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Cellular systems biology identifies dynamic trophoblast populations in early human placentas

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ABSTRACT

Introduction: The human placenta is accessible in early developmental stages and affords a unique opportunity to investigate human organogenesis and the dynamics of transitory cell populations in human placenta development.

Methods: The cell surface proteomic profile of early trophoblast cells of first trimester human placentas was quantified using a high throughput flow cytometry screen of 370 Cluster of Differentiation (CD) antigens. Targeted investigation of candidate trophoblast progenitor populations was done through immunohistochemistry, multi-color flow cytometry, and genome wide expression analysis.

Results: Using a novel batch correction and normalization methodology, we identified 23 increasing and 13 decreasing markers of dynamic populations between the week 6 and week 10 of placenta development. We identified and characterized two transient populations expressing either EpCAM (CD326) or CDCP1 (CD318). Immunohistochemistry revealed these CD antigens are expressed by discrete cells with EpCAM localized to the proximal villi columns and CDCP1 to distal columns. Flow analysis confirmed independence of these populations and identified EpCAM cells as positive for EGFR. Microarray analysis indicated EpCAM + /EGFR + and EFGR + cells showed high degree of gene expression similarities to villus cytotrophoblast but loss of EpCAM expression was concomitant with exit from the cell cycle. Similarly, CDCP1 positive trophoblast are enriched in cell cycle gene sets and expressed genes with significant similarity to extravillous cytotrophoblast.

Discussion: Our study indicates at least two distinct subpopulations of cytotrophoblasts exist in the early first trimester within the column that likely maintains pools of actively dividing progenitor cells giving rise to the developing placenta villous tree.

1. Introduction

Defects in development affect the placenta's role as an endocrine organ and transporter of nutrients and wastes. Developmental errors of placentation underlay disorders of pregnancy, such as preeclampsia and intrauterine growth restriction that affect up to 10% of all pregnancies and are a cause of poor fetal development and preterm delivery [1]. The effect of adverse pregnancy on society is two-fold, as over half a million women worldwide die each year from pregnancy related issues [2] and children and women who experience adverse pregnancies are at an elevated risk of chronic health problems such as hypertension, metabolic syndrome, and cardiovascular disease [3,4].

The placenta originates from a single layer of cells in the pre-implantation mammalian embryo called the trophectoderm. These cells drive blastocyst implantation and placenta development that, in humans, generates a highly branched structure termed the chorionic villous tree to bring fetal and maternal blood systems in close proximity for exchange. Within the placenta, three main types of human trophoblast cells are described: (1) cytotrophoblast cells line the villous trees and contribute to the generation of either (2) syncytiotrophoblast, the outer layer of syncytial cells that directly contact the maternal blood and are essential for hormonal and immunological regulation during pregnancy or (3) extravillus trophoblast cells (EVT) that emerge from anchoring points of the villi with the maternal uterine decidua and invade into the endometrial and myometrial tissues. However, there is morphological diversity among the same trophoblast cell types as a function of both location in the proximal-distal axis of the villous tree and gestational age [5]. During early development, cytotrophoblast can

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Abbreviations

EpCAM	Epithelial Cell Adhesion Molecule/CD326
CDCP1	CUB domain-containing protein 1/CD318
CD	Cluster of Differentiation
CTB	Cytotrophoblast
EVT	Extravillous Trophoblast

be observed as a tightly associated monolayer while they are sparse at later stages [5]. Recent single cell RNA-sequencing analysis of term and first trimester placentas are identifying the diversity of the cell population [6–8]. We have taken an alternate approach to identify cell populations though protein expression in addition to staging of placentas at different developmental windows within the first trimester.

The largest changes in placental morphology and development occur during the first trimester (< 12 weeks gestation). The villous tree is developed through a process of branching morphogenesis but does not follow stereotypic patterns as observed in the lung or kidney [5,9]. Several groups have isolated primary human cytotrophoblasts from first trimester human placentas and characterized their growth potential and gene expression using microarrays towards the goal of assessing cellular heterogeneity of the trophoblast cells composing the human placenta [10–12].

To gain insight into the trophoblast cell populations of the early stages of villous tree development we applied a cellular systems biology method of high throughput flow cytometry to quantify shifts in the cell surface proteome. We sampled human placentas at gestational week 6 in the early phase of branching and at week 10 during fetal vascular invasion and development of the placenta. We reasoned that transient or progenitor populations will be more prevalent and enriched at earlier stages and these populations will be identifiable by differential expression of cell surface markers. Understanding the cellular composition is essential to defining the process of human placenta development and understanding how cellular populations signal to form a complex organ.

