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Genome-wide expression profile of first trimester villous and extravillous human trophoblast cells

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ABSTRACT

We have examined the transcriptional changes associated with differentiation from villous to extravillous trophoblast using a whole genome microarray. Villous trophoblast (VT) is in contact with maternal blood and mediates nutrient exchange whereas extravillous trophoblast (EVT) invades the decidua and remodels uterine arteries. Using highly purified first trimester trophoblast we identified over 3000 transcripts that are differentially expressed. Many of these transcripts represent novel functions and pathways that show co-ordinated up-regulation in VT or EVT. In addition we identify new players in established functions such as migration, immune modulation and cytokine or angiogenic factor secretion by EVT. The transition from VT to EVT is also characterised by alterations in transcription factors such as STAT4 and IRF9, which may co-ordinate these changes. Transcripts encoding several members of the immunoglobulin-superfamily, which are normally expressed on leukocytes, were highly transcribed in EVT but not expressed as protein, indicating specific control of translation in EVT. Interactions of trophoblast with decidual leukocytes are involved in regulating EVT invasion. We show that decidual T-cells, macrophages and NK cells express the inhibitory collagen receptor LAIR-1 and that EVT secrete LAIR-2, which can block this interaction. This represents a new mechanism by which EVT can modulate leukocyte function in the decidua. Since LAIR-2 is detectable in the urine of pregnant, but not non-pregnant women, trophoblast-derived LAIR-2 may also have systemic effects during pregnancy.

1. Introduction

In the early weeks of human pregnancy the embryo implants completely into the uterine wall, surrounded by trophoblast cells. Trophoblast progenitors located at the basement membrane of the placental villi differentiate into distinct lineages with specialized functions [1]. Villous trophoblast (VT) cells proliferate and cover the mesenchyme and fetal vessels that form the placental villous tree. Subsequent fusion of VT generates an overlying syncytium of villous syncytiotrophoblast, which provides a large surface area for nutrient and gas exchange as well as synthesis of hormones such as progesterone. A distinct lineage of extravillous trophoblast (EVT) cells arises from VT cells in trophoblast columns at the tip of villi anchoring the placenta to the uterine wall. EVT do not divide but, during the first trimester of pregnancy, they detach from the placental villi and invade directly through the decidual stroma as individual cells. These interstitial EVT can penetrate as far as the inner third of the myometrium before forming sessile, multinucleated giant cells. Endovascular EVT advance along the luminal endothelium of decidual spiral arteries, against the direction of flow. EVT invasion is associated with dramatic remodeling of decidual spiral arteries. Interstitial EVT destroy the muscular coat and endovascular EVT displace endothelial cells. Together these trophoblast cells convert the spiral arteries into distensible low-resistance channels to increase blood flow to the growing feto-placental unit. Failure to transform arteries is associated with poor trophoblast invasion and results in inadequate placental perfusion [2]. Clinically, this may present as pre-eclampsia, fetal growth restriction or recurrent miscarriage. Understanding the factors controlling the differentiation of VT to EVT and the extent of EVT

Abbreviations: VT, villous trophoblast; EVT, extravillous trophoblast; uNK, uterine NK; ECM, extracellular matrix.

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migration is therefore crucial if we are to improve the diagnosis and management of these complications of pregnancy.

