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Differentiation of first trimester cytotrophoblast to extravillous trophoblast involves an epithelial–mesenchymal transition

Sonia DaSilva-Arnold [a, 1], Joanna L. James [b, 1], Abdulla Al-Khan [a], Stacy Zamudio [a], Nicholas P. Illsley [a, *]

[a] Department of Obstetrics and Gynecology, Center for Abnormal Placentation, Hackensack University Medical Center, Hackensack, NJ, USA
[b] Department of Obstetrics and Gynecology, University of Auckland, Auckland, New Zealand

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ABSTRACT

The transformation of cytotrophoblast (CTB) to extravillous trophoblast (EVT) is an essential process for placental implantation. EVT generated at the tips of the anchoring villi migrate away from the placenta and invade the endometrium and maternal spiral arteries, where they modulate maternal immune responses and remodel the arteries into high-volume conduits to facilitate uteroplacental blood flow. The process of EVT differentiation has several factors in common with the epithelial-to-mesenchymal transition (EMT) observed in embryonic development, wound healing and cancer metastasis. We hypothesized that the generation of invasive EVT from CTB was a form of EMT. We isolated paired CTB and EVT from first trimester placentae, and compared their gene expression using a PCR array comprising probes for genes involved in EMT. Out of 84 genes, 24 were down-regulated in EVT compared to CTB, including epithelial markers such as E-cadherin (−11-fold) and occludin (−75-fold). Another 30 genes were up-regulated in EVT compared to CTB including mesenchymal markers such as vimentin (235-fold) and fibronectin (107-fold) as well as the matrix metalloproteinases, MMP2 and MMP9 (357-fold, 129-fold). These alterations also included major increases in the ZEB2 (zinc finger E-box binding homeobox 2, 198-fold) and TCF4 (transcription factor 4, 18-fold) transcription factors, suggesting possible stimulatory mechanisms. There was substantial up-regulation of the genes encoding TGFβ1 and TGFβ2 (48-fold, 115-fold), which may contribute to the maintenance of the mesenchymal-like phenotype. We conclude that transformation of CTB to EVT is consistent with an EMT, although the differences with other types of EMT suggest this may be a unique form.

1. Introduction

Cytotrophoblasts (CTB) are the primary placental cells derived from the trophectoderm of the blastocyst. Formation of the placental villous structure requires the differentiation of the CTB into the multinuclear syncytium that forms the epithelial covering of the villous tree, the primary barrier between maternal and fetal circulations and the site of nutrient and gas exchange. At the tips of the anchoring villi, where the placenta attaches to the endometrium, CTB undergo a different transformation into a non-proliferative cell type that migrates away from the placenta, invades into the endometrium and colonizes the maternal spiral arteries. These invasive cells, the EVT, play a number of crucial roles in placental development. These include modulation of maternal immune response and conversion of maternal spiral arteries into the dilated non-reactive vessels essential for high-volume maternal blood flow to the placental intervillous space.

The differentiation of CTB into EVT has been a subject of great interest following the discovery that shallow placental implantation and defective spiral artery conversion due to impaired invasion were implicated in the etiology of major placental pathologies, most notably preeclampsia and intrauterine growth restriction [1,2]. Investigators have mapped some of the molecular changes that take place as the epithelial CTB leave the terminal ends of the anchoring trophoblast columns and move individually into the endometrium. One of these changes is the increased secretion of

* Corresponding author. Department of Obstetrics and Gynecology, Hackensack University Medical Center, 40 Prospect Ave, Hackensack, NJ 07601, USA.
E-mail address: nillsley@hackensackumc.org (N.P. Illsley).
[1] These authors should be regarded as joint First Authors.

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the metalloproteinases that break down the extracellular matrix (ECM), enabling the EVT to migrate through the endometrium [3]. Another is the switch in the repertoire of ECM factors such as the integrins to types that are capable of interacting with the ECM of the endometrium, enabling invasion [4,5]. As a result, several groups have suggested that the transformation of the epithelial-like CTB to the invasive, mensenchymal-like EVT is akin to the epithelial–mesenchymal transition (EMT) that occurs in embryonic development, wound healing and cancer metastasis [6]. Data showing integrin and metalloproteinase changes [5,7,8], changes in cadherin expression [9], alterations in Wnt signaling [10] and trophoblast expression of mesenchymal markers [11] or EMT biomarkers [12] support this hypothesis. These results are summarized in several reviews [13–16] where the CTB-EVT conversion process has been described as “pseudo EMT” or “metastable EMT”.

