1 α (Cayman Chemical, Ann Arbor, MI), mouse anti-HLAG (Novus Biologicals, Littleton, CO), rabbit anti-cleaved caspase 3 (Cell Signaling), mouse anti- β -actin (Sigma-Aldrich), and rabbit anti-ILK (BethylLab, Montgomery, TX). After three washes in 1X Tris-buffered saline containing 0.1% Tween, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature, then washed with 1X Tris-buffered saline containing 0.1% Tween three times. The labeled proteins were visualized using the enhanced chemiluminescence kit (Thermo Fisher Scientific).

Measurement of Secreted Total hCG and MMP2

Cell culture supernatants were collected and stored in -80°C. Levels of total human chorionic gonadotropin (hCG) were quantified using the hCG ELISA Kit (Calbiotech Inc., Spring Valley, CA), according to the manufacturer's protocol. Levels of secreted matrix metalloproteinase 2 (MMP2) were quantified using MMP2 ELISA kit (Abcam). The results were normalized to DNA content, using DNA isolated from the DNEasy Blood and Tissue Kit (Qiagen, Valencia, CA).

RNA Isolation, RT-qPCR, and Microarray-Based Gene Expression Profiling

Total RNA was extracted with the mirVana RNA Isolation Kit (Ambion, Austin, TX), according to the manufacturer's protocol. For quantitative RT-PCR (RT-qPCR), purity and concentration of the samples were assessed with NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). cDNA was prepared from 500 ng RNA using iScript (Bio-Rad) in a 20 µL reaction, and diluted 1:5 with nuclease-free water. RT-qPCR was performed using 4 µL of the diluted cDNA, along with 625 nmol/L of each primer and POWER SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) in a total reaction volume of 20 µL. RT-qPCR was performed using a System 7300 instrument (Applied Biosystems) and a one-step program: 95°C, 10 minutes; 95°C, 30 seconds, 60°C, 1 minute, for 40 cycles. Relative mRNA expression levels were determined by the $\Delta\Delta C_T$ method using 18S rRNA as a housekeeping gene and normalized to day 0 unless otherwise stated. Data are shown as relative mRNA expression with SD. All primer pairs (Table 1) were checked for specificity using BLAST

Table 1 List of qPCR Primers

Gene name	Primer sequence
<i>18S</i>	F: 5'-CGCCGCTAGAGGTGAAATTCT-3' R: 5'-CGAACCTCCGACTTTTCGTCT-3'
<i>GCM1</i>	F: 5'-CTCGCAGAGGAGGGGCGCAA-3' R: 5'-ATAAAGCGTCCGTGCTGCCTCC-3'
<i>TEAD2</i>	F: 5'-CAGGCCTCTGAGCTTTTCCA-3' R: 5'-GGAGGTGAGTAGATGGGGGA-3'
<i>ASCL2</i>	F: 5'-CACTGCTGGCAAACGGAGAC-3' R: 5'-AAAACCTCCAGATAGTGGGGGC-3'
<i>EGFR</i>	F: 5'-CTAAGATCCCGTCCATCGCC-3' R: 5'-GGAGCCCAGCACTTTGATCT-3'
<i>HLA-G</i>	F: 5'-CAGATACCTGGAGAACGGGA-3' R: 5'-CAGTATGATCTCCGCAGGGT-3'
<i>HIF1A</i>	F: 5'-CGGCGCAACGACAAGAAAA-3' R: 5'-TCCAGAAGTTTCCTCACACGC-3'
<i>TPM1</i>	F: 5'-TCATCATTGAGAGCGACCTG-3' R: 5'-TTAATTGTTCTTCCAGCTGTTCG-3'
<i>IGFBP3</i>	F: 5'-AGAGCACAGATACCCAGAACT-3' R: 5'-GGTGATTCAGTGTCTTCCATT-3'

F, forward; qPCR, real-time quantitative PCR; R, reverse.

analysis and melting curve to ensure amplification of a single product with the appropriate size and melting temperature.

For RNA to be used for gene expression microarray, total RNA was purified using the MirVana RNA Isolation Kit (Ambion), quantified using the Ribogreen reagent (Lifetechn, Inc., Carlsbad, CA) and a Qubit fluorometer (Thermo Fisher Scientific), and quality controlled on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Only those RNA samples with an RNA integrity number > 8.0 were subjected to further gene expression analysis. Total input RNA (200 ng) was amplified and labeled using the TotalPrep kit (Ambion). The labeled product was then hybridized to Illumina HT12 arrays and scanned on a BeadArray Reader (Illumina, Inc., San Diego, CA), according to the manufacturer's instructions.

Analysis of Gene Expression Profiling Data

Probes were filtered with a detection *P* value cutoff of 0.01, and normalized by using the LUMI package in R with the robust spline normalization method. QluCore Omics Explorer software version 3.1 (QluCore AB, Lund, Sweden) was used to explore and analyze microarray gene expression data sets and to generate heat maps. Sorted vCTB (EGFR⁺) and pcEVT (HLA-G⁺) gene expression profiles were compared and specific gene signatures were defined with a variance <0.01, two-group comparison (equivalent to paired *t*-test) *q* < 0.05 and fold change (FC) \geq 1.5. These specific gene signature lists were then subjected to Gene Ontology analysis using Metascape web application (<http://metascape.org>, last accessed November 9, 2016) and signaling pathway analysis using Qiagen's Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA).

Separately, expression profiles of freshly isolated vCTBs, and Scramble and sh-ARNT transduced vCTBs, cultured for 4 days in either 2% or 20% oxygen were also compared. Statistically significant genes were defined by variance < 0.01 , multigroup comparison (equivalent to analysis of variance) $q < 0.05$ and $FC \geq 1.3$. To look at the effect of ARNT knockdown, probes specifically up-regulated in hypoxia in Scramble shRNA-treated cells were compared to probes up-regulated in hypoxia in ARNT shRNA-treated cells. The resulting groups were then compared to the gene signatures of sorted vCTBs and pcEVTs (as explained above). For this comparison, we used probes that had an FC of 1.3 or higher for cultured vCTBs, or an FC of 2.0 or higher for the sorted vCTB and pcEVT samples.

The list of probes up-regulated in hypoxia in Scramble samples but not in shARNT samples was then subjected to Gene Ontology analysis using Metascape and signaling pathway analysis in IPA. Probe lists, with FC data, are provided in [Supplemental Tables S1–S3](#).

All microarray data were submitted to the Gene Expression Omnibus repository (<http://www.ncbi.nlm.nih.gov>) with the accession numbers GSE80996 (for sorted vCTB

and pcEVT) and GSE80995 (for freshly isolated vCTB, and vCTB transduced with Scramble and ARNT-specific shRNA and cultured for four days).

Statistical Analysis

Unless otherwise stated, data presented are means \pm SD of five separate first-trimester placentas. Paired *t*-test was performed, and *P* values < 0.05 were taken to indicate a statistically significant difference between the populations sampled.

