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ORIGINAL ARTICLE

Human endometrial cell-type-specific RNA sequencing provides new insights into the embryo–endometrium interplay

Mariann Koel [1,2], Kaarel Krjutškov[1,3], Merli Saare [1,3], Külli Samuel[1], Dmitri Lubenets[2], Shintaro Katayama[4,5], Elisabet Einarsdottir[4,6], Eva Vargas [7,8,9], Alberto Sola-Leyva [7,8], Parameswaran Grace Lalitkumar [10], Kristina Gemzell-Danielsson [10], David Blesa[11], Carlos Simon[12,13], Fredrik Lanner[14,15], Juha Kere[4,5], Andres Salumets[1,3,14,†], and Signe Altmäe [1,7,8,14,*,†]

[1]Competence Centre on Health Technologies, Tartu, Estonia [2]Department of Cell Biology, Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia [3]Department of Obstetrics and Gynaecology, Institute of Clinical Medicine, University of Tartu, Tartu, Estonia [4]Stem Cells and Metabolism Research Program, Research Programs Unit, University of Helsinki, and Folkhälsan Research Center, Helsinki, Finland [5]Department of Biosciences and Nutrition, Karolinska Institutet, Huddinge, Sweden [6]Science for Life Laboratory, Department of Gene Technology, KTH-Royal Institute of Technology, Solna, Sweden [7]Department of Biochemistry and Molecular Biology, Faculty of Sciences, University of Granada, Granada, Spain [8]Instituto de Investigación Biosanitaria ibs.GRANADA, Granada, Spain [9]Systems Biology Unit, Department of Experimental Biology, Faculty of Experimental Sciences, University of Jaén, Jaén, Spain [10]Department of Women's and Children's Health, Division of Obstetrics and Gynecology, Karolinska Institutet, and Karolinska Univeristy Hospital, Stockholm, Sweden [11]Department of Product Development, IGENOMIX, Valencia, Spain [12]Department of Obstetrics and Gynecology, Valencia University and INCLIVA in Valencia, Valencia, Spain [13]Department of Obstetrics and Gynecology, BIDMC, Harvard University, Boston, MA, USA [14]Department of Clinical Science, Intervention and Technology, Division of Obstetrics and Gynecology, Karolinska Institutet, Stockholm, Sweden [15]Ming Wai Lau Center for Reparative Medicine, Stockholm node, Karolinska Institutet, Stockholm, Sweden

*Correspondence address. Department of Biochemistry and Molecular Biology, Faculty of Sciences, University of Granada, Fuente Nueva S/N, 18071 Granada, Spain. E-mail: signealtmae@ugr.es https://orcid.org/0000-0002-0708-1865

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STUDY QUESTION: Which genes regulate receptivity in the epithelial and stromal cellular compartments of the human endometrium, and which molecules are interacting in the implantation process between the blastocyst and the endometrial cells?

SUMMARY ANSWER: A set of receptivity-specific genes in the endometrial epithelial and stromal cells was identified, and the role of galectins (LGALS1 and LGALS3), integrin β1 (ITGB1), basigin (BSG) and osteopontin (SPP1) in embryo–endometrium dialogue among many other protein–protein interactions were highlighted.

WHAT IS KNOWN ALREADY: The molecular dialogue taking place between the human embryo and the endometrium is poorly understood due to ethical and technical reasons, leaving human embryo implantation mostly uncharted.

STUDY DESIGN, SIZE, DURATION: Paired pre-receptive and receptive phase endometrial tissue samples from 16 healthy women were used for RNA sequencing. Trophectoderm RNA sequences were from blastocysts.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Cell-type-specific RNA-seq analysis of freshly isolated endometrial epithelial and stromal cells using fluorescence-activated cell sorting (FACS) from 16 paired pre-receptive and receptive tissue samples was performed. Endometrial transcriptome data were further combined *in silico* with trophectodermal gene expression data from 466 single cells originating from 17 blastocysts to characterize the first steps of embryo implantation. We constructed a protein–protein interaction network between endometrial epithelial and embryonal trophectodermal cells, and between endometrial stromal and trophectodermal cells, thereby focusing on the very first phases of embryo implantation, and highlighting the molecules likely to be involved in the embryo apposition, attachment and invasion.

†These authors share last authorship and contributed equally to this work.