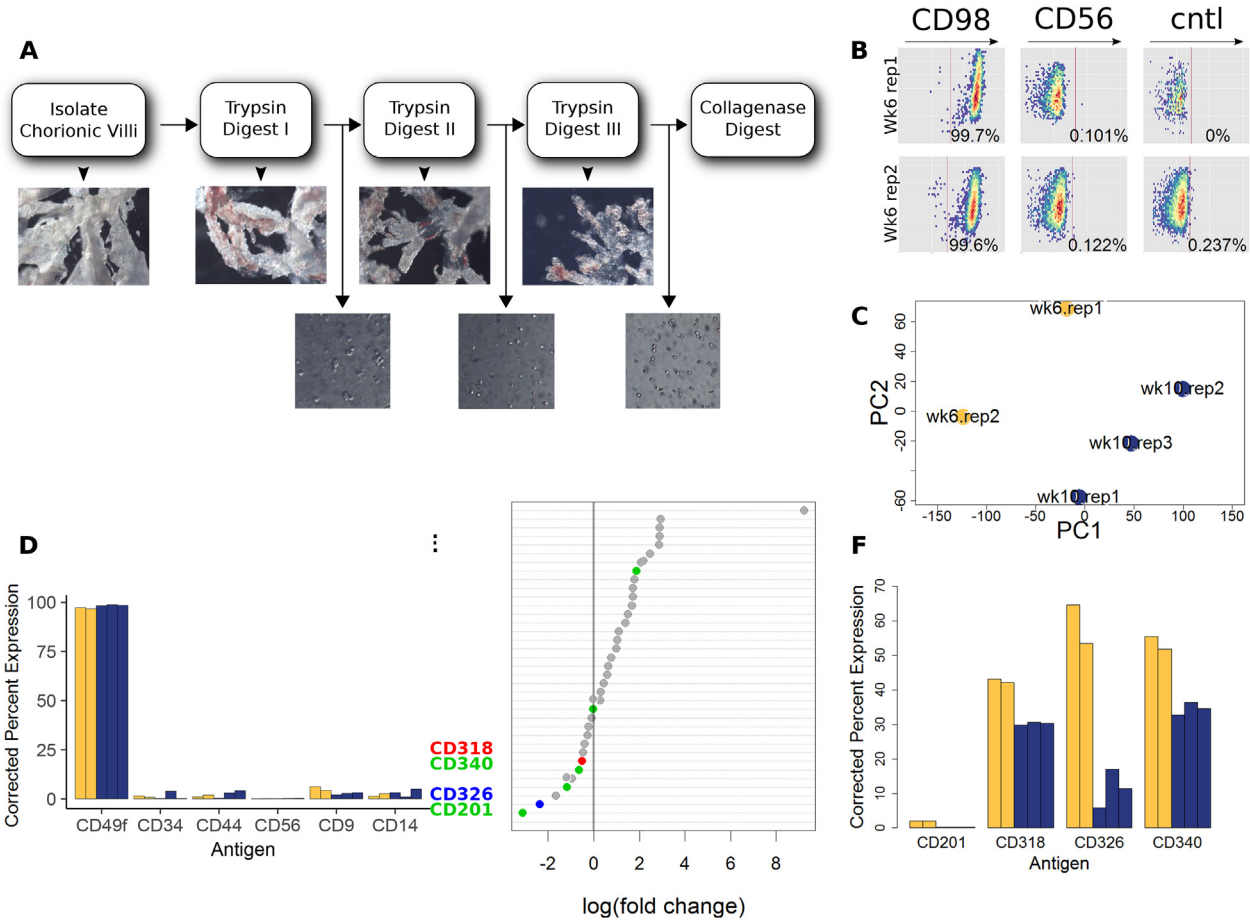

Fig. 1. Overview of methods and results of cell surface marker screen of trophoblast enriched fractions from placentas at gestational week 6 and 10.(A) Workflow overview with brightfield images to monitor dissociation. Chorionic villi are subject to three light rounds of trypsin mediated digestion to release the outer syncytiotrophoblast and cytotrophblast cells. Brightfield images show the villi initially surrounded by a loose layer of trophoblast cells but by the third trypsin digest, the villi are translucent and the basement membrane is exposed. Corresponding images of cell fractions at each stage are shown, with fractions II and III having the highest percentage of trophoblast cells. (B) Representative density plots of flow cytometry data for CD98 (high expression, trophoblast marker), CD56 (low expression, natural killer cell marker), and Fluorescence Minus One (FMO) negative controls used to calculate the percentage of positive labeled cells. The red line represents the gate for positive expression with cells to the right of the line expressing the antigen of interest.(C) Principal component analysis of batch corrected data show good grouping of samples by gestation week.(D) Normalized population percentages of known cell lineage markers confirm our fraction is enriched for trophoblast cells (CD49F) and depleted of other cell lineages including endothelial, fibroblast, natural killer, extravillous trophoblast (EVT), and Hofbauer cells marked by CD34, Cd44, CD56, CD9, and CD14, respectively. (E) Shown are the 36 CD antigens that label significantly differential cell populations between weeks 6 and 10 of development, with 13 decreasing and 23 increasing. CD318 (CDCP1) and CD326 (EpCAM) are both significantly decreasing. Two additional markers, CD340 and CD201 were selected for further validation with immunohistochemistry (IHC)·(F) Normalized population percentages in each replicate of antigens selected for further investigation. All show significant differentiation populations ($p < 0.05$).

2. Methods

2.1. Differential placenta digest

Human placentas (fourth to eleventh week of gestation) were collected through the Research Center for Women's and Infant's Health (RCWIH) Biobank approval from the Mount Sinai Hospital Research Ethics Board #13-0273-E. To simplify the analytical complexity of multiple cell types, we used a two phase protocol to dissociate placentas into separate trophoblast and stromal cell enriched fractions adapted from Aboagye-Mathiesen et al. and Petroff et al. [13,14] For additional description of samples used and a detailed protocol for isolation, see Supplemental Methods.

2.2. CD antigen screen

Between 12 and 15 million viable trophoblast cells were pooled from 6 to 8 week 6 placentas or 1–3 week 10 placentas and submitted to the SickKids-UHN Monoclonal Antibody Core. In total, 14 placentas between ages 5.5 and 6.6 were pooled for the two week 6 replicates and 6 placentas between weeks 10.0 and 10.6 were pooled for the three week 10 replicates. Each sample was probed with approximately 370 antibodies. Five Fluorescence Minus One (FMO) controls were randomly distributed throughout the plates for negative controls. Data has been deposited in Flow Repository under repository IDs FR-FCM-ZY4S, FR-FCM-ZY4V, FR-FCM-ZY4R, FR-FCM-ZY4T, and FR-FCM-ZY4U.

2.3. Statistics

Raw FCS files from five CD antigen screens were analyzed using the FlowCore and OpenCyto packages [15] in the R bioconductor [16], detailed workflow in Supplemental Methods. Significantly dynamic populations were identified by a *t*-test where fold change expression was significant (p-value < 0.05).

2.4. Immunohistochemistry

Detailed experimental procedure provided in Supplemental Experimental Procedures. Briefly, placenta villi were fixed in 4% paraformaldehyde, placed in the Automated Tissue Processor then transferred to the Tissue Embedder for arranging into arrays. Antigen retrieval was achieved with sodium citrate. Sections were blocked before incubation with the primary antibodies, as outlined in Supplementary Table 2. Slides were probed with a biotinylated secondary antibody, incubated with horseradish peroxidase (HRP) tagged streptavidin, visualized with DAB, and counterstained with hematoxylin.

2.5. Multi-channel flow cytometry analysis and sort

Approximately 1 million live trophoblast cells were analyzed in each run. Cells were stained with fluorescently labeled primary antibodies outlined in Supplementary Table 2 and labeled with propidium iodine. Single color control and FMO controls were also prepared. Flow cytometry was performed on a BD LSR II Flow Cytometer System at the Flow Cytometry Facility in the Medical Science Building. Raw FCS files were analyzed using the statistical language R (version 3.5.0) and packages including flowCore [15] (version 1.46.2) and flowViz [17].

For Fluorescence Activated Cell Sorting (FACS), cells were prepared as per the flow cytometry protocol and target populations are gated and diverted into different collection tubes. Cells were immediately spun down, suspended in Trizol, and frozen in −70 to await RNA extraction.

2.6. RNA extraction and microarray

RNA extraction from samples in Trizol were carried out using the Norgen Biotek Total RNA extraction kit as per manufacturer instructions. RNA samples were submitted to the UHN Microarray Center for hybridization to Affymetrix Human Gene 2.0ST Arrays and data was analyzed with R (version 3.5.0) packages *oligo* [18] and *limma.* [19] Batch correction was applied using removeBatchErrect from the *limma* package. Data was deposited in Gene Expression Omnibus (GEO) under accession number GSE90646. Expression values were normalized between the sample with the lowest and highest expression within each gene. Samples were also compared using Gene Set Enrichment Analysis (GSEA) with a p-value = 0.01 and a FDR Q-value cutoff of 0.25 [20]. Heat maps were constructed using *gplots* package in R and network analysis was performed using Cytoscape and the Enrichment Map plugin [21].

3. Results

3.1. Surface proteomic profile of early first trimester trophoblast cells

The high throughput flow cytometry analysis requires a minimum of 15 million viable single cells per assay. We determined that a week 6 placenta yields 3–5 million viable trophoblast cells, while a week 10 placenta yielded between 9 and 12 million. Cells were collected from fractions two and three of our cell isolation methods (Fig. 1A). Two independent replicates using a total of 16 placentas between week 6–6.6 and three independent replicate samples using a total of nine placentas between week 10 and 10.6 were isolated and analyzed.

Each of the five replicate CD antigen screens generated 370 flow cytometry files totalling 1850 analyses. Each FCS file was gated for single viable cells and positive/negative antibody labelling relative to unstained controls (Fig. 1B). The five data sets showed marked batch affects resulting in poor Pearson correlation coefficients between replicate time points (Supplemental Fig. 1A). Batch correction greatly improved the general correlation between replicates and samples from 0.28 to 0.92 for week 6 and from 0.41 to 0.90 for week 10 (Supplementary Fig. 1B). As most proteins measured were unchanged over all gestational stages, we anticipated a high correlation between different time points. Key proteins are detected as differentially regulated and these drove grouping of replicates and separation of the time points in a principal component analysis (PCA) of the batch corrected data (Fig. 1C).

To assess the purity of the cells in each replicate, we extracted expression of known cell lineage markers. All fractions showed strong positive expression ($> 95\%$) of CD49f, a known marker of cytotrophoblast (CTB) and small populations positive for CD9 (5–10%), a marker of extravillous trophoblast (EVT), and CD14 ($< 5\%$), a marker of Hofbauer cells(Fig. 1D). There was very limited contamination from the stromal layer, as minimal positive cell populations ($< 2.5\%$) for CD34, CD44 or CD56 (known markers of endothelial cells, fibroblast cells or natural killer cells, respectively) in all five samples in our screen [22–25].

3.2. Novel dynamic populations between weeks 6 and 10 of development

Linear model analysis identified 36 markers that labelled differing percentages of cell populations at a P-value less than 0.05 (Fig. 1E and Supplemental Table 2). Of these, 23 markers were increasing while 13 were decreasing (complete table of normalized values in Supplementary Table 3. Of the markers down regulated with development, CD326 (EpCAM) and CD318 (CDCP1) showed consistently decreasing expression in both replicates of our CD antigen screen (Fig. 1F) and no evidence exists for their expression in healthy term placentas (via Protein Atlas) [26]. Additional decreasing markers, CD340 (ERBB2) and CD201 (EPCR) were also selected for investigation.

3.3. Immunolocalization of candidate markers in first trimester placentas

The dissociation of placentas into single cell suspension means that

positional information of cell location within the placenta was lost. To determine what structures the cell populations labelled by EpCAM, CDCP1, ERBB2, and EPCR, localized, we employed immunohistochemistry of serial sections of a second cohort of placentas from weeks 4.0–9.3 of gestation (3–5 independent placentas) arrayed on a single slide (Fig. 2, additional images in Supplemental Fig. 2). After surveying the stained slides, we noted consistent patterns of expression of different markers were detected related to expression changes over time, the anchoring villus columns villus branch points and the villus trunk cytotrophoblasts.

3.4. Anchoring columns

Cells in anchoring villi columns showed enrichment of all 4 proteins: EpCAM, CDCP1, ERBB2, and EPCR (Fig. 2A). We observed EPCAM restricted to the proximal column regions while CDCP1, ERBB2, and EPCR enriched in the distal regions. EpCAM expression was strongest in early week 4–5 placentas and quickly diminished with minimal expression by late first trimester. ERBB2 expression persisted throughout the first trimester in a pattern previously reported. Additionally, we noted that ERBB2 co-localized with HLA-G indicating that EVT cells were ERBB2 positive, similar to previous reports [27].

CDCP1 and EPCR expression were diffuse throughout the distal columns while a subset of single cells or small groups of cells in the anchoring columns strongly expressed CDCP1 (Fig. 2A). These cells clearly mark a subpopulation of trophoblasts distinct from EpCAM but overlapping with ERBB2 expressing cells. CDCP1 was also detected in the stromal layer of the placenta by gestational week 7 labeling fibroblasts and endothelial cells. However, our quality control assessment of endothelial and fibroblast markers indicated very little contamination of these cell types in the CD antigen screen.

3.5. Villus trunk and branches

EpCAM is strongly expressed by villous trunk cytotrophoblasts (vCTB) with no expression in syncytiotrophoblast and stromal cells (Fig. 2B). EPCAM expression is strongest at the earliest time points (week 4–5) and becomes greatly reduced by late time points (Week 8–9; Fig. 2B), supporting our observation in the screen as EPCAM positive populations decrease with increasing gestational age. CDCP1 and EPCR have diffuse expression in the syncytiotrophoblast but not villous cytotrophoblast layer while ERRB2 has no expression. Interestingly we also observed EpCAM and ERBB2 as co-expressed in CTB at newly emerging branch points. The expression was observed in the smaller of the branches at the junction with the parent column of the branch.

3.6. Floating villi tips

Cytotrophoblasts in the floating villi tips had expression of ERRB2 in distal cells, EpCAM in proximal cells, and co-expression of CDCP1 and EPCR diffusely in distal cells. (Fig. 2C). These tips often had a trophoblastic sprout (a projection of the syncytiotrophoblast), suggesting active morphogenesis is occurring at the EPCAM/ERBB2 co-labelled tips [9].

3.7. Multi-color flow cytometry analysis of EpCAM, CDCP1, and EGFR

We further investigated EpCAM and CDCP1 because of their strong down regulation within first trimester development and distinct populations labelled. To confirm that EpCAM and CDCP1 mark distinct cell populations, a co-labeling flow cytometry analysis of a third cohort of week 5, 8 and 11 placentas using EpCAM (CD326), CDCP1 (CD318) and EGFR as a marker of mature villus cytotrophoblast [28]. EpCAM and CDCP1 mark two distinct single positive populations in trophoblast cells isolated from early week 5 placentas, confirming our observations with histology (Fig. 3A). By weeks 8 and 11, most trophoblast cells form a single double negative population supporting the results of our CD antigen screen and immunohistological analysis. In contrast, EpCAM and EGFR, a marker of mature trophoblast cells, identify a large double positive population and a smaller double negative population in week 5 trophoblasts (Fig. 3B) that diminishes by week 8 and 11. Three-channel flow analysis determined that the EpCAM-/EGFR-population is CDCP1 + (data not shown).

3.8. Identification of differentially expressed genes in novel populations

We repeated the above flow analysis and sorted duplicates of four populations of cells based on EGFR, EpCAM and CDCP1 labeling for

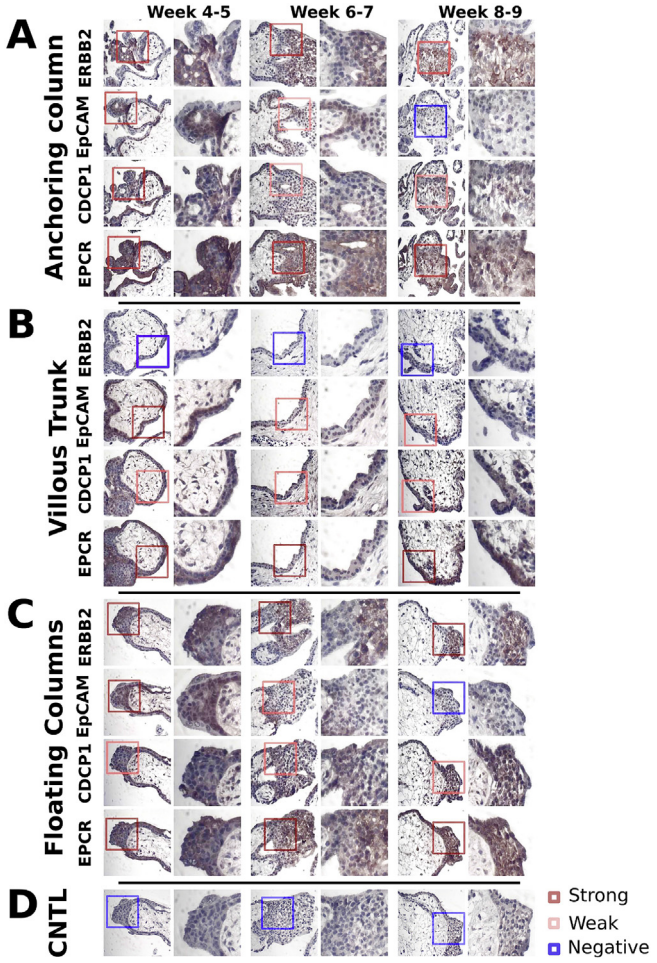

Fig. 2. EpCAM, ERBB2 and CDCP1 localization to different trophoblast structures in vivo during gestational weeks 5 through 9.(A) Anchoring columns. Representative images form anchoring villus columns for staining of EPCAM, CDCP1 and ERBB2 from multiple placentas biopsies. EpCAM expression (brown) is reduced in villus cytotrophoblasts with gestational age but maintained in the proximal column. CDCP1 expression (brown) only labels trophoblast cells of the column more distal to the stromal layer, singly or in clusters. ERBB2 (brown) also labels cells of the distal columns and does not have significant overlap with EPCAM in serial sections at weeks 6–7.(B) Villus trunk. Representative images of EPCAM and ERBB2 (brown) in the villus trunk. EPCAM shows specific staining of the villus column cytotrophoblasts while ERBB2 staining is absent. EPCAM staining weakens with increasing gestational age. (C) Floating column tips. EPCAM is expressed in the floating tips of developing villi, indicated by the multiple layers of cytotrophoblasts and overlying syncytiotrophoblast. Imaged at week 4–5 and week 8–9. At week 8–9 ERBB2 is co-expressed in the tip. The Week 8–9 tip has a syncytiotrophoblast sprout. At week 6–7 representative image is shown of a branch point of the villous tree that has higher expression of both EPCAM and ERBB2 than surrounding trunk villus cytotrophoblasts. CDCP1 does not stain these structures.