Results

Isolation and Characterization of vCTB and pcEVT from First-Trimester Human Placenta

Immunostaining of first-trimester human placentas with EGFR and HLAG antibodies confirmed that vCTBs are characterized by EGFR, whereas proximal column extravillous trophoblasts (pcEVTs) are characterized by surface expression of HLA-G ([Figure 1A](#)). Placenta samples, aged 6

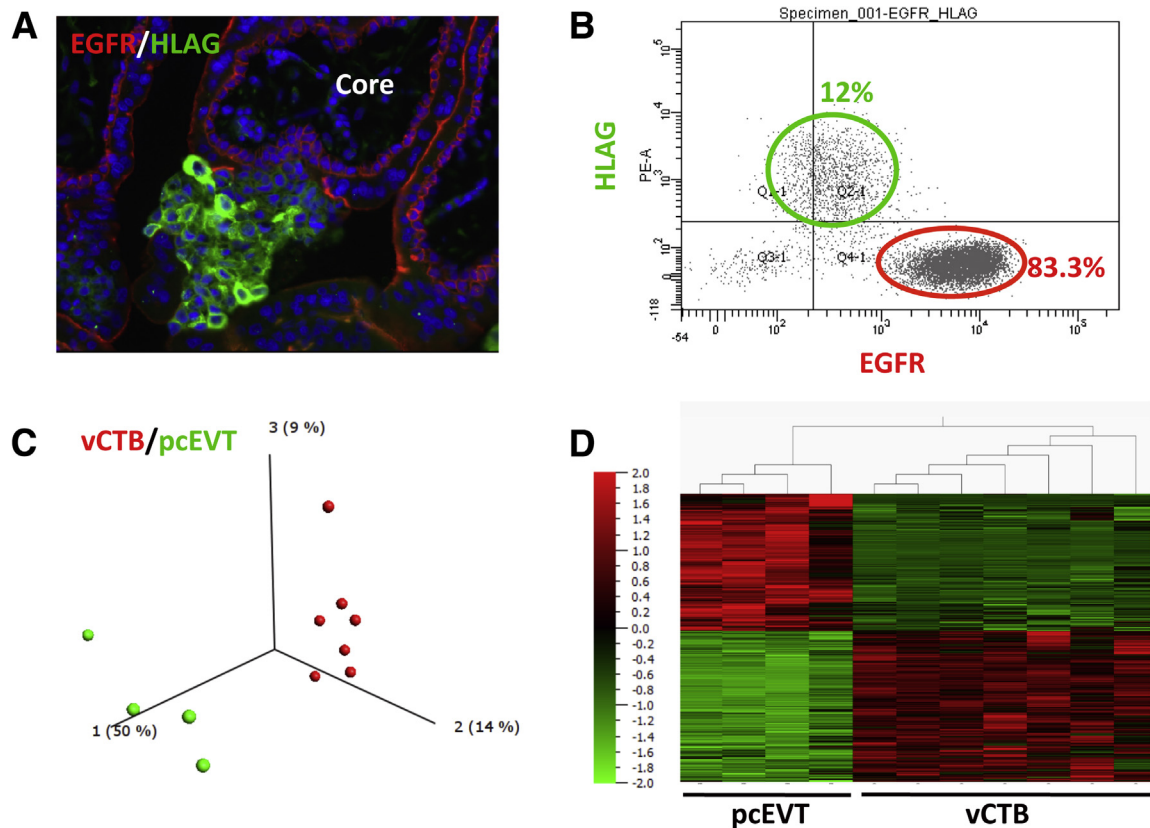


Figure 1 Characterization of the first step of villous cytotrophoblast (vCTB) differentiation into a distinct proximal column extravillous trophoblast (pcEVT) population. **A:** Immunohistochemistry of first-trimester placenta showing epidermal growth factor receptor (EGFR) expression (red) in vCTB and human leukocyte antigen (HLA)-G expression (green) in pcEVT. Core indicates mesenchymal villous core. **B:** Representative flow cytometric analysis of freshly isolated first-trimester trophoblast stained with antibodies to EGFR and HLA-G. The $EGFR^{high}/HLA-G^{-}$ vCTB and $EGFR^{low}/HLA-G^{+}$ pcEVT cell populations were subsequently purified to $>99\%$ by fluorescence activated cell sorting. **C:** After microarray-based gene expression profiling, principle component analysis shows separation, on the basis of the first principal component, of the sorted pcEVT (green) and vCTB (red) subpopulations. **D:** Hierarchical clustering shows distinct clusters of genes up-regulated in pcEVTs and vCTBs ($v < 0.01$, two group comparison $q < 0.05$, fold change ≥ 1.5). Original magnification, $\times 315$ (A), PE, phycoerythrin.

to 9 weeks, were subjected to three steps of trypsin digestion (*Materials and Methods*), and the released single cells were immediately stained with EGFR and HLAG antibodies and subjected to fluorescence activated cell sorting. Analysis by flow cytometry confirmed that in this gestational age range, the trophoblasts are composed of two subpopulations: a larger (80% to 90%) EGFR^{high}/HLA⁺ vCTB population and a smaller (10% to 20%) EGFR^{low}/HLA⁺ pcEVT population (Figure 1B). Seven separate placentas were subjected to the above cell isolation and sorting, followed by RNA isolation directly from the sorted cells; in three cases, RNA from the pcEVT samples was of insufficient quality (RNA integrity number <7.0) to be used for further analysis, so we proceeded to microarray analysis with the remaining seven vCTB samples and four pcEVT preparations. After data preprocessing, principal component analysis showed distinct clustering of these two cell populations (Figure 1C). Statistical analysis (*Materials and Methods*) revealed clusters of genes highly expressed in each cell population, representing distinct gene signatures for vCTBs (1733 gene probes) and pcEVTs (1546 gene probes) (Figure 1D and Supplemental Tables S1 and S2). We analyzed each gene signature for gene ontology (GO) and signaling pathways, using Metascape and IPA, respectively. Supplemental Figure S1 shows the most highly enriched 10 GO and signaling pathways in vCTB and pcEVT, respectively. For vCTB, the most highly enriched GO categories and signaling pathways were related to cellular metabolism, protein ubiquitination, and the cell cycle. For pcEVT, the most highly enriched GO categories and signaling pathways included those involved in hypoxia, cytoskeletal remodeling, cell-cell and cell-matrix junctions, and interferon γ signaling.