MAIN RESULTS AND THE ROLE OF CHANCE: In total, 499 epithelial and 581 stromal genes were up-regulated in the receptive phase endometria when compared to pre-receptive samples. The constructed protein–protein interactions identified a complex network of 558 prioritized protein–protein interactions between trophectodermal, epithelial and stromal cells, which were grouped into clusters based on the function of the involved molecules. The role of galectins (LGALS1 and LGALS3), integrin β1 (ITGB1), basigin (BSG) and osteopontin (SPP1) in the embryo implantation process were highlighted.

LARGE SCALE DATA: RNA-seq data are available at www.ncbi.nlm.nih.gov/geo under accession number GSE97929.

LIMITATIONS, REASONS FOR CAUTION: Providing a static snap-shot of a dynamic process and the nature of prediction analysis is limited to the known interactions available in databases. Furthermore, the cell sorting technique used separated enriched epithelial cells and stromal cells but did not separate luminal from glandular epithelium. Also, the use of biopsies taken from non-pregnant women and using spare IVF embryos (due to ethical considerations) might miss some of the critical interactions characteristic of natural conception only.

WIDER IMPLICATIONS OF THE FINDINGS: The findings of our study provide new insights into the molecular embryo–endometrium interplay in the first steps of implantation process in humans. Knowledge about the endometrial cell-type-specific molecules that coordinate successful implantation is vital for understanding human reproduction and the underlying causes of implantation failure and infertility. Our study results provide a useful resource for future reproductive research, allowing the exploration of unknown mechanisms of implantation. We envision that those studies will help to improve the understanding of the complex embryo implantation process, and hopefully generate new prognostic and diagnostic biomarkers and therapeutic approaches to target both infertility and fertility, in the form of new contraceptives.

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Key words: blastocyst / endometrial epithelium / embryo implantation / endometrial receptivity / endometrial stroma / interactome

WHAT DOES THIS MEAN FOR PATIENTS?

This study investigates the molecular dialogue between the human embryo and the endometrium, i.e. the inner lining of the uterus. As it is ethically and technically impossible to study embryo implantation in the uterus in real life, the detailed process remains unknown, and we require alternative tools to predict which molecules could interact in the embryo implantation process. With the analysis of RNA sequencing techniques, we were able to predict the potential proteins involved in the embryo–endometrium cross-talk during successful implantation. We identified a molecular network of 558 protein–protein interactions between the embryo and the endometrium. Several of the previously known molecular interactions were confirmed but also new molecules in the interplay were detected. Detailed knowledge of the molecular embryo–endometrium dialogue is important for understanding human reproduction and some underlying causes of female infertility.

Introduction

One of the most elegant and fascinating interactions in human physiology takes place between an embryo and the endometrium, in order to initiate and maintain pregnancy. For successful embryo implantation, the development of an embryo into a blastocyst and the differentiation of the endometrium into the receptive phase need to be synchronized. The first physical contact between an implantation-competent blastocyst and the receptive-phase endometrium involves sequential stages of apposition, attachment and invasion, which together steer the entire process of implantation (Norwitz *et al.*, 2001; Haller-Kikkatalo *et al.*, 2014; Evans *et al.*, 2016; Ashary *et al.*, 2018). The first step of implantation, apposition, is characterized by unstable adhesion where the surface of the blastocyst touches the endometrial epithelium (Wang and Dey, 2006; Hernández-Vargas *et al.*, 2020). During this phase, the blastocyst rolls on the epithelial lining, anchors itself into the mucus and orients

itself so that the polar trophectodermal part of the embryo, located nearest to the inner cell mass, faces the uterine decidua (Pellicer *et al.*, 2002; Deglincerti *et al.*, 2016). During the attachment phase, the embryo adheres to the endometrial epithelial cells via the polar trophectodermal cells (Meistermann *et al.*, 2021). This phase is characterized by specific receptor–ligand binding and signalling via integrins and E-cadherins (Aplin, 1997). In the early invasion stage, the embryo dislodges epithelial cells, penetrates the basal lamina, establishes contact with the endometrial stromal cells and finally invades into the uterine decidua (Norwitz *et al.*, 2001). The events that orchestrate these processes in implantation are coordinated by numerous factors under the influence of the ovarian hormones oestrogen and progesterone (Abbas *et al.*, 2020). Identification of the molecular events, however, remain hardly accessible for ethical reasons, making human embryo implantation a true 'black box' in developmental biology. Thus, the molecular dialogue taking place between the embryo and the endometrium is still poorly understood and the proteins involved in the implantation process are largely unknown.

We have previously modelled the human embryo–endometrium molecular crosstalk by studying the transcriptome of the whole embryos and full-thickness endometrial biopsies (Altmäe *et al.*, 2012). Gene expression analyses of whole tissues neglect the cell-type specific profiles and proportions of the cell populations within the sample, thereby likely confounding the results of differential transcriptome analyses (Suhorutshenko *et al.*, 2018). With a novel approach, we set out to analyse the cell-type-specific transcriptome of freshly isolated pre-receptive and receptive stage endometrial epithelial and stromal cells and combine the data with transcriptome data from trophectodermal cells of blastocyst stage embryos in order to identify the first molecular events taking place in the embryo–endometrium dialogue.

Materials and methods

Endometrial samples

The study was approved by the Research Ethics Committee at the University of Tartu (Estonia) and Instituto Valenciano de Infertilidad (Spain), and written informed consent was obtained from all participants. All methods were performed in accordance with the relevant guidelines and regulations.

Altogether, 16 healthy fertile women from Estonia and Spain were recruited. The average age of the women was 29.7 ± 3.2 years, all had normal BMI (average BMI: 22.8 ± 2.3), a regular menstrual cycle (25–35 days), had not used hormonal medication for 3 months before recruitment, had normal serum levels of progesterone, prolactin and testosterone, negative screening results for sexually transmitted diseases, no uterine pathologies, no endometriosis nor polycystic ovary syndrome and had at least one live-born child. Menstrual cycle dating was confirmed by combining menstrual cycle history and luteinizing hormone (LH) peak estimation (BabyTime, Pharmanova, Beograd, Serbia), vaginal ultrasound and endometrial histology by Noyes' criteria (Noyes *et al.*, 1975).

Each participating woman provided two endometrial biopsy samples within the same menstrual cycle: from early secretory phase (2 days after the LH peak, LH + 2, defined as pre-receptive phase) and from mid-secretory phase (LH + 7/+8, defined as receptive phase) (Fig. 1).

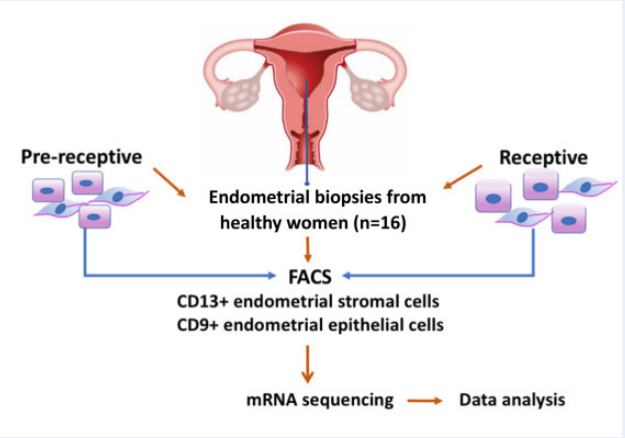

Figure 1. Overview of endometrial sample collection and study design. The preparation of the endometrial biopsies for the cell sorting has been described in detail (Krjutškov *et al.*, 2016). Briefly, the biopsied tissue samples were thawed and dissociated; endometrial stromal cells were stained with fluorochrome-conjugated mouse anti-human CD13 monoclonal antibody (clone TUK1, R-Phycoerythrin, Invitrogen, CA, USA); epithelial cells were stained simultaneously with fluorochrome-conjugated mouse anti-human CD9 monoclonal antibody (clone MEM-61, FITC, Novus Biologicals, Littleton, USA); and all dead cells were stained with DAPI (final concentration of 0.5 μg/ml) (Invitrogen). CD9 or CD13 positive and DAPI low signal or negative cells were sorted by fluorescence-activated cell sorting (FACS) and collected separately into QIAzol Lysis Reagent (Qiagen). The percentages of both epithelial (CD9 positive) and stromal (CD13 positive) cells varied considerably from patient to patient: in the early secretory phase CD9 positive (epithelial) cells composed 26.4–87.7% of the cells, while the number in mid-secretory samples was 8.2–61.8%. Divergence of CD13-positive (stromal) cells was 3.4–50.2% in early secretory biopsy samples, and 6.6–59.0% in mid-secretory phase samples. To verify the purity of our isolated cell populations, we performed additional FACS analysis using a leukocyte-specific CD45 antibody (BD Pharmingen, cat: 555485). CD45 is present on all human leukocytes including lymphocytes, monocytes, granulocytes, eosinophils and thymocytes. We detected that only 0.2% of isolated CD13+ and only 1.1% of CD9+ cells were CD45 positive. As the fractions of CD45+ cells were small and all our isolated cells from early and mid-secretory endometria were manipulated in the exact same way, we believe that the purity of the cell populations between different samples is comparable and does not affect the overall expression profile.

The receptivity status of all analysed samples was confirmed using the beREADY endometrial receptivity test (www.beready.ee) that is based on the transcriptomic profiling of receptivity biomarkers (Altmäe *et al.*, 2017). The biopsies were obtained using a Pipelle catheter (Laboratoire CCD, Paris, France) and the samples were frozen at –80°C using cryopreservation media to keep cells alive, as described in our previous study (Krjutškov *et al.*, 2016). The preparation of the endometrial biopsies after thawing for sorting of CD9-positive epithelial and CD13-positive stromal cells is summarized in Fig. 1; a detailed protocol has been described previously (Kato *et al.*, 2007; Krjutškov *et al.*, 2016; Masuda *et al.*, 2016). Total RNA was isolated immediately from the sorted cells using the RNeasy Micro kit (Qiagen, Hilden, Germany).

RNA-seq analysis

Full transcriptome analysis of the sorted endometrial cells was performed using the single-cell tagged reverse transcription (STRT) protocol (Krjutškov et al., 2016) with modifications that are required for bulk RNA analysis (RNA-seq data are available at www.ncbi.nlm.nih.gov/geo under accession number GSE97929). Samples of 10 ng of high-quality RNA extracted from enriched epithelial and stromal cell populations was converted into cDNA and amplified using 15 plus 10 PCR cycles to form an Illumina-compatible sequencing library with single 8 bp indexes. The STRTprep pipeline, available at https://github.com/shka/STRTprep/tree/v3dev, was used to process the raw RNA-seq reads, aligning to the hg19 genome, quantitating the expression levels and running the differential expression tests. Significance of fluctuation on the gene expression was tested by comparison with fluctuation of spike-in levels as described before (Krjutškov et al., 2016). Differential expression between sample types was tested by SAMstrt (https://github.com/shka/R-SAMstrt) (Katayama et al., 2013), which is based on Wilcoxon statistics with multiple Poisson resampling to equalize the spike-in depths, with the diffexp score representing the average of the Wilcoxon statistics among the resampling. Significantly differentially regulated genes were defined by Benjamini–Hochberg adjusted *P*-value <0.05 for the fluctuation and *Q*-value <0.05 for the differential expression. The gene expression was compared between the pre-receptive and receptive phase endometria for the enriched fraction of CD9-positive epithelial cells and CD13-positive stromal cells. The endometrial genes that were identified as significantly up-regulated in receptive versus pre-receptive phase, but were detectable (expression value > 0) in $<75\%$ of the receptive phase samples were filtered out.

The transcripts expressed at the polar trophectoderm of the blastocyst stage embryo were obtained from our previously published human embryo transcriptional mapping, which was based on RNA-seq of 466 single embryonal cells from 17 human blastocyst-stage embryos from days 6 and 7 (Petropoulos et al., 2016). Briefly, cDNA libraries were generated using the Smart-seq2 protocol (Picelli et al., 2014). Gene expression levels were estimated in terms of reads per million mapped reads (RPKM). Based on the fact that human embryo attaches to the endometrial surface with the polar side (Johnson, 2012), we used the subset of genes that were expressed on the polar trophectodermal cells and filtered in the transcripts with mean expression of >10 RPKM. As confirmation, the recently identified maturation marker of polar trophectoderm, NR2F2 (Meistermann et al., 2021), was detected in our dataset of polar trophectodermal cells.

Construction of protein–protein interaction networks

Human protein–protein interactions (PPI) were downloaded from the UniHI version 7.1 database (http://www.unihi.org) (Kalathur et al., 2014) and STRING 11.0 database (http://version11.string-db.org/) (Szklarczyk et al., 2015). These two databases involve the largest number of experimentally verified interactions to date (Bajpai et al., 2020). The most relevant interactions from the STRING database were attained by retaining only interactions with confidence score of ≥ 0.7. PPI between (i) endometrial epithelial and stromal cells and (ii) the embryo and the endometrium were defined between the genes that were significantly up-regulated in the receptive phase endometrial epithelial and stromal cells and all identified genes expressed in the polar trophectodermal cells. The detected transcript identifiers were converted into corresponding proteins and only these genes whose expression was positively correlating (Spearman $R > 0$) with the protein expression profile in the previously published proteome encyclopaedia (Nusinow et al., 2020) were added to the subsequent analyses. To explore the possible epithelium–stroma interactions within receptive endometrium and embryo–endometrium interaction partners, we focussed only on the genes that encode proteins localized at the cell surface, extracellular matrix or secreted by the cells, according to the UniProt database (Accessed date: 10 April 2020). Only the PPI that took place between different cell types were described. To delineate the most relevant PPI for implantation processes, predicted interactions were clustered using the Cytoscape application AutoAnnotate (http://apps.cytoscape.org/apps/autoannotate) using the default settings (Kucera et al., 2016). Enrichment analyses for the interacting molecules within endometrium were performed for the three largest clusters ($n > 11$) and analyses for the embryo–endometrium interaction were performed for the five largest clusters ($n > 18$) by g:Profiler (Raudvere et al., 2019). Enrichment analyses were conducted by hypergeometric test and the resulting enrichment *P*-values were further adjusted for multiple testing using the g: SCS (Set Counts and Sizes) method, developed to address the non-independent structure of tested GO terms (Raudvere et al., 2019).

RNA-seq validation by quantitative real-time PCR

Endometrial cell-type-specific RNA-seq was validated by quantitative real-time PCR (qRT-PCR) on a selected set of genes (*APOC1*, *CLU*, *RGS16*, *RORC* and *TEX40*), using flow cytometer isolated endometrial LH + 2 epithelial ($n = 10$) and stromal ($n = 8$) cells, and LH + 8 epithelial ($n = 15$) and stromal ($n = 12$) cells obtained from the same individuals used in the RNA-seq analyses. qRT-PCR primers are listed in Supplementary Table SI. Samples of 10 ng of DNase-treated (TURBO DNA-free™ kit, Ambion Inc., Austin, TX, USA) RNA was converted into cDNA using RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific Inc. MA, USA). qRT-PCR was performed using 1× HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Estonia) according to the conditions specified by the manufacturer. The list of primers is provided in Supplementary Table SI. *SDHA* was used as endogenous control and the expression differences between LH + 2 and LH + 8 cell populations were calculated using Student's t-test, using a *P*-value cut-off of <0.05. The 2-ΔΔCt method was used for calculating the relative gene expression and to determine the fold change in gene expression between LH + 2 and LH + 8 samples. The comparisons with RNA-seq fold change values are provided in Supplementary Table SII.

Results

Gene expression patterns of epithelial and stromal cells through the secretory phase

To identify the cell-type-specific transcriptome changes between the pre-receptive and receptive endometria, we conducted separate differential expression analyses on endometrial epithelial and stromal cells. Out of 12 339 gene transcripts detected in the receptive phase

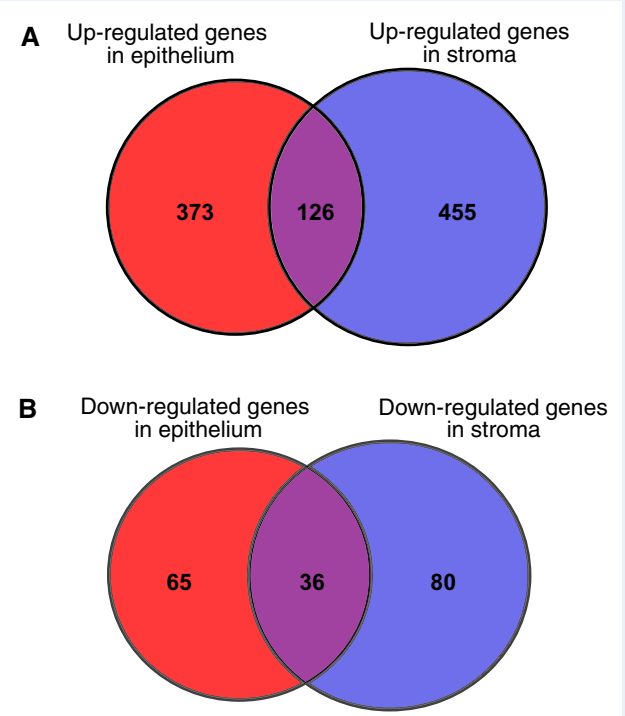

Figure 2. Cell-type-specific gene expression. (**A**) Up-regulated genes in epithelial and stromal cells in receptive versus pre-receptive phase endometrium. (**B**) Down-regulated genes in epithelial and stromal cells in receptive versus pre-receptive phase endometrium. Gene lists are provided in Supplementary Table SIII.

endometrial epithelial cells, 499 genes were up-regulated and 101 genes were down-regulated when compared to the pre-receptive phase samples (Fig. 2, Supplementary Table SIII). Out of the 12 944 genes detected in the stromal cells, 581 genes were significantly up-regulated and 116 genes were down-regulated when compared to the pre-receptive phase samples. We compared the up- and down-regulated genes between two cell types to identify the unique epithelium- and stroma-specific genes. As 126 receptivity phase genes were up-regulated and 36 genes were down-regulated in both cell types, 373 genes (74.7%) remained as uniquely up-regulated and 65 genes (64.4%) remained as uniquely down-regulated in epithelial cells, while 455 genes (78.3%) remained as uniquely up-regulated and 80 genes (69.0%) remained as uniquely down-regulated in stromal cells (Fig. 2, Supplementary Table SIII). In the following analyses, we considered all differentially expressed genes as potential endometrial receptivity-specific genes.

Validation by qRT-PCR confirmed the RNA-seq expression change for five genes selected for validation: *APOC1*, *CLU*, *RGS16*, *RORC* and *TEX40* (Supplementary Table SII).

Predicted PPI within the receptive phase endometrium

The list of endometrial cell-type-specific molecules enabled us to predict potential interactions between epithelial and stromal cells in the receptive phase endometrium. In the *in silico* analyses of the RNA-seq data, we prioritized the interactions with tested gene–protein correlation in the previously published proteome encyclopaedia (Nusinow *et al.*, 2020). Among the up-regulated 499 endometrial epithelial genes, 418 (83.8%) and among the up-regulated 581 endometrial stromal genes, 458 (78.8%) showed positive correlation between the gene and protein expression (Supplementary Table SIV). These genes were mapped to respective protein products and the epithelial–stromal protein–protein interaction networks were constructed. This epithelium–stroma network contained 194 predicted interactions, where 36 (18.6%) were unique to the epithelial cells and 67 (34.5%) to the stromal cells (Supplementary Table SV). Next, we clustered the constructed PPI networks, which resulted in 21 clusters, where the three biggest clusters represented 53% of all the nodes (Supplementary Fig. S1). Enrichment analyses were performed for the three biggest clusters: Cluster A that integrated 27 proteins and processes involved in wound healing and regulation of cell proliferation; Cluster B that integrated 13 proteins and antigen processing and presentation process; and Cluster C that integrated 11 proteins and regulation of immune response process (Supplementary Table SV).

Predicted PPI between trophectoderm and endometrial cell types

The unique list of cell-type-specific molecules of the receptive phase endometrium allowed us to predict potential interactions that could take place between embryonic trophectodermal cells and endometrial epithelial or stromal cells at the late apposition, attachment and initiation of invasion processes. To find interacting proteins, we used cell-type-specific endometrial transcriptomic RNA-seq data and single-cell transcriptomic data of polar trophectodermal cells, and prioritized the interactions where there is also evidence of gene–protein correlation (Nusinow *et al.*, 2020). Of the up-regulated endometrial epithelial genes, 83.8%, and of the up-regulated endometrial stromal genes, 78.8% showed positive correlation between the gene and protein expression. Similarly, 4812 out of 5578 (86.3%) polar trophectodermal genes showed positive correlation between gene and protein levels (Supplementary Table SIV). Those genes were mapped to their respective protein products and used for the construction of embryo–endometrial protein–protein interaction networks. This network contained 558 predicted interactions, encompassing 222 proteins (Supplementary Table SVI). Within the PPI network, 125 (56.3%) were unique to the polar trophectodermal cells, 16 proteins (7.2%) were detected in trophectodermal and stromal cells and 13 proteins (5.9%) were detected in trophectodermal and epithelial cells (Supplementary Table SVI). Only seven proteins (3.2%) (HLA-B, TIMP1, APOL1, AGR2, SