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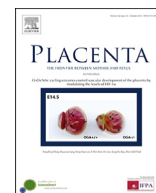

# Differentiation of first trimester cytotrophoblast to extravillous trophoblast involves an epithelial–mesenchymal transition

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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.

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## 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

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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.

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