Transcription Factors Up-Regulated in pcEVT

We next evaluated the transcription factors (TFs) significantly up-regulated ($v < 0.01$, q value < 0.05 , $FC \geq 1.5$) in pcEVTs compared to vCTBs (Table 2). The most highly up-regulated TF was ASCL2 (12.4-fold), followed by TEAD2 (11.5-fold). Other transcription factors that were significantly up-regulated in pcEVTs included TCF4 and STAT4 (4.1-fold), HEY1 (3.8-fold), and HIF1A (2.6- and 2.4-fold for two different probes); these TFs have been

Table 2 List of Most Highly Up-Regulated Transcription Factors in pcEVT

Gene name	Fold change in pcEVT/vCTB
ASCL2	12.4
TEAD2	11.5
TCF4	4.1
STAT4	4.1
HEY1	3.8
HIF1A	2.6
HIF1A	2.4
GCM1	1.8

pcEVT, proximal column extravillous trophoblast; vCTB, villous cytotrophoblast.

previously associated with EVT.^{5,10} GCM1, a TF usually associated with differentiation of STB, was also significantly up-regulated in pcEVT (1.8-fold). We measured expression of ASCL2, TEAD2, HIF1A, and GCM1 in three independent samples each of vCTB and pcEVT by RT-qPCR (Figure 2A), and were able to confirm up-regulation of these TFs in pcEVT. To further validate the up-regulation and localization of these TFs in pcEVT, we performed *in situ* hybridization on first-trimester placental

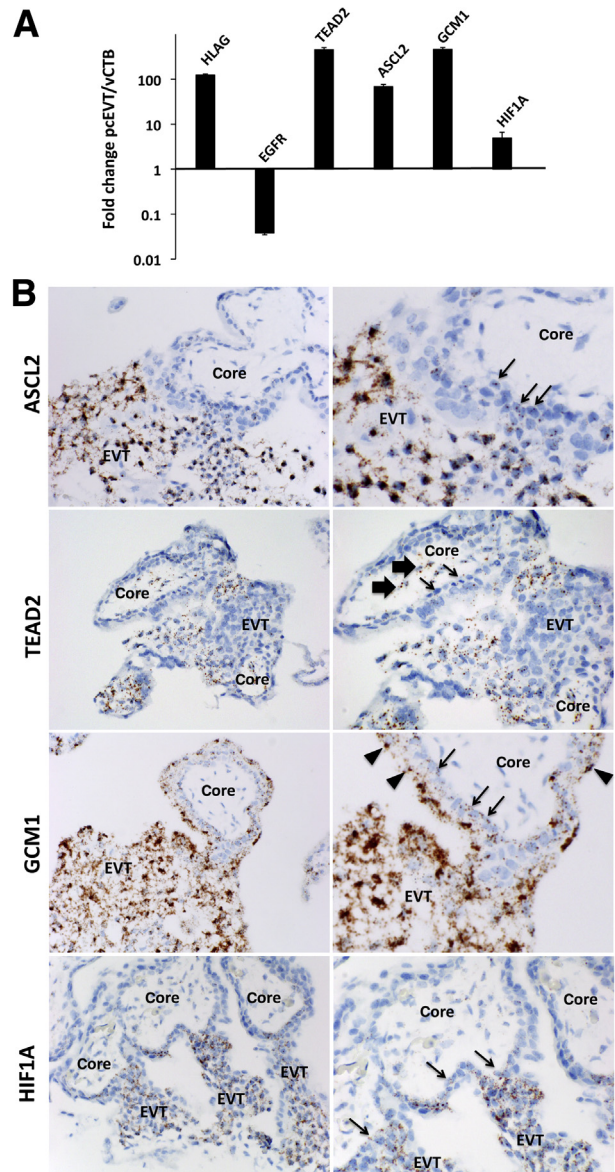


Figure 2 Validation of gene expression profiling. **A:** Quantitative RT-PCR analysis of the indicated genes in RNA extracted from sorted proximal column extravillous trophoblast (pcEVT) and villous cytotrophoblast (vCTB). **B:** *In situ* hybridization for the indicated genes in first-trimester placental tissues. Each dot corresponds to a single mRNA; larger dots may be multiple mRNAs closely localized. Villous core and EVT are labeled; vCTB are highlighted by thin arrows, villous core cells by thick arrows, and syncytiotrophoblast by arrowheads. Original magnification: ×200 (B, left column); ×300 (B, right column).

tissues using gene-specific probes (Figure 2B). All three gene products were abundantly found in pcEVTs, compared to vCTBs. Rare ASCL2 and HIF1A transcripts were also noted in vCTB near cell columns, whereas TEAD2 transcripts were also noted in vCTB and villous stromal cells (Figure 2B). GCM1 transcripts were also present in both vCTBs and STBs, although expression in vCTBs appeared significantly lower than in either STBs or pcEVTs (Figure 2B). We confirmed expression of HIF1A at the protein level in scattered EVT (Supplemental Figure S2); however, we were unable to obtain specific antibodies to the other three transcription factors to use in IHC.

Culture in Low Oxygen Tension Increases Differentiation of Isolated CTBs into pcEVTs

Interestingly, hypoxia was noted as the top third group of GO terms most enriched in pcEVTs (Supplemental Figure S1). We therefore evaluated the effect of hypoxia on this initial transition of vCTBs into HLA-G⁺ pcEVTs. For all these experiments, to minimize contamination with pcEVTs, we used 8- to 10-week gestation placentas and only proceeded when the preparations contained >88% EGFR^{high}/HLA-G⁻ vCTBs at the time of isolation.

We initially tested plating of these cells on fibronectin, collagen IV, and laminin, and found that isolated CTBs

adhered to all but laminin-based matrices (Supplemental Figure S3). We tested differentiation of CTBs in 20%, 5%, or 2% oxygen on both fibronectin- and collagen IV-coated dishes, and found that lowering oxygen tension increased the proportion of HLA-G⁺ EVT on both of these matrices (Supplemental Figure S3). We chose fibronectin for the remainder of the experiments, because this matrix is most commonly used for studying EVT differentiation.^{5,24} We also chose to go forward with 2% oxygen in the remaining experiments as this gave the maximal response of EVT differentiation, on the basis of proportion of HLA-G⁺ cells.