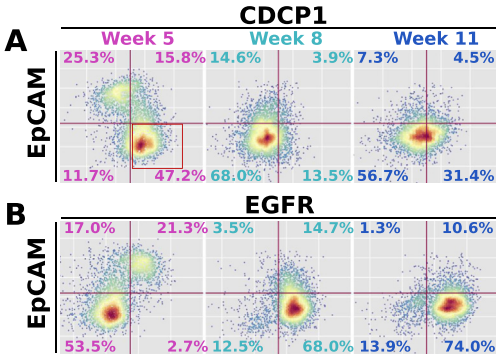

Fig. 3. Developmentally regulated subpopulations of trophoblast cells can be identified through EpCAM, EGFR, and CDCP1 expression. Tri-color flow-cytometric analysis presented as density plots comparing EpCAM and CDCP1 or EpCAM and EGFR expression in trophoblast cells isolated from week 5, 8, and 11 placentas.(A) Trophoblast cells are initially either EpCAM or CDCP1 single positive and become predominantly double negative by week 8 through 11.(B) Double positive and double negative populations initially separated by EpCAM and EGFR at week 5.0 become EpCAM-/EGFR + at week 8, persisting until week 11.

genome wide gene expression analysis using two placentas from weeks 5.5 and 6.6. Each isolated population showed high correlation between mRNA and protein expression of EpCAM, CDCP1, or EGFR (Fig. 4A). PCA of the microarray data indicated four sets of paired cell populations containing one of each sort using different pairs of antibodies (Fig. 4B).

Given the novelty of the trophoblast populations, we compared gene expression to other vCTB and EVT data sets purified through traditional methods, such as villous cytotrophoblast cells purified by a percoll gradient. PCA analysis of the three microarray datasets (current study, Bilban, and Apps; Fig. 4C) revealed the CTB populations of Bilban and Apps data sets were similar to our EGFR + populations but our novel CDCP1 + trophoblast subpopulation agreed more strongly with the Bilban data set clustering distinctly from the EVTs isolated in Apps et al.'s study [29,30].

When only considering the 50 genes Bilban et al. identified as most differentially expressed by vCTB and EVT cell types, hierarchical clustering produced similar groupings to PCA (Fig. 4D). Similarly, the 885 cytotrophoblast enriched genes identified in the Apps data set indicated a stronger relationship to the mature EGFR + populations of our study (Fig. 4E). Consequently, we sought to understand differences among our novel populations and between the cells isolated from previous