The EMT label includes a broad range of potential changes in cellular phenotype. There is general agreement that cells transition from an epithelial phenotype adherent to a basal lamina and coupled through tight junctions to a separated, invasive mesenchymal phenotype. Within this general context however there are substantial differences between the EMT process, even between metastatic cell types, no matter those involved in embryonic gastrulation or wound healing. Analysis of well-recognized EMT markers can reveal contradictory results between cell types undergoing EMT, despite the similarities of a general progression from the epithelial to a mesenchymal phenotype.

Individual EMT markers have been examined in prior research but there has been no systematic investigation to determine whether a placental EMT mechanism plays a role in the CTB differentiation into EVT, nor efforts to compare the process to EMT in other cell types. The progressive acquisition of a migratory/invasive cell phenotype during CTB differentiation resembles the general EMT process whereby epithelial cells lose their junctional contacts, are remodeled to a mesenchymal phenotype and migrate away from the originating tissue. We decided to investigate the CTB to EVT differentiation to determine if it could be characterized as an EMT and to see how it compares to other EMT processes. As cellular sources, we used purified CTB and EVT, isolated in a paired manner from first trimester human placenta. Using a PCR array for multiple genes known to be involved in EMT in other systems, we compared gene expression between paired primary CTB and EVT. We hypothesized that the conversion of CTB to EVT could be characterized as an epithelial–mesenchymal transition.

2. Methods

2.1. Tissue source

First trimester (6–10 week gestation) placental tissue was obtained following termination of pregnancy performed by dilation and curettage. Tissue was obtained with written informed consent from the Auckland Medical Aid Centre (AMAC), Auckland, NZ. The tissue acquisition protocol was approved by the Northern Regional Ethics Committee (NTX/12/06/057/AM01), Auckland, New Zealand. The samples were obtained from singleton pregnancies with no known gross morphological or other abnormalities.

2.2. Cell preparation

CTB and EVT were isolated from the same placental tissue sample, as previously described [17]. First trimester placentae from 6 to 10 weeks of gestation were washed in phosphate buffered saline (PBS, pH 7.4) to remove maternal blood. Villi were dissected from the membranes and incubated with 0.25% trypsin (Gibco, Auckland), 0.02% DNAse I (Sigma, Auckland) in PBS (10 mL per gram of tissue) in a 37°C water bath for 10 min. The supernatant was removed and villi were washed a further 8 times with 20 mL of PBS to remove the EVT and much of the syncytiotrophoblast layer.

To isolate EVT, the combined supernatant and wash solution was filtered through a 70 μm cell strainer into tubes containing FBS (final concentration 10%) then centrifuged at 450 × g for 8 min. The cell pellet was resuspended in DMEM/F12 (Life Technologies, Auckland) containing 5% FBS. The majority of the cells (90%) were then incubated with an FITC-conjugated monoclonal anti-HLA-G antibody for 30 min at 37°C (5 μg/mL; clone MEM-G/9, AB7904, Abcam, Cambridge, UK). The remaining 10% of the cells were incubated with DMEM/F12 containing 5% FBS only (negative control). Cells were centrifuged at 450 × g for 8 min, resuspended in PBS, stained with propidium iodide (1 μg/mL; PI, Invitrogen, Auckland) for 5 min at 4°C then washed with PBS. HLA-G (FITC)-positive cells were sorted using a Becton Dickinson FACSAria II SORP cell sorter. FITC detection was performed using 488 nm excitation with 505LP and 530/30 BP emission filters. Dead cells and doublets were excluded. We have previously demonstrated the purity of similar EVT preparations as quantified by immunocytochemistry. Cells were ≥95% CK-7 (cytokeratin-7) positive [18,19], thus we have a high level of confidence that the cells sorted by flow cytometry as HLA-G positive from this population represent a pure EVT population.

To isolate CTB, residual villous tissue from the first trypsin digest described above was incubated with 0.25% trypsin, 0.02% DNAse I in PBS (10 mL per gram of tissue) on a rocker at 4°C for 7 min, then stationary at 4°C for 16 h. Villi were then washed a further 10 times in PBS and supernatant and washes were filtered through 70 μm cell strainers into FBS (final concentration 10%). The filtrate was centrifuged at 450 × g for 8 min and pellets were resuspended in DMEM/F12 containing 5% FBS. Cells were incubated in a 10 cm petri dish in a humidified 37°C environment with 5% CO_2 for 10 min to deplete contaminating mesenchymal cells by adhesion to the plastic. The cell suspension was removed, and the dish gently washed twice with DMEM/F12 containing 5% FBS. Cells from the combined suspension and wash were incubated for 30 min at 37°C with an FITC-conjugated monoclonal anti-β4 integrin antibody (10 μg/mL; clone 450-9D, AB22486, Abcam) in DMEM/F12 containing 5% FBS. The cell preparation was stained with PI as described above to identify dead cells. We have previously demonstrated that this methodology produces a ≥95% pure trophoblast population as demonstrated by expression of CK-7 and lack of expression of vimentin [19]. From this 95% pure population, an even greater level of CTB purity was achieved through flow sorting, as described above, to isolate a pure β4 integrin (FITC)-positive CTB population.

2.3. Sample preparation and analysis

EVT and CTB were sorted into 2 mL RNAse-free tubes containing 0.5 mL RNAlater (Life Technologies). RNA was extracted using the Invitrogen PureLink RNA Mini Kit (Life Technologies) according to the manufacturer's instructions. RNA quality and quantity were determined using an RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions, employing an Agilent 2100 Bioanalyzer. For each sample cDNA was transcribed from 10 ng of RNA using an RT^2 PreAmp cDNA Synthesis Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, using the RT^2 PreAmp Pathway Primer Mix specific for the Epithelial–Mesenchymal Transition (EMT) RT^2 Profile PCR Array (Cat. #PAHS-090Z, Qiagen). The PCR Array was analyzed using an ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems Inc., Foster City, CA) under cycling conditions recommended by the manufacturer.

The data from the PCR Array was analyzed using $\Delta\Delta C_T$ methodology to obtain the fold-change in relative gene expression between CTB and EVT. Out of a choice of 5 potential housekeeping genes (B2M, GAPDH, ACTB, HPRT1, RPLP0) we chose to use the combination of B2M, GAPDH, RPLP0 and HPRT1 to normalize our samples [20]. These genes demonstrated no significant changes across our sample groups ($p < 0.05$, Student's t test; [21]). The PCR Array also contains built-in controls for the detection of genomic DNA contamination, a reverse transcription control (which tests the efficiency of the reverse transcription reaction) and a positive PCR control that tests the efficiency of the polymerase chain reaction itself. The statistical significance of the difference between the CTB and EVT gene expression was determined from the $2^{(-\Delta CT)}$ values using a two-tailed Mann–Whitney U test. A p value of ≤ 0.05 was taken as indicating a significant difference. PCR Array data has been deposited in the NCBI's Gene Expression Omnibus (GEO) Database with Accession Number GSE74040; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE74040.

3. Results

3.1. Samples

EVT and CTB were obtained from the placental tissue of pregnancy terminations ranging from 6 to 10 weeks gestational age. The EVT and CTB were isolated using the EVT-specific marker HLA-G and the CTB-specific marker integrin β4. An average of 1.8×10^5 and 1.2×10^5 cells were isolated from the CTB and EVT samples respectively (from 1 to 3 g of tissue). Extraction of the cell samples produced RNA for CTB and EVT with concentrations of 4.2 ± 1.0 and 4.1 ± 1.1 ng/μL respectively (mean ± SEM, n = 6 placentae); total RNA extracted was 251 ± 60 ng (range 89–515 ng) for the CTB and 248 ± 65 ng (range 96–494 ng) for the EVT. The average yield of RNA per cell number was 1.4 pg/cell for CTB and 2.1 pg/cell for EVT. The quality of the RNA extracted from the isolated cells was assessed using the RIN (RNA Integrity Number) score. The CTB samples had a mean RIN score of 8.1 ± 0.2 (n = 6) while the score for the EVT was 9.0 ± 0.3 (n = 6), demonstrating a high degree of RNA integrity for all the extracted samples and their suitability for qPCR analysis.

3.2. PCR array

We used a PCR Array to examine expression of a panel of 84 genes associated with the EMT. The built-in controls showed that none of the samples had genomic DNA contamination and that the efficiency and reproducibility of both the reverse transcription and PCR were sufficient and appropriate.

We compared gene expression between villous cytotrophoblast (CTB, n = 6) and extravillous trophoblast from the same placental samples (EVT, n = 6). Out of the 84 genes potentially associated with the EMT, 54 showed significant changes ($p \leq 0.05$). There were 30 up-regulated genes (Table 1) and 24 down-regulated genes (Table 2).

Multiple genes demonstrated significant changes consistent with an EMT as described for other cell types. These changes include decreases in the expression of epithelial/junctional adhesion markers (Fig. 1(a)) such as OCLN (−75-fold), a gene that encodes occludin, the primary protein maintaining tight junction stability, CDH1 (−11-fold), the gene coding for E-cadherin, a transmembrane protein involved in cell–cell adhesion, EFGR (−33-fold), the epidermal growth factor receptor and CTNNB1 (-6-fold), β-catenin. This is in contrast to the increase in the expression of the mesenchymal markers (Fig. 1(b)) such as FN1 (107-fold), encoding fibronectin 1, an ECM protein involved in cell adhesion and migration, vimentin (VIM; 235-fold), a mesenchymal intermediate filament protein and the extracellular matrix integrins α5 (ITGA5; 140-fold) and β1 (ITGB1; 4-fold). The change in the nature of these cells from an epithelial to a mesenchymal phenotype is emphasized by the increases in the metalloproteinases (MMP2, MMP3, MMP9; 357-, 129-, 160-fold respectively; Fig. 1(c)) necessary for invasion. Another important group of genes showing changes in expression are the transcription factors (Fig. 1(d)). Several, including SMAD2, TCF3, SNAI1, SNAI2 and TWIST1 show decreases in expression (−2, −2, −3, −24, −64-fold), contrary to the changes reported in other EMT types. Several others however, show increases, including a robust increase in ZEB2 (198-fold), often described as a "master EMT regulator".

Two other groups are of note. The first are those genes that show changes opposite to those previously observed in the EMT. In this

Table 1
Genes down-regulated in EVT compared to CTB.

Gene	Fold change	p value	Description
FOXC2	−121.90	0.036	Forkhead box C2 (MFH-1, mesenchyme forkhead 1)
OCLN	−75.02	0.002	Occludin
TWIST1	−64.04	0.050	Twist homolog 1 (Drosophila)
BMP7	−61.92	0.002	Bone morphogenetic protein 7
RGS2	−49.61	0.004	Regulator of G-protein signaling 2, 24 kDa
JAG1	−40.12	0.002	Jagged 1
EGFR	−33.38	0.005	Epidermal growth factor receptor
SNAI2	−23.61	0.009	Snail homolog 2 (Drosophila)
WNT5A	−20.25	0.002	Wingless-type MMTV integration site family, member 5A
FZD7	−14.38	0.002	Frizzled family receptor 7
CDH1	−10.82	0.002	Cadherin 1, type 1, E-cadherin (epithelial)
TSPAN13	−9.98	0.002	Tetraspanin 13
TMEFF1	−9.91	0.002	Transmembrane protein with EGF-like and two follistatin-like domains 1
SERPINE1	−6.45	0.004	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
CTNNB1	−5.72	0.002	Catenin (cadherin-associated protein), beta 1, 88 kDa
COL5A2	−4.88	0.004	Collagen, type V, alpha 2
COL1A2	−2.92	0.026	Collagen, type I, alpha 2
VPS13A	−2.79	0.009	Vacuolar protein sorting 13 homolog A (S. cerevisiae)
SNAI1	−2.75	0.004	Snail homolog 1 (Drosophila)
PTP4A1	−2.52	0.016	Protein tyrosine phosphatase type IVA, member 1
NUDT13	−2.47	0.041	Nudix (nucleoside diphosphate linked moiety X)-type motif 13
TCF3	−1.92	0.041	Transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)
SMAD2	−1.69	0.005	SMAD family member 2
DESI1	−1.50	0.025	PPPDE peptidase domain containing 2

Table 2
Genes up-regulated in EVT compared to CTB.

Gene	Fold change	p value	Description
F11R	1.58	0.026	F11 receptor
STAT3	2.01	0.005	Signal transducer and activator of transcription 3 (acute-phase response factor)
KRT7	2.40	0.041	Keratin 7
WNT5B	2.95	0.009	Wingless-type MMTV integration site family, member 5B
BMP1	3.37	0.002	Bone morphogenetic protein 1
CAV2	3.64	0.009	Caveolin 2
CALD1	3.79	0.009	Caldesmon 1
PLEK2	3.89	0.002	Pleckstrin 2
SNAI3	3.92	0.041	Snail homolog 3 (Drosophila)
ITGB1	4.34	0.002	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
SPARC	4.94	0.002	Secreted protein, acidic, cysteine-rich (osteonectin)
KRT19	6.17	0.002	Keratin 19
NOTCH1	6.39	0.009	Notch 1
MSN	12.72	0.005	Moesin
TCF4	18.40	0.004	Transcription factor 4
GNG11	24.30	0.008	Guanine nucleotide binding protein (G protein), gamma 11
TFPI2	24.38	0.002	Tissue factor pathway inhibitor 2
TIMP1	24.50	0.002	TIMP metallopeptidase inhibitor 1
KRT14	41.44	0.002	Keratin 14
TGFB1	47.69	0.004	Transforming growth factor, beta 1
FN1	106.94	0.005	Fibronectin 1
TGFB2	115.10	0.005	Transforming growth factor, beta 2
MMP3	128.93	0.002	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)
ITGA5	139.68	0.002	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
MMP9	160.43	0.002	Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)
SPP1	186.25	0.005	Secreted phosphoprotein 1
ZEB2	198.48	0.004	Zinc finger E-box binding homeobox 2
IL1RN	230.62	0.002	Interleukin 1 receptor antagonist
VIM	235.15	0.008	Vimentin
MMP2	356.83	0.002	Matrix metallopeptidase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)

category are the increases in the cytokeratins KRT14 (41-fold), KRT19 (6-fold) and KRT7 (2-fold), shown in Table 1. The second category comprises those genes that might be expected to change but showed no significant alteration in expression. In this category are ERBB3 and NODAL. Thus while the majority of gene expression changes support an EMT as the primary process of CTB to EVT differentiation, there are clear differences with the EMT observed in other cell types and conditions (for a complete listing of gene expression changes see Supplementary Table 1).

4. Discussion

To our knowledge this is the first study in which paired CTB and EVT obtained from the same first trimester placentae have been compared for multiple genes associated with the EMT. Taken together the data support our hypothesis that differentiation of CTB into EVT is an EMT. There were significant and substantial changes in gene expression in over half of the 84 genes examined. Of the 30 up-regulated and 24 down-regulated genes, 40 are altered in a manner consistent with the EMT-associated changes observed in other cell types. These include changes in the expression of genes associated with the junctional interactions between epithelial cells, cytoskeletal (re)organization, migration and invasion as well as alterations in the genes for ECM factors permissive of EMT. There were alterations in multiple cellular processes that contribute to the transformation from an adherent epithelial phenotype to an anchorage-independent, invasive mesenchymal phenotype. While there were some changes in gene expression that do not conform or are opposite to prior published patterns of EMT, the majority support the conclusion that the process whereby CTB differentiate into EVT can be classified as an EMT, albeit one that differs in part from previously defined types of EMT.

The cell types used in this study are defined by the presence or absence of specific cell markers that are utilized in the isolation procedure, such as HLA-G. The presence of these markers denotes a particular grouping of cells, but it is by no means clear that these groups are homogeneous. Our prior data suggests that CTB that differentiate into EVT may be a subpopulation of the overall CTB population in the first trimester [18]. This subpopulation of CTB acts as EVT progenitors whereas the remainder of the CTB differentiate into syncytiotrophoblast. Cells expressing the HLA-G marker have been shown to be spatially separated from the trophoblast cell columns [18,22], meaning that cells isolated using HLA-G in this study are unlikely to simultaneously display characteristics of cells normally attached to the basal lamina.

This study is limited to the genes contained in the PCR Array. These were chosen based on literature reports on other forms of EMT, most frequently involving metastatic cancer cells. Undoubtedly there are other genes that form part of the CTB to EVT transformation which are not examined in this profile. However consistent evidence from gene groups strongly support the characterization of CTB/EVT as an EMT. The genes involved in cell–cell interaction form one such group. Our results show down-regulation of several genes crucial for the epithelial adherens junction and desmosome structures [15], including a reduction in the expression of CDH1 (E-cadherin), OCLN (occludin) and CTNNB1 (β-catenin). Loss of E-cadherin is associated with increased invasiveness in multiple forms of EMT [6]. Loss of E-cadherin and β-catenin in first trimester EVT is associated with increased trophoblast invasiveness [9,23–25]. Another gene group of interest shows changes in ECM components permissive for the EMT, including up-regulation of FN1 (fibronectin 1), VIM (vimentin), ITGA5 (integrin α5) and ITGB1 (integrin β1) [26,27]. These changes are similar to the ECM component/adhesion receptor switch observed as villous CTB transition to the invasive EVT [4,24,25] and are consistent with an EMT process.

Another set of components associated with EMT, ECM proteases and their inhibitors, also show significant changes in our CTB/EVT

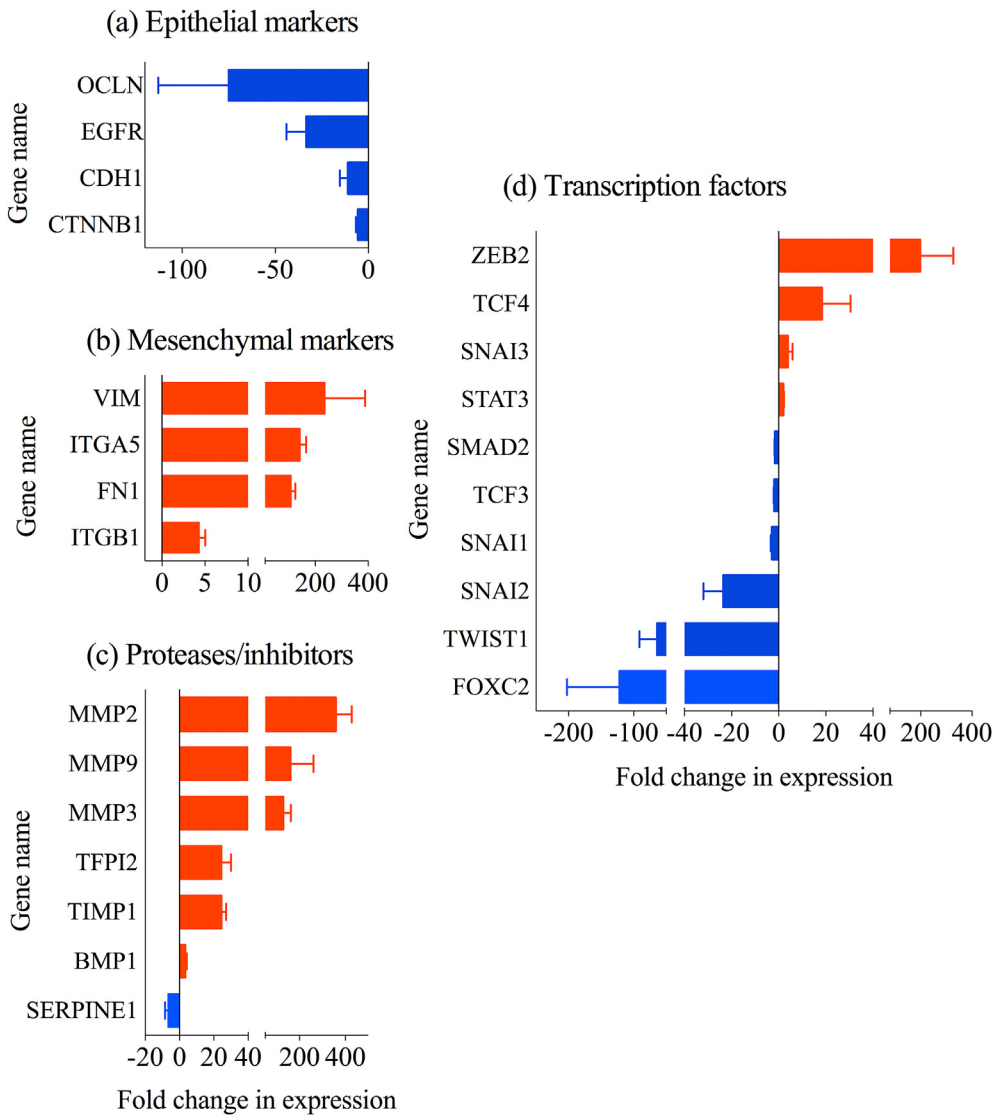

Fig. 1. Specific groups of genes altered in the comparison of EVT and CTB gene expression. The figure shows the changes in groups of genes associated with specific functions or phenotypes that demonstrate a significant ($p < 0.05$) alteration in EVT compared to CTB. The fold-change in gene expression is shown as mean ± SEM for 6 placentae. (a) Junctional and epithelial marker genes, (b) genes encoding mesenchymal markers, (c) genes for matrix metalloproteinases and inhibitors, (d) genes encoding transcription factors.

comparison. There was substantial up-regulation of MMP2, MMP3 and MMP9, a group of metalloproteinases involved in breaking down the ECM to enable EVT invasion. Prior investigations examining MMP2 and MMP9 expression and activity suggested that MMP2 was the more important protease for initial EVT invasion events, whereas by 9 weeks of gestation MMP9 assumes a more prominent role [8,28]. The presence of MMP3 in both CTB and EVT confirms prior observations, however we found an increase in EVT compared to CTB, contrary to the prior report [29]. Changes consistent with an EMT were also observed in protease inhibitors in the CTB/EVT comparison; TIMP1 (tissue inhibitor of metalloproteinase 1) and TFPI2 (tissue factor pathway inhibitor 2) were both up-regulated (25-fold and 24-fold respectively), suggesting tightly coupled protease/inhibitor gene expression that nonetheless favors ECM proteolysis. The protease inhibitor, SERPINE1 (plasminogen activator inhibitor-1, PAI-1) showed a significant decrease (-6-fold), contrary to what might be expected, however it has also been shown to be an inhibitor of cell migration via its effect in reducing integrin-mediated adhesion [30,31], thus its reduction here may be viewed as an alteration that promotes the EMT.

Clues to the factors driving the CTB/EVT transformation can be obtained by looking at some of the regulatory components that have significance for the EMT in other cell types. Previous analysis of EMT processes identified a list of master transcriptional regulators for EMT that were included on the PCR-array [32–34]. The decreases we observe in FOXC2, SNAI1 (Snail), SNAI2 (Slug), TCF3 and TWIST1 (Twist) suggest these pathways may not be functional in the CTB/EVT transformation. Up-regulation of Twist is associated with repression of E-Cadherin in the trophoblast syncytialization process [35], but the opposite is true in the data reported here; the EVT show a substantial decrease in Twist expression concurrent with a loss of E-Cadherin. The Wnt pathway is a major signaling system both stimulatory for EMT and involved in CTB/EVT differentiation [36,37]. We found a decrease in the antagonistic Wnt 5A ligand in EVT (−20-fold), consistent with a prior report [37] however the 6-fold decrease in the nuclear co-activator CTNNB1 (β-catenin) raises questions about the functioning of the pathway.

The other transcription factor which shows a marked increase between CTB and EVT is ZEB2 (zinc finger E-box-binding homeobox 2), also known as SIP1 (Smad interacting protein 1); expression is

increased 198-fold in the EVT vs. CTB. There is close homology between ZEB2 and ZEB1 (also measured here) that is reflected in their strong binding affinity for E-box DNA sequences. Both are associated with the EMT and both share many of the same regulatory factors and downstream targets [38]. Mechanisms used by both ZEB1 and ZEB2 in embryonic development are also involved in the pathological onset of EMT in carcinogenesis [32], suggesting both are basic components of the EMT process. That we see no change in the expression of ZEB1 between the two cell types, suggests that CTB-EVT differentiation may be specifically regulated by ZEB2. ZEB2 is likely a fundamental component of the EMT process since it has been implicated not only with the EMT characteristic of advanced stages of carcinoma [38] but also with developmental neural crest EMT [39].

The primary transcriptional function of ZEB2 in the EMT is coordinated suppression of junctional genes such as E-cadherin and occludin [40], although it may also be directly involved with the simultaneous up-regulation of mesenchymal and other EMT elements such as vimentin and integrin α5 [41,42]. Prior reports show that the induction and maintenance of a stable mesenchymal phenotype requires the establishment of autocrine TGFβ signaling to drive sustained ZEB expression [34,43]. Consistent with this is the significant increase in TGFB1 (48-fold) and TGFB2 (115-fold) expression in EVT compared to CTB, suggesting that this autocrine loop may be functioning to sustain the mesenchymal EVT phenotype.

Our data provides broad support for the designation of the CTB-EVT transformation as an EMT event, displaying characteristics typical of EMT observed in other conditions as well as unique changes that may mark it out as a novel form of EMT. Having identified a primary mechanism responsible for CTB differentiation into EVT, it will now be possible to explore the changes in this process that occur in aberrant invasion pathologies such as pre-eclampsia and placenta accreta.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2015.10.013.

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