We found that culture in 2% oxygen reproducibly resulted in a 1.5-fold increase in proportion of surface HLA-G⁺ cells (Figure 3A). In fact, HLA-G protein was induced as early as day 1 after plating, along with stabilization of HIF1A protein (Supplemental Figure S4, A and B); no Ki-67⁺ cells were noted in either condition, even early after plating (Supplemental Figure S4C). Induction of HLA-G in 2% oxygen was accompanied by increased levels of MMP2 secretion, another marker of pcEVT²⁵ (Figure 3B), and decreased hCG secretion, a marker of STB (Figure 3C). We also noted decreased formation of syncytiotrophoblasts in 2% oxygen (Figure 3D). Finally, we assessed the effect of low oxygen tension on CTB differentiation by RT-qPCR for pcEVT-specific markers, including HLAG, ASCL2, and TEAD2, all of which were significantly higher at 2%

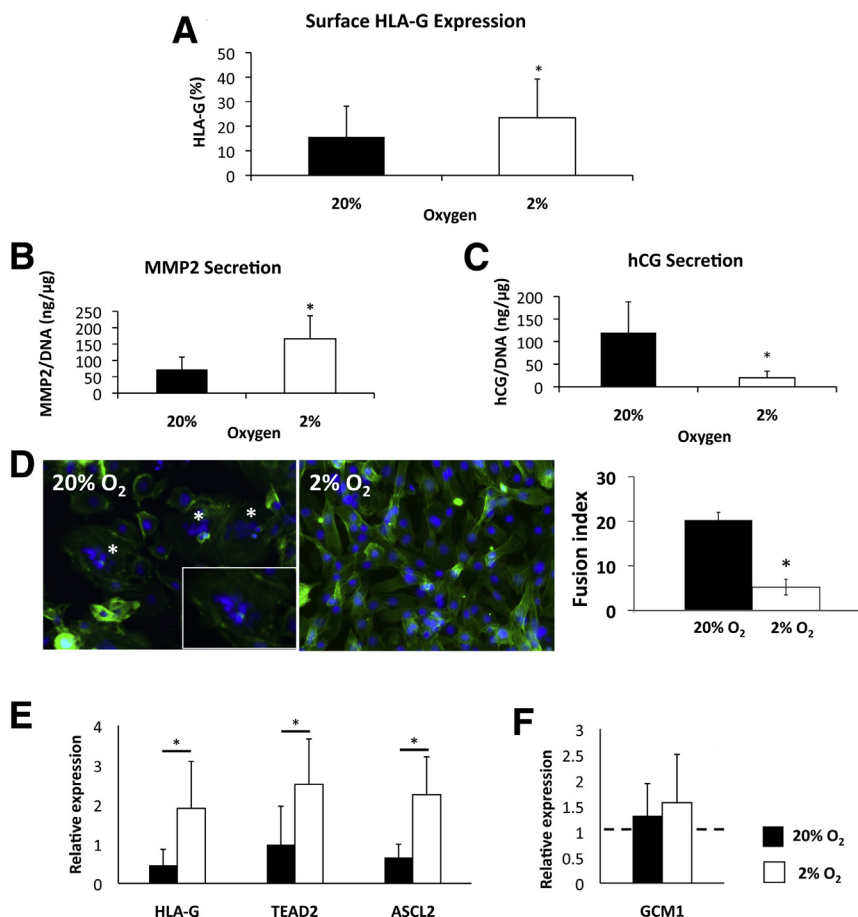


Figure 3 Effect of hypoxia on differentiation of villous cytotrophoblast (vCTB) into proximal column extravillous trophoblast (pcEVT). **A:** Proportion of surface human leukocyte antigen (HLA)-G⁺ cells after 4 days of culture in 20% or 2% O₂. **B:** Matrix metalloproteinase 2 (MMP2) secretion of vCTB cultured in 20% or 2% O₂, normalized to DNA content. **C:** Human chorionic gonadotropin (hCG) secretion of first-trimester vCTB cultured in 20% or 2% O₂, normalized to DNA content. **D:** Cultured vCTB after 4 days of culture in either 20% or 2% oxygen, stained with phalloidin (green) and DAPI (blue). Multinucleated syncytiotrophoblast are noted by asterisks; one syncytiotrophoblast is shown at higher magnification in the inset. Fusion index is shown graphically. **E** and **F:** Quantitative RT-PCR analysis of pcEVT-associated genes (**E**) and GCM1 (**F**) in vCTB cultured in 20% or 2% O₂. Values adjusted to freshly isolated (day 0) cells and normalized to 18S ribosomal RNA. **Dotted line** in **F** represents value in freshly isolated (day 0) cells. *n* = 3 (**D**); *n* = 5 (**E** and **F**). **P* < 0.05. Original magnifications: ×100 (**D**, main images); ×200 (**D**, inset).

oxygen (Figure 3E). Interesting, HIF1A also increased at the RNA level under 2% oxygen (Supplemental Figure S4D). Levels of GCM1 mRNA, confirmed to be highly expressed by *in situ* hybridization in both pcEVTs and STBs, compared to vCTBs (Figure 2B), were not significantly different in 20% versus 2% oxygen (Figure 3F).

Low Oxygen Tension Promotes pcEVT and Inhibits STB Differentiation in an HIF-Dependent Manner

To determine whether an intact and functioning HIF complex is required for differentiation of primary vCTBs toward pcEVTs, we transduced isolated CTBs with lentivirus expressing either ARNT-specific or a negative control (scramble) shRNA. Efficiency of ARNT knockdown was assessed by Western blot (Figure 4A). ARNT was expressed in isolated CTBs (day 0) and in scramble-shRNA-transduced cells, cultured for 4 days in both 20% and 2% oxygen, but was significantly down-regulated in shARNT-transduced cells. HIF1 α , on the other hand, was not present in freshly isolated vCTBs, but was up-regulated

on culture in 2% oxygen, and more significantly so, in shARNT-transduced cells (Figure 4A). No apoptosis was noted in cells cultured in either condition, or after ARNT knockdown (Supplemental Figure S5). Down-regulation of transcripts for ARNT and various HIF target genes, including *SLC2A1*, *GAPDH*, and *ANKRD37*, in shARNT-transduced cells was confirmed by microarray analysis (Figure 4B). Microarray analysis also showed induction of EVT-associated markers (*HLA-G*, *TEAD2*, and *ASCL2*) and down-regulation of STB markers (*CGA* and *CGB*) in 2% oxygen; both of these effects were attenuated in shARNT-transduced cells. To further assess the effect of ARNT knockdown on vCTB differentiation, we evaluated both the expression and functional secretion of specific markers. We found that, with ARNT knockdown, the hypoxia-induced increases of both the percentage of HLA-G⁺ cells and MMP2 secretion were attenuated (Figure 4, C and D). In addition, hypoxia-induced reduction of hCG secretion (Figure 4E) and syncytialization (Figure 4F) were also partially abrogated by knockdown of ARNT. Finally, shARNT-transduced vCTBs showed reduced expression of