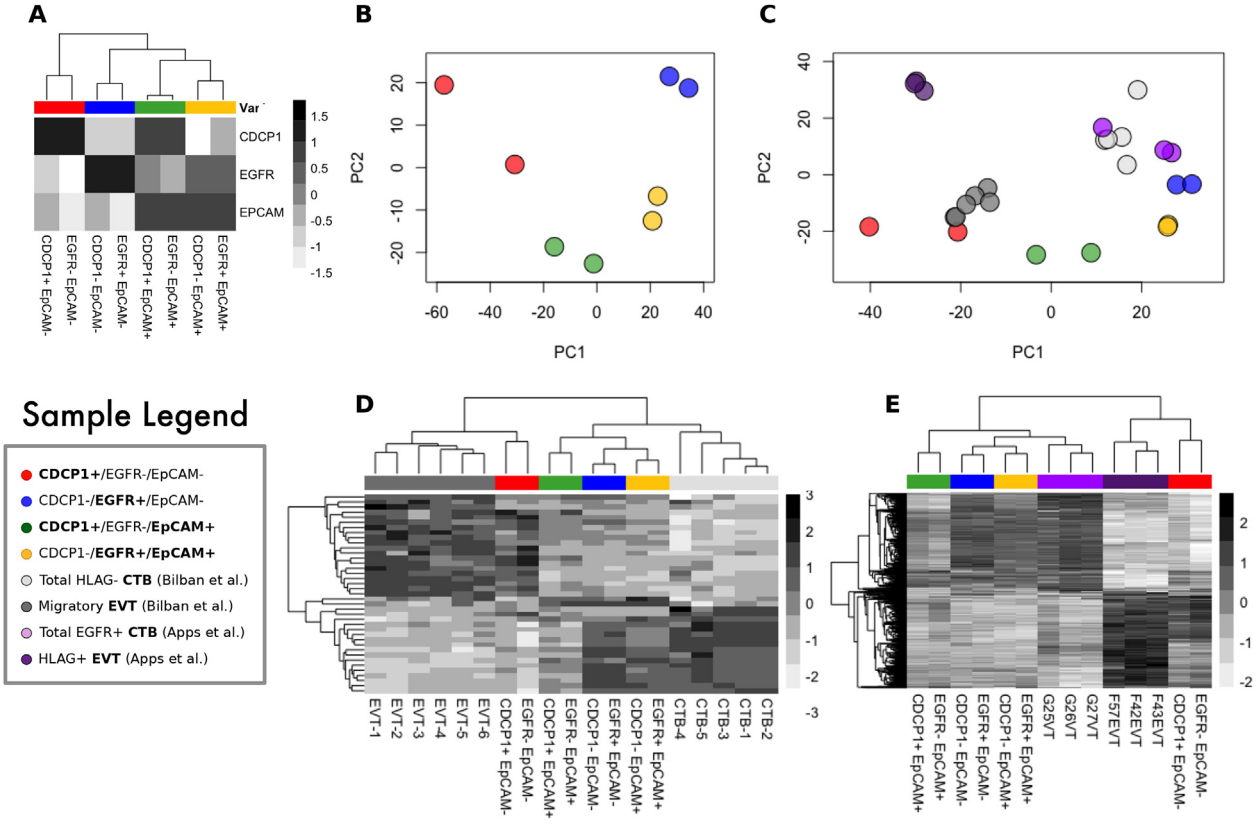

Fig. 4. Genome wide expression analysis week 5–6 trophoblast cell populations marked by EpCAM, EGFR, and CDCP1 expression and external validation with published datasets. Trophoblast cell isolates were labelled with EPCAM and EGFR or EPCAM and CDCP1 in independent flow cytometry sorts. Cells populations were gated into four quadrants for each experiment. Based on three-colour sort in Fig. 3 the expression of the third marker can be imputed. Samples are grouped by color and consistent throughout the figure as defined by the legend.A) Heatmap of CDCP1, EpCAM, and EGFR mRNA expression in all eight sorted populations correlates to cell surface marker proteins combinations used for isolation of the cell populations. Cellular populations are paired by colour in the hierarchical clustering dendrogram.B) Principal component analysis of the 8 isolated cell fraction indicates samples also group based on genome wide expression profiles. The matched pairs separate in component 1 by anti-correlated expression of CDCP1 and EGFR while component 2 separates by expression of EpCAM.(C) Principal component analysis following merging data from this current dataset with two previous microarray datasets comparing cytotrophoblasts and extravillous cytotrophoblast [29,30]. While the trophoblast populations from these two datasets show similarity with our EGFR positive populations, the CDCP1 positive populations show greater similarity to extravillous trophoblasts isolated by Bilban et. Al compared to the extravillous trophoblasts in Apps et al.'s study. (D) Hierarchical cluster of samples based on a selected set of the top 25 genes enriched to each of CTB and EVT (50 genes in total) identified in Bilban et al.'s study [29]. (E) Hierarchical cluster of samples based on a selected set of the top 885 genes most differentially expressed between CTB and EVT identified in Apps et al.'s study [30].

studies through gene set enrichment analysis (GSEA, full outputs in Supplemental Tables 5–8).

GSEA identified a cluster of gene ontologies termed “dna cell cycle” and “spindle microtubule organizing as enriched to our EpCAM +/EGFR + cells relative to the EGFR+/EpCAM-populations (Fig. 5A). We find a similar enrichment with terms related to “dna polymerase replication” in our EpCAM + relative to the total vCTB sample of Bilban et al. (Fig. 5B). The total cytotrophoblasts of Bilban et al. and our EGFR+/EpCAM + are enriched in Gene Ontology terms related to “immune production response” and “female pregnancy multi”, indicating a greater level of maturity in the cells of Bilban cells.

The distinct functions of our novel populations are highlighted by their respective gene ontology enrichments: EpCAM+/EGFR + cells are enriched for sets including “transmembrane transporter activity” and “junction complex cells” as expected of an epithelial cell layer whereas the CDCP1+ cells with more contact with the decidua is enriched for sets such as “extracellular region matrix” (Fig. 5C). Compared to the migratory EVTs isolated by Bilban et al. our CDCP1+ cells are enriched in gene sets suggestive of active proliferation such as “phase mitotic cycle” and “DNA checkpoint replication” (Fig. 5D). The total migratory EVTs are enriched in terms related to extracellular matrix and cytoplasmic vesicles. The Bilban and Apps data sets utilized two distinct methods to generate EVT cells (migration from column explants versus differentiation on fibronectin and HLAG + selection), which may account for developmental or even subtype differences.

To further investigate CDCP1+ cells as EVT progenitors we compared are gene expression data with markers identified by Haider et al. of Notch1 positive mitotically active extravillous trophoblast progenitors [31]. Our two CDCP1 positive populations expressed Notch1 (the novel marker of progenitor extravillous trophoblasts), HLA-G (a classic marker of extravillous trophoblasts), and CDH5 and MYC (markers of column cytotrophoblast) (Fig. 6A). CDCP1+ cells had low expression of TP63 and TEAD4, markers of villous cytotrophoblasts.

Lastly, we functionally investigated the enrichment of cell cycle related gene sets in EpCAM + or CDCP1+ labelled cells using flow cytometry analysis of DNA content to model the percentage of cells in each phase of the cell cycle [32]. We observed that cells with high expression of either EPCAM (Fig. 6C) or CDCP1 (Fig. 6E) have over a two-fold higher level of cells in M phase, indicating a higher rate of cell division. Cells with low expression of these markers (Fig. 6 B, D) have a

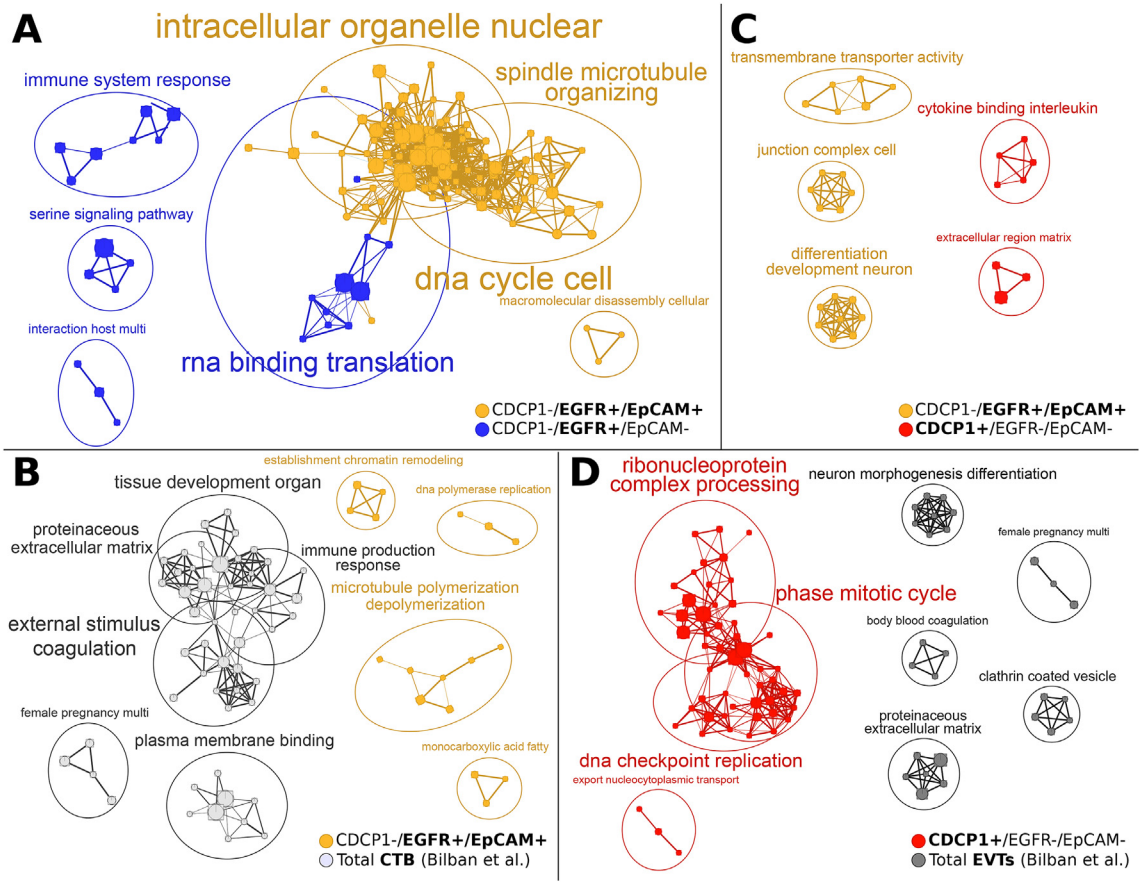

Fig. 5. Gene Set Enrichment Analysis (GSEA) reveals EGFR/EpCAM + and CDCP1 + subpopulations contribute to distinct cell lineages.A) Network graph of significantly different ontology terms identified by GSEA of microarray data based on EPCAM + populations differentially isolated by EGFR co-expression. CDCP1-/EpCAM+/EGFR + cells have enrichment of terms related to cell cycle (Cell Cycle and Spindle Organization) and epigenetic regulation (Methylatransferase Activity and Chromatin Organization), while EGFR+/EpCAM-cells show increase in genes related to epithelium and molecular transport. Terms (nodes) are grouped by gene similarity and labelled by common annotation words. Complete table of GSEA outputs listed in Supplementary Table 5(B) Enrichment map of GSEA comparison of EpCAM+/EGFR+/CDCP1- to the CTB fraction isolated by Bilban et al. shows enriched genes with functions in the cell cycle and chromatin organization, while general CTB show greater enrichment/expression of other functional classes. Complete table of GSEA outputs listed in Supplementary Table 6C) Network graph of significantly different ontology terms identified by GSEA of microarray data for CDCP1+/EpCAM-/EGFR-compared to CDCP1-/EpCAM+/EGFR + populations. Terms (nodes) are grouped by gene similarity and labelled by common annotation words. Of not is the enrichment of cell junctions and transporter activity in CDCP1-/EpCAM+/EGFR + populations and extra cellular matrix and cytokine/interleukin activity in CDCP1+/EpCAM-/EGFR-populations. Complete table of GSEA outputs listed in Supplementary Table 7 (D) Enrichment map of GSEA comparison of EpCAM-/EGFR-/CDCP1+ to migratory EVT populations identified enrichment in genes with functions related to cell cycle, DNA replication and RNA processing. EVT sample are enriched in genes with functions in migration, extracellular matrix. Complete table of GSEA outputs listed in Supplementary Table 8.

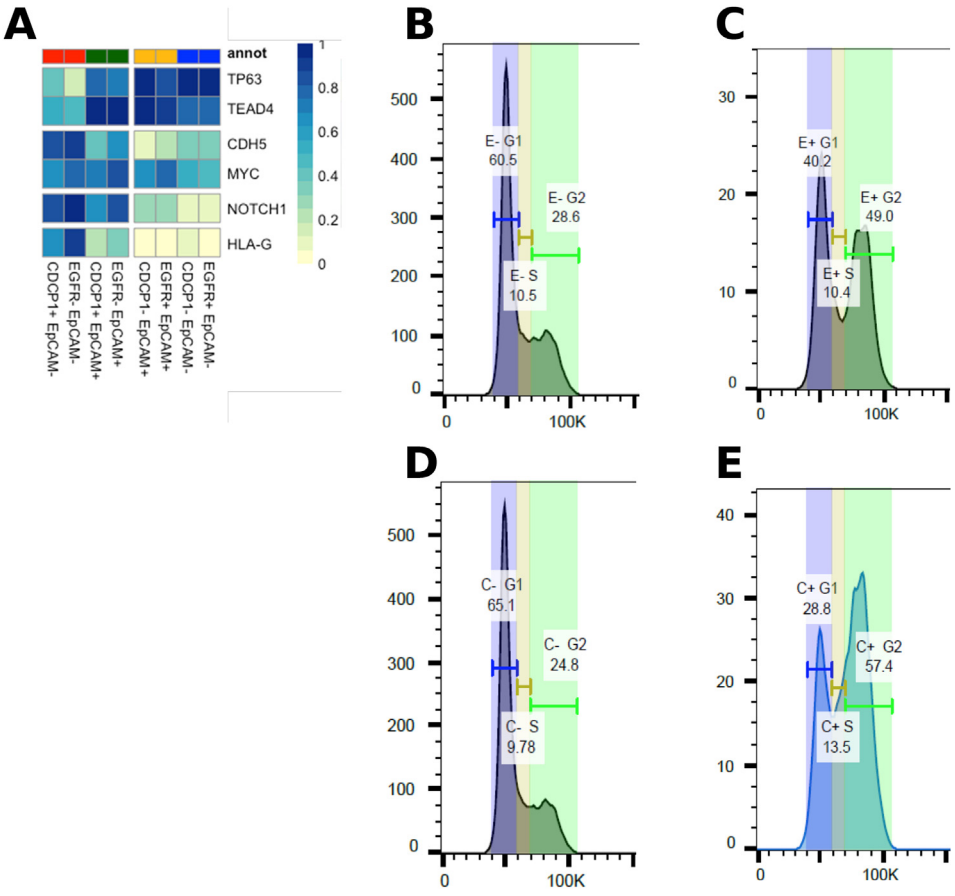

Fig. 6. Cell cycle analysis of EPCAM + and CDCP1 + cell populations.(A) Heatmap of cell lineage specific genes and Notch1, a novel extravillous trophoblast progenitor marker identified by Haider et al.(B) EPCAM negative EGFR + cells have higher G1 and lower G2 phase cells compared to (C) EPCAM positive cells. This indicates that EPCAM expression correlates to increased DNA replication.(D) CDCP1- cells have higher G1 and lower G2% populations compared to (E) CDCP1+ cells. This indicates that CDCP1 expression also correlated to increased DNA replication.

nearly two-fold increase in cells in G1/0 phase indicating quiescence.

4. Discussion

Through our integrated analysis of florescent cytometry, immunolohistology and transcription expression, we identified two novel populations of proliferative trophoblast cells. An EpCAM population of early cytotrophoblasts and a CDCP1 population of extravillus trophoblasts. A significant finding was that the EpCAM positive population of cells are enriched in cell cycle genes as compared to EGFR+/EpCAM- cells within our study and to the total vCTB population assessed by Bilban and colleagues. Although the role of EpCAM in human placentas is still to be elucidated, EpCAM is known to mark highly proliferative tumour cells in many cancers [33–35]. EpCAM is also a marker of proliferative and undifferentiated embryonic cells [36,37] and we believe it is similarly labeling a proliferative subpopulation of human trophoblast cells.

Beyond cell cycle control, work in mice has shown EpCAM is expressed by a progenitor cell type that can give rise to both the labyrinth trophoblast and the sinusoidal giant cells of the labyrinth [38]. Our findings are intriguing as they indicate a conserved role of EpCAM in early placental development of both mice and humans, although the human equivalent of sinsusoidal giant cells is not known.

We also observed enrichment of genes involved in cell cycle regulation in CDCP1 positive cell population as compared to the migratory cell populations of EVTs assessed by Bilban and colleagues [29]. CDCP1 expression was identified on cancerous cells isolated from various organs and is associated poor prognosis in renal and pancreatic cancers [39]. Knockdown experiments in cells lines indicate that CDCP1 can repress epithelial and promotes mesenchymal states. This function fits well with our observed expression of CDCP1 in the distal trophoblast and association of CDCP1 positive cells with EVT lineage that is mesenchymal and migratory. Mouse knock out studies did not identify a phenotype for CDCP1 in placental development, but it is a cell surface marker of the mouse pre and post implantation trophoblast [40].

ERBB2 was described as localized to the distal column trophoblast cells and co-localizing with HLA-G, indicating that it may mark emerging EVTs [27]. A novel finding in our study was the co-localization of ERBB2 and EPCAM at regions of villus sprouting and branching. This expression pattern has not previously been reported and may indicate a role in EPCAM and ERBB2 in placenta morphogenesis beyond differentiation of villus cytotrophoblasts and EVT lineages.

Many of the genes observed to label developmentally regulated populations of cells have known functions and may provide additional information about these cell populations. CD105, more commonly known as Endoglin, is observed as decreasing and has been heavily studied in many human placental cell lineages including endothelial, syncytiotrophoblast, and extravillous trophoblast cells [41]. CD144 (VE-Cadherin) was identified in fetal endothelial cells as a homophilic adherence protein and has also been observed as expressed in term and near term placentas [22]. Markers such as CD107A (LAMP1), CD63 (MLA1), and CD81 (TAPA1) are known to be expressed from endosomes and exosomes [23,25,42]. Endosome transport is an essential function of the human placenta as this is the interface for exchange between maternal and fetal systems [43–45] Of significance was our finding of a population labeled by CD121b (IL1R2) that has previously been identified as a marker of side population cells isolated from first trimester human placenta [11]. High efflux rates of nuclear dyes are more typically observed in stem cells. This property results from specific expression of ABC family transporters that likely function to prevent differentiation by removing small molecule ligands such as steroid hormones. CD122 and CD223 show almost no expression in term human placentas indicating a strong down regulation of expression following first trimester, also making these candidates as markers of

progenitor populations. Our results should encourage others to pursue the isolation and characterization of these different cell populations.

While others have employed flow cytometry screens on human cells and tissues, to our knowledge, our dataset is one of the largest collections of flow cytometry data and one of the first to utilize the automated analysis work flow of openCyto. Additionally, our integration of batch correction and normalization from other high throughput assays to our flow cytometry analysis pipeline has not been reported. Past studies have used flow screens as a qualitative assessment of gene expression [46]. Using our analysis workflow we can treat these large data sets as one would other gene expression data sets, enabling statistical analysis of differences in cell populations.

A caveat to interpretation of flow cytometry data is that we can only assess the change in a cell population as a percentage of the sampled population. We cannot determine if the absolute numbers of cells in a subpopulation change. Our results could reflect an increase in another subpopulation causing a dilution. However, our histological assessment of the markers EPCAM, CDCP1 and ERRB2 indicates that there is a reduction in the total number of cells with these labels. Another caveat of this study is we did not validate our microarray results with further qPCR because the data correlated reasonably with our flow cytometry and histology data, and is supported by previously published datasets.

Isolation protocols for specific cell lineages should be carefully considered because they may have population biases. Histological analysis of the CDCP1 + trophoblast cells indicates that they are embedded within the trophoblast cell column. Bilban and colleagues isolated EVTs based on their ability to migrate out of the columns into matrigel cultures. Apps et al. derived EVT cells by isolation as HLA-G positive cells from primary human trophoblasts allowed to differentiate on fibronectin for 12hrs. The different strategies used by Bilban et al. and Apps et al. to isolate extravillous trophoblast may have contributed to differences in gene expression profiles. There are many EVT subpopulations including endovascular and interstitial EVTs which all express HLA-G therefore the differences seen by Apps et al. may be a result of capturing a different subpopulation or a heterogeneous mixture. It would be interesting to further investigate the EVTs captured by Apps et al. to access their functional capabilities and determine what cells are captured.

An important consideration for past and future studies on human placental development is our observation of rapid changes in cell populations between weeks 4–8 of gestation. Our observation of near ubiquitous expression of EpCAM at weeks 4 and 5 in the villous cytotrophoblasts indicates the early placenta may be broadly proliferating and become more restricted as the branching of the villous tree becomes more elaborate. Human placentas tend to be broadly grouped in to three trimesters with “First Trimester” placentas spanning week 1–12. Our results suggest that there may be earlier phases of development during the first trimester that should be considered. Specifically, our targeted flow cytometry and histology analysis confirmed EpCAM and CDCP1 as having reduced heterogeneity in cell populations by week 10 and significantly changed before week 7. Two recent reports of human trophoblast stem cells isolated from human placentas indicate that these cells can only be isolated from early first trimester, less than 7–8 weeks [47,48]. Intriguingly, this stem cell population aligns with our observed timing of high expression of EpCAM.

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Appendix A. Supplementary data

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