The factors that control exit of VT from the cell cycle, differentiation into motile EVT and the extent of migration are unclear. The spontaneous differentiation of VT to EVT cells when cultured *in vitro*, strongly suggests that this process is largely controlled by an intrinsic differentiation program. However, interactions with extracellular matrix (ECM), growth factors secreted by cells in the decidua and even oxygen tension also influence trophoblast differentiation [3]. Differentiation of VT to EVT is accompanied by complex changes in phenotype that include the expression of adhesion molecules, such as integrins, metalloproteases and cathepsins which permit EVT migration. Conversely, decidual cells express tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitor (PAI) which function to limit invasion. How these interactions are co-ordinated has until recently been unclear. There is now strong evidence that uterine NK (uNK) cells, the dominant lymphocyte in the decidua during early pregnancy, also play a role in regulating trophoblast invasion and vascular conversion [4]. Unlike VT, which lack surface HLA class I or class II, migrating EVT express a unique repertoire of HLA class I: HLA-C, HLA-E and HLA-G. Corresponding receptors for these molecules are present on uNK cells and decidual macrophages at the implantation site [2]. Immunogenetic data suggests that certain combinations of genes from the Killer Immunogobulin-like Receptor (KIR) family expressed on maternal uNK cells and their HLA-C ligands on trophoblast, are associated with an increased risk of pre-eclampsia or recurrent miscarriage [5,6]. Although recognition of trophoblast MHC by receptors such as KIR, LILR and NKG2/CD94 on uNK cells is clearly important, activating and inhibitory receptors that recognise non-MHC self molecules also regulate NK cell functions and cytokine secretion. NK cells integrate the signals from receptors for MHC and non-MHC ligands and responses stimulated by non-MHC receptors can modify those induced by the conventional MHC receptors. Over 30 potential receptor/ligand pairs have been described and uNK cells have been shown to express many of these [7,8]. Understanding of their role in trophoblast/uNK interactions is limited because a systematic screen for known and novel ligands expressed by EVT, has not yet been undertaken.

Efforts to define how VT to EVT differentiation and subsequent migration is controlled have also been hampered by the difficulty in obtaining primary VT and EVT cells from the first trimester. Here we present a microarray analysis of the transcriptome in primary VT and EVT cells, isolated from healthy first trimester pregnancies to a high degree of purity. The aim of this whole genome analysis was to define what transcriptional changes underlie the transition from the villous to the extravillous phenotype. We also examined the expression by trophoblast of potential ligands for receptors on NK cells and other decidual leukocytes to better understand the interactions between EVT and the maternal immune system. Microarrays of the JAR and JEG-3 choriocarcinoma cell lines are also included, to characterise the validity of these models for VT and EVT, since they are frequently used due to the inaccessibility of primary trophoblast cells.

2. Methods

2.1. Clinical samples and cell lines

Decidual and placental tissues were obtained from elective terminations of normal pregnancies between 8 and 12 weeks gestation. Ethical approval for the use of these tissues was obtained from the Cambridge Local Research Ethics Committee. For histological staining, blocks of tissue were frozen and 5 μm serial sections cut before fixing in acetone and storage at −20 °C. For flow cytometry, single cell preparations of trophoblast or leukocytes were isolated as previously described [9]. Briefly, placental villi were identified macroscopically, digested with trypsin and collected by density gradient to obtain cells which are 50–80% EGF-R+. VT is the only cell type at the implantation site that expresses EGF-R [10]. EGF-R+ cells representing VT were obtained at high purity by staining these trophoblast cells immediately, followed by flow cytometry sorting. Alternatively, the primary trophoblast cells were cultured for 12 h on fibronectin in Hams F12 with 20% fetal calf serum, after which 50–80% of the cells express HLA-G, a marker specific for EVT [9,11]. The resulting HLA-G positive EVT cells were then analysed or purified by flow cytometry as described below. Leukocytes were isolated by collagenase digestion of maternal decidual tissue and stained for flow cytometry immediately. Villous mesenchyme or decidual stromal cells were obtained by at least 4 passages of the adherent placental or decidual cells isolated. Peripheral blood leukocytes were isolated on Lymphoprep (Axis-Shield) from fresh venous blood of normal adult volunteers. Urine was obtained with informed consent from healthy local donors who were not pregnant, or throughout gestation, and were stored at −20 °C until use. Urine from pre-eclamptic subjects was provided by the Pregnancy Exposures and Preeclampsia Prevention (PEPP) study from women at 10 weeks gestation (11 of whom went on to develop pre-eclampsia and 11 healthy controls) and by the SCOPE project from women at 15(±1) weeks gestation (20 of whom went on to develop pre-eclampsia and 20 healthy controls). Both studies were approved by Ethical Board review. JAR and JEG-3 cells were purchased from the American Type Culture Collection and cultured according to their instructions (ATCC reference numbers HTB-144 and HTB-36).