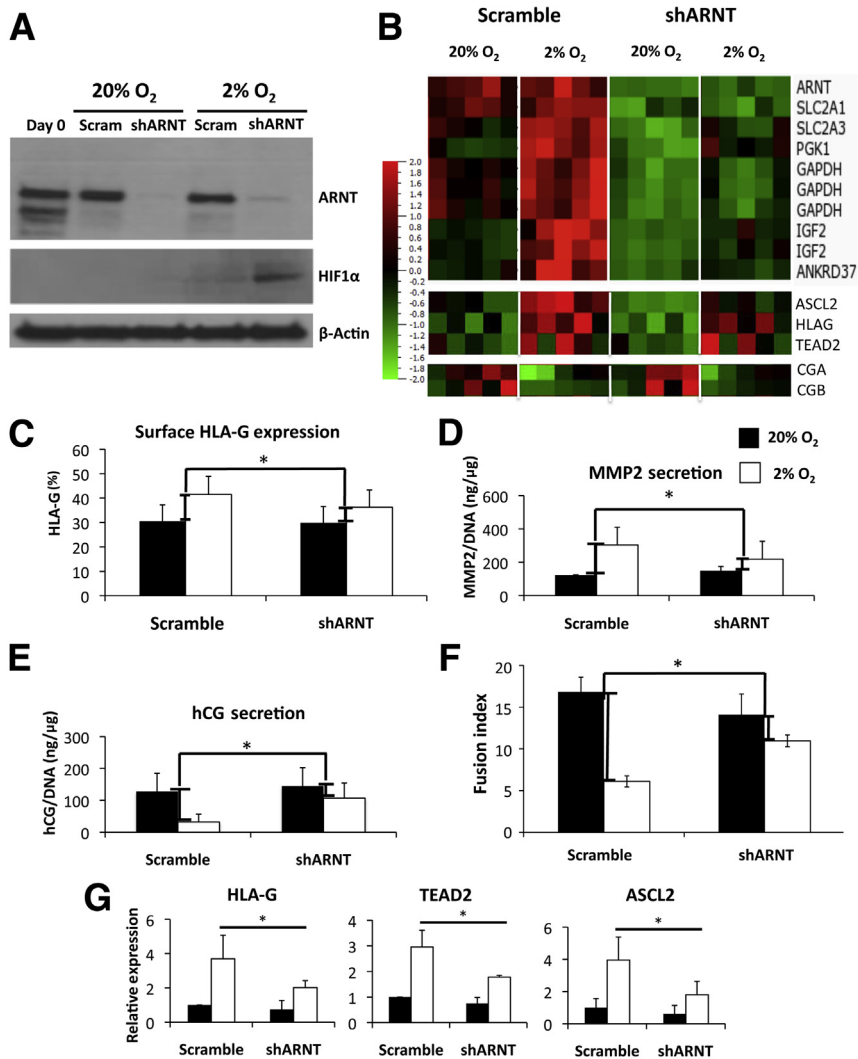


Figure 4 Hypoxia induces proximal column extravillous trophoblast (pcEVT) differentiation in a hypoxia-inducible factor (HIF) complex-dependent manner. **A:** Aryl hydrocarbon receptor nuclear translocator (ARNT) and HIF1 α Western blots of lysates from freshly isolated cytotrophoblasts (CTBs) (day 0) and CTBs transduced with lentivirus expressing either scramble (Scram) shRNA or ARNT-specific shRNA, then cultured in 20% or 2% O₂ for 4 days. Actin was used as loading control. **B:** Gene expression of ARNT and HIF complex targets, EVT-associated genes (*HLA-G*, *TEAD2*, and *ASCL2*), and syncytiotrophoblast-associated genes (*CGA* and *CGB*) in scramble or shARNT transduced cultured CTBs. **C:** Surface expression of HLA-G, analyzed by flow cytometry, of scramble- and shARNT-transduced cultured CTBs. **D:** Matrix metalloproteinase 2 (MMP2) secretion of scramble and shARNT-transduced cultured CTBs, normalized to DNA content. **E:** Human chorionic gonadotropin (hCG) secretion of scramble and shARNT-transduced cultured CTBs, normalized to DNA content. **F:** Fusion index of scramble and shARNT-transduced cultured CTBs, under 20% and 2% oxygen, calculated after staining with phalloidin and DAPI. **G:** Quantitative RT-PCR analysis of EVT-associated genes in RNA extracted from scramble and shARNT-transduced cultured CTBs. Values adjusted to freshly isolated cells (day 0) and normalized to 18S ribosomal RNA. *n* = 3 (F); *n* = 5 (G). **P* < 0.05.

pcEVT-associated transcripts, including *HLAG*, *TEAD2*, and *ASCL2* (Figure 4G).

RNA Profiling Reveals Distinct Patterns of HIF-Dependent and HIF-Independent Gene Expression under Hypoxia

We subjected the CTBs, transduced with scramble or ARNT shRNA-expressing lentivirus, and cultured in 20% or 2% oxygen, to microarray-based gene expression analysis. Within each group, we focused on genes that were significantly up-regulated only after culture in 2% oxygen. We determined that 747 genes were significantly up-regulated in 2% oxygen in the presence of ARNT, whereas only 223 genes were significantly up-regulated in 2% oxygen in the ARNT knockdown cells (Figure 5). Of the 747 genes that were up-regulated in response to hypoxia in the presence of

ARNT, 588 (79%) were no longer up-regulated with ARNT knockdown, and were considered to be HIF dependent (Figure 5); only 159 (21%) remained up-regulated in the ARNT knockdown cultures and were considered to be HIF independent (Figure 5).

Next, we compared these 588 HIF-dependent and 159 HIF-independent hypoxia-inducible genes (Supplemental Table S3) to the gene expression profiles of sorted vCTBs ($EGFR^{\text{high}}/HLAG^{-}$) versus sorted pcEVTs ($EGFR^{\text{low}}/HLAG^{+}$). We found that both the HIF-dependent and HIF-independent groups shared the most genes with sorted pcEVT (21% and 9% of pcEVT-associated genes, versus 1% and 0.5% of vCTB-associated genes, respectively). We then characterized the potential role(s) for the HIF-dependent subset of hypoxia-inducible genes by subjecting them to functional enrichment analysis (Metascape), and to pathway analysis using IPA. The top 10 most significantly

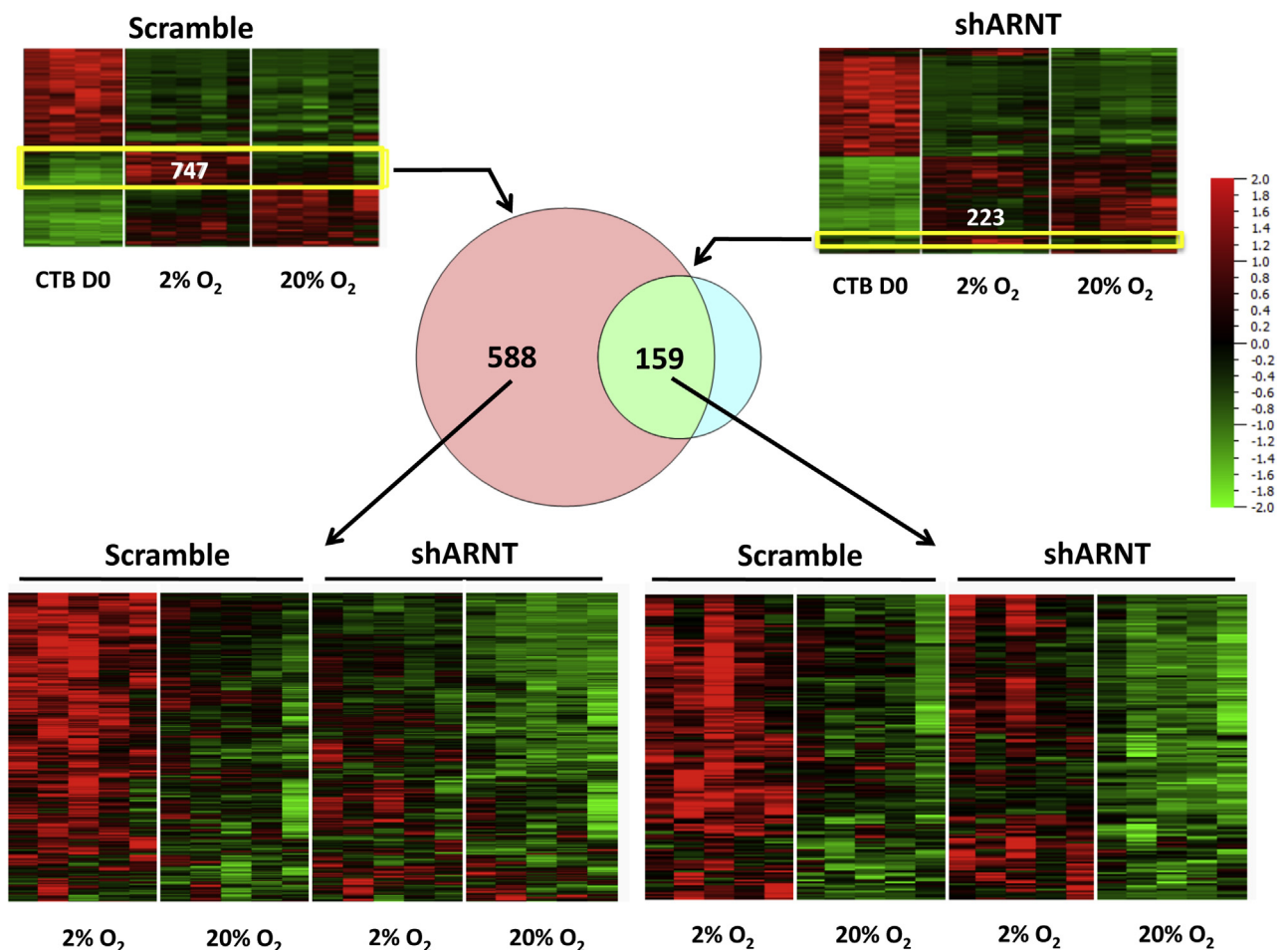


Figure 5 Analysis of hypoxia-inducible factor (HIF)—dependent hypoxia-inducible genes in cultured cytotrophoblasts (CTBs). Gene expression patterns in Scramble or shARNT-transduced cultured villous CTBs (vCTBs), compared to freshly isolated vCTBs. We performed statistical analysis ($\nu < 0.01$, multigroup comparison $q < 0.05$) on Scramble and shARNT-transduced cells, separately. Genes up-regulated in 2% O_2 in each group are bracketed in yellow: 747 genes in Scramble and 223 genes in shARNT-transduced cells. The overlap between these two gene groups is shown in the Venn diagram. One hundred fifty-nine genes remain up-regulated in the absence of aryl hydrocarbon receptor nuclear translocator (ARNT) and are labeled as HIF independent. Five hundred eighty-eight genes are lost (no longer up-regulated) in the absence of ARNT and labeled as HIF dependent. Heat maps below the Venn diagram show gene expression patterns of the 588 HIF-dependent (left two panels) and the 159 HIF-independent (right two panels) genes in both Scramble and shARNT-transduced samples. D0, day zero.

enriched GO groups and top 10 pathways are listed in [Supplemental Figure S6](#). Reassuringly, the most significantly enriched GO term was hypoxia. Other functions include cell adhesion and cytoskeletal remodeling, with multiple integrin and integrin-associated pathways. Interestingly, one of the pathways identified by IPA analysis was ILK signaling, a pathway similarly represented in the same analysis of pcEVT signature genes ([Supplemental Figure S1](#)). *ILK* is a known HIF complex–regulated gene with a strong role in promotion of epithelial-mesenchymal transition (EMT) in cancer cells.²⁶ We first evaluated the

expression of ILK itself. ILK protein was expressed specifically in EVT in the first-trimester placenta ([Figure 6A](#)). Although RNA and protein levels were not significantly changed after culture ([Figure 6B](#) and data not shown), protein levels were significantly reduced after ARNT knockdown ([Figure 6B](#)). We next inspected the members of the ILK pathway gene list in IPA, which were induced by hypoxia in an HIF-dependent manner. Among these genes, we identified genes associated with both remodeling of the actin cytoskeleton (cofilin/*CFL1*, paxillin/*PXN*, and *RHOA*) and with ILK-mediated EMT (β -parvin/*PARVB*)