2.2. Monoclonal antibodies

Monoclonal antibodies (mAbs) used which bind HLA-G were G233 [12] made in our own laboratory and MEM-G/9-FITC [13] from Serotec. Additional unconjugated mAbs were to IL-15Rα (clone JM7A4) from BioLegend, cytokeratin (clone PKK-1) from Labsystems, IL-2Rβ (clone TU27) from Prof. K. Sugamura and LAIR-2 (clone FMU-LAIR 2.1) from Dr. Boquan Jin. Conjugated mAb purchased were EGF-R-FITC (clone EGF-R1) from Insight Biotechnologyl; HLA-DR-FITC (clone L243), CD56-Alexa Fluor 488 (clone B159), CD3-FITC (clone SK7), CD14-PE (clone MφP9), CD132-PE (clone AG184) and LAIR-1-PE (clone DX26) all from Becton Dickinson; LILRB1-PE (clone HP-F1) from Beckman Coulter; LILRB3-PE (clone *293623*) from R&D Systems; CD45-PE-Cy5 (clone HI30) and IL-2Rβ-APC (clone TU27) both from BioLegend. Isotype controls used were the mAbs HIB19 and X40 (unconjugated and PE-conjugated) from Becton Dickinson, MOPC-21-APC from Biolegend and 20102-PE from R&D. Where indicated, binding of unlabelled mAbs was detected by polyclonal PE-conjugated secondary antibody to murine IgG (Sigma–Aldrich). Biotinylated polyclonal goat anti-LAIR-2 IgG was also obtained from R&D Systems.

2.3. Flow cytometry

Freshly isolated cells or those harvested by trypsin digestion of adherent trophoblast after overnight culture were resuspended in FACS buffer (1% FCS in PBS), incubated with human IgG (Sigma–Aldrich) followed by unlabelled primary monoclonal antibodies and then fluorochrome-conjugated polyclonal secondary antibodies. Free secondary antibody binding sites were blocked with murine immunoglobulin (Sigma–Aldrich) before staining with directly-conjugated mAb to identify leukocyte or trophoblast cell populations. Cells were analysed using FACScan or FACSCalibur flow cytometers with CellQuest software (Becton Dickinson), or sorted using a DakoCytomation MoFlo cytometer and Summit software.

2.4. Expression profiling by microarray

2.4.1. RNA preparation

Total RNA was isolated from primary trophoblast or choriocarcinoma cell lines by lysis in Trizol (Invitrogen) followed by cleanup and DNAse treatment using RNeasy Micro kits (Qiagen). Biotinylated cRNA was synthesized using 100 ng of this total RNA using the Illumina RNA amplification kit (Ambion) according to the manufacturer's instructions.

2.4.2. Array hybridization

Labelled cRNA was hybridised to Illumina Human HT-12 V3 BeadArrays using the manufacturer's standard protocol (*http://www.illumina.com/*). Illumina *BeadStudio* output, comprising background corrected and summarised expression scores for each microarray probe-set, was imported using functions of the *BeadArray* package for the *Bioconductor* (*http://bioconductor.org*) suite of software in the *R* statistical programming environment (*http://www.r-project.org*).

2.4.3. Data processing

Signal intensities were converted to log2 expression units. Quantile normalisation [14], implemented in the *limma* package for Bioconductor, was employed to equalise summarised expression intensity distributions across all sample profiles. Probe-sets were annotated to gene targets using annotations from the *illuminaHumanv3.db* package for Bioconductor and the manufacturer's own annotation package. Raw and processed data are available from the ArrayExpress microarray data repository (*http://www.ebi.ac.uk/arrayexpress/*) under accession number E-MTAB-429.