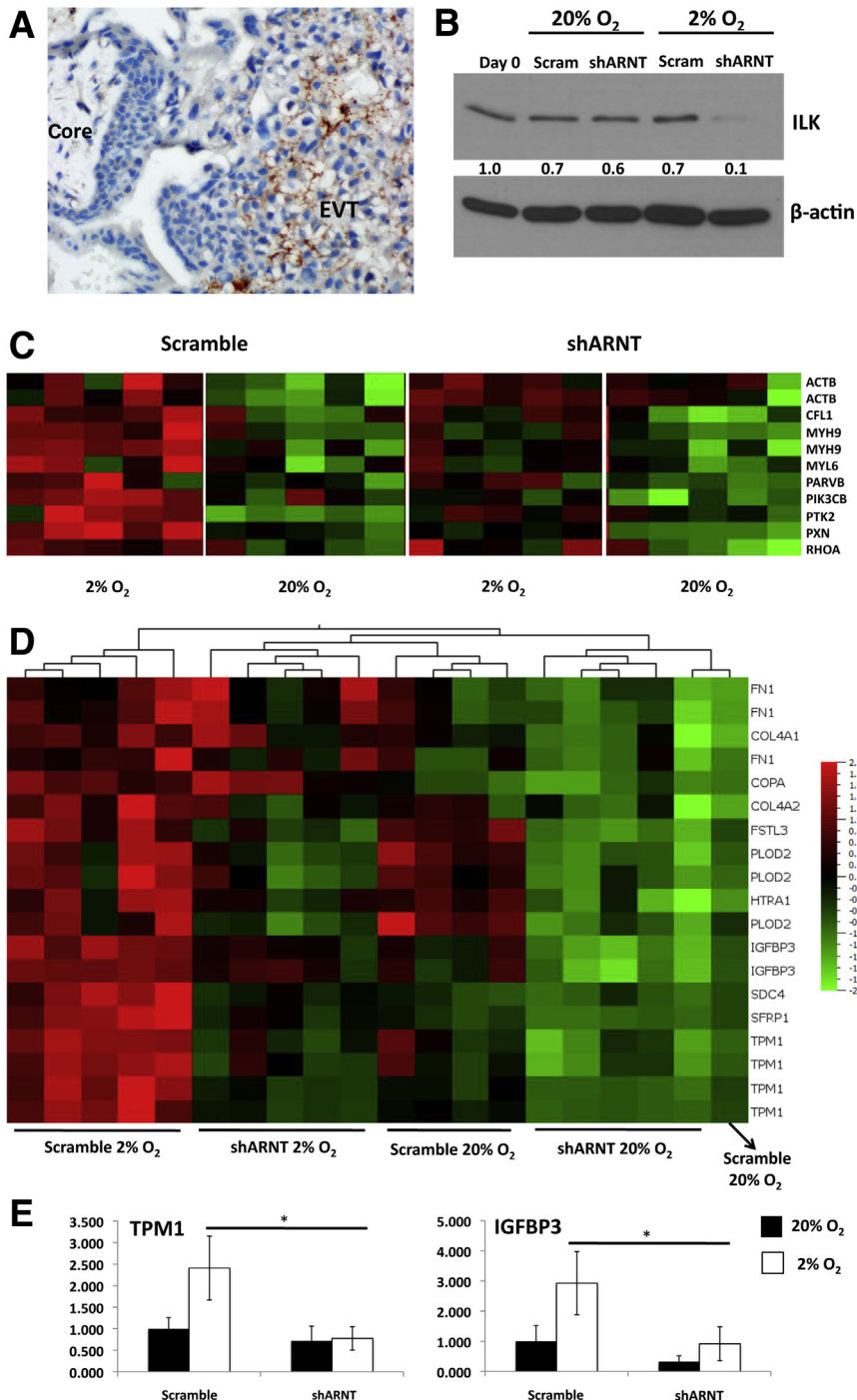


Figure 6 Integrin-linked kinase (ILK) pathway in cultured cytotrophoblasts (CTBs). **A:** ILK immunostaining of first-trimester placental tissue. Villous core and extravillous trophoblast (EVT) are labeled. **B:** ILK Western blot of lysates from freshly isolated CTBs (day 0) and CTBs transduced with lentivirus expressing either scramble (Scram) shRNA or *ARNT*-specific shRNA, then cultured in 20% or 2% O₂ for 4 days. Actin served as loading control. Numbers under the ILK blot indicate relative protein expression, by densitometry, normalized to actin and then to day 0. The expression of ILK is drastically reduced in shARNT-transduced samples, but only when cultured in 2% O₂. **C:** ILK pathway–associated genes differentially expressed in cultured villous CTBs under indicated conditions. Note that the listed genes are most highly expressed in scramble-shRNA–transduced cells cultured in 2% O₂, and are down-regulated both in 20% O₂ and after aryl hydrocarbon receptor nuclear translocator (ARNT) knockdown. **D:** Hierarchical clustering, using differentially expressed epithelial-mesenchymal transition (EMT)–associated genes, separates scramble-shRNA–transduced CTB cultured in 2% O₂ from the other three groups of cells (both groups of CTBs cultured in 20% O₂, and *ARNT*-shRNA–transduced CTBs cultured in 2% O₂) ($v < 0.01$, multigroup comparison $q < 0.05$). **E:** Validation of changes in expression of two EMT-associated genes, *TPM1* and *IGFBP3*. Values adjusted to scramble-shRNA–transduced CTB cultured in 20% O₂, and normalized to 18S ribosomal RNA. $n = 3$ (E). * $P < 0.05$. Original magnification, $\times 200$ (A).

(Figure 6C). Other EMT-associated genes also showed a distinct hypoxia-induced HIF-dependent pattern of expression (Figure 6D), which we confirmed by real-time quantitative PCR (Figure 6E). These results suggest that the HIF complex may promote differentiation of pcEVT through maintenance of ILK and ILK-induced EMT.

Discussion

This study evaluated the first step in differentiation of villous cytotrophoblast (vCTB) into HLA-G⁺ proximal column extravillous trophoblast (pcEVT). A few previous studies used similar microarray-based gene expression profiling to evaluate EVT-specific gene signatures.^{27–29} However, two of these studies used cultured cells or explants, thereby introducing potential culture-induced artifact and/or evaluating potentially later stages of EVT differentiation.^{27,28} A third study evaluated the EVT gene signature, but with a focus on their interaction with the maternal immune system.²⁹ By sorting EGFR^{high}/HLA-G⁻ and EGFR^{low}/HLA-G⁺ cells directly from digested villous tissue of early gestation placentas, we aimed to evaluate the first step toward differentiation of vCTB into the fully invasive EVT, focusing on the changes in regulatory transcription factors. Differentiation into EVT takes place in trophoblast cell columns, in anchoring villi of the early placenta,³ and, as we have previously found, is characterized by loss of p63, a transcription factor expressed exclusively in vCTB, and lost quickly when these cells are removed from the underlying villous mesenchyme, either *in vivo*³⁰ or *in vitro*.³¹ We identified multiple transcription factors up-regulated quickly during the transition into pcEVT, including two previously described factors, ASCL2 and GCM1. ASCL2 was originally characterized in the mouse, shown to be required for proper development of the placenta and particularly of the spongiotrophoblast,^{32,33} and later found to be expressed in EVT in the human placenta and maintained in CTB cultured in 2% oxygen.^{34,35} Herein, we confirmed its expression in EVT by *in situ* hybridization and its up-regulation on EVT differentiation of primary vCTB *in vitro*.

After ASCL2, the most highly expressed pcEVT-specific transcription factor was TEAD2. This protein is a member of the TEA domain family of transcription factors; the best known member of this family in the context of trophoblast differentiation in mice is Tead4, whose association with Yap1 and induction of Cdx2 is one of the earliest events in establishment of trophoblast in mice.^{36,37} In fact, we noted enrichment of TEAD4 mRNA in the vCTB fraction, but also discovered TEAD2 as a novel pcEVT-associated transcription factor. Although a specific function for TEAD2 has not yet been identified, along with other TEA domain proteins, it has been shown to play a role in both cell growth and promotion of EMT³⁸; the latter pathway has been implicated in differentiation of EVT in the human placenta (see below).

Other transcription factors identified in our sorted pcEVT population included TCF4, a Wnt-inducible factor, previously identified as a marker of human EVT,^{5,39} and GCM1, a transcription factor originally described as a labyrinth (villous) trophoblast-specific marker, required for branching morphogenesis, in the mouse placenta.^{40,41} Although more is known about its expression in villous trophoblast, where it regulates expression of *SYNCYTIN* genes,^{42,43} GCM1 has also previously been localized to EVT.^{44,45} We also noted high levels of GCM1 RNA in both STB and EVT, and comparatively lower levels in vCTB; that GCM1 induction is involved in differentiation into both lineages was further confirmed on *in vitro* differentiation of vCTB, where GCM1 levels were equally increased in 20% and 2% oxygen.

Finally, HIF1A was another transcription factor enriched in the pcEVT fraction. This finding, along with the known role of the HIF complex in differentiation of the rodent EVT lineage (spongiotrophoblast and trophoblast giant cells), led us to further evaluate the role of oxygen tension during induction of the EVT lineage. We noted induction of HIF1A at the RNA level, in pcEVT *in situ*, in freshly isolated pcEVT, and following *in vitro* culture of vCTB in low oxygen; a similar induction of HIF1A RNA takes place during differentiation of mouse trophoblast stem cells into spongiotrophoblast/trophoblast giant cell lineages *in vitro*.¹⁶ That the protein was not abundantly expressed in pcEVT *in situ* is likely because of rapid degradation of this protein after exposure to room air; after vCTB culture in low oxygen, the protein was stabilized and easily detectable by Western blot.

Our findings on the effect of low oxygen on CTB may appear to diverge from the literature; however, in fact, there is much agreement between our results and other publications. That low oxygen (2%) increases the proportion of HLA-G⁺ cells in the cell column of first-trimester explants can be clearly seen in the study by Genbacev et al⁷; however, these cells were referred to as cytotrophoblast and the data interpreted as no change in level of HLA-G expression in the individual cells, on the basis of immunostaining.⁷ Subsequent studies of similar gestation explants by Caniggia et al¹⁰ showed expansion of integrin- α_5 /ITGA5⁺ cell column cells in low oxygen (3%), in contrast to explants maintained in 20% oxygen. Most similar to our experimental model of isolated primary cytotrophoblast, however, is the study by Robins et al,¹² showing that although isolated CTB fused and formed a syncytium at 20% oxygen, under low oxygen conditions (1%), they remained mononuclear and instead expressed HLA-G abundantly. Similar to the latter study, our results point to high oxygen promoting villous trophoblast differentiation, and low oxygen directing differentiation along the extravillous pathway. Our results further expand on the Robins et al¹² study, showing that an intact HIF complex is required, both for inhibition of villous differentiation and promotion of the EVT lineage.

Although this exciting new finding matches with rodent data on HIF dependence of the EVT lineage specification, it does not resolve the role of oxygen tension in trophoblast

invasion. In rodents, not only has HIF been shown to be required for specification of this lineage,^{14–16,18} low oxygen and stabilization of HIF promote the invasive properties of these cells, leading to greater remodeling of uterine spiral arteries.^{17,18} Such *in vivo* promotion of trophoblast invasion by low oxygen has also been shown in rhesus monkeys, where aortic strictures increased the depth of invasion by interstitial EVT, although no change in endovascular invasion was observed.⁴⁶ *In vitro* experiments, however, using both explants and isolated CTB from first-trimester human placental tissues, point to inhibition of trophoblast invasion under low oxygen, using integrin α_1 /ITGA1 as a surface marker of differentiated, invasive EVT and Matrigel invasion as a functional assay.^{7–11} It is possible that early during implantation, low oxygen initiates differentiation of the EVT lineage, and even promotes proliferation of cells with this proximal column phenotype, but that a gradient toward higher oxygen levels promotes maturation of these cells, into fully differentiated, invasive EVT. Future experiments will need to test this hypothesis, using either an explant system with various oxygen gradients or isolated cells, which can be differentiated into early EVT, then replated to complete maturation and test invasive ability at various oxygen tensions.

Similarly, our current study does not fully resolve the role of oxygen tension on trophoblast proliferation. In fact, previous studies have also noted increased proliferation of primary trophoblast under low oxygen, particularly in the context of first-trimester explants,^{7,10} but also in isolated CTBs.^{8,47} As in our previous study,³¹ and similar to results from other groups,²⁹ we did not observe any proliferation of isolated CTBs, even at early time points after plating. However, it is possible that *in vivo*, in addition to promotion of pcEVT differentiation, low oxygen tension also promotes proliferation of vCTB and/or pcEVT, before differentiation into more mature EVTs.

Our results also point to the initial differentiation of vCTB into pcEVT having features in common with EMT. Although previous studies have focused on changes in expression of specific genes during the CTB-to-EVT transition,^{48,49} our study evaluated global gene expression changes in these two populations, then applied gene ontology and pathway analysis to identify changes in expression patterns of genes with similar functions. This led to the identification of EMT, along with many associated pathways involving cytoskeletal remodeling and cell adhesion. One of these was a pathway regulated by ILK, which was also found to be induced during pcEVT differentiation under low oxygen, in an HIF-dependent manner. ILK was originally discovered as a component of the focal adhesion kinase and has since been found to play numerous roles during embryonic development and tissue homeostasis.^{50,51} Although expression of ILK itself was not induced in the cells we used under low oxygen, its maintenance under these culture conditions appeared to be HIF dependent. In addition, multiple ILK pathway-associated genes,

including cofilin (*CFL1*) and β -parvin (*PARVB*), were induced by low oxygen, also in an HIF-dependent manner.

The association of the ILK pathway with EVT differentiation is interesting in the context of the specific type of EMT occurring in the human placenta, which has been described as partial EMT. This is because EVT differentiation does not appear to be accompanied by complete loss of epithelial markers (eg, cytokeratin 7), nor the gain of hallmark mesenchymal markers (eg, vimentin).^{48,49,52} In fact, differentiation into endovascular EVT is even accompanied by reactivation of E-cadherin expression.⁵³ In our study, we found the initial step in EVT differentiation to be associated with a high expression of TEAD2, a transcription factor shown to promote EMT.³⁸ Even more interesting, however, was the involvement of hypoxia/HIF-regulated ILK pathway, which has also been shown to be a hallmark of cancer stem cells. Cancer stem cells are thought to be a subpopulation of cells within a tumor, maintained in a hypoxic microenvironment, and characterized by an EMT-like phenotype.⁵⁴ ILK plays a role in maintenance of these cancer stem cells by linking integrins to the underlying actin cytoskeleton via β -parvin and cofilin,⁵⁵ genes within the ILK pathway that were also up-regulated in pcEVT. Given that these cells are precursors to the highly invasive interstitial and endovascular EVT, we propose that proximal column EVTs are akin to cancer stem cells, and could potentially be studied as a model in this context.

In summary, we have presented a detailed molecular profile of the first stage of EVT differentiation in the human placenta and determined that low oxygen promotes this lineage in an HIF-dependent manner. In future studies, we plan to dissect out the mechanisms by which hypoxia-induced EMT induces EVT differentiation, specifically focusing on the role of the newly identified transcription factors, such as TEAD2, in this context.

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M.M.P. is the guarantor of this work, had full access to all data, and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2016.11.018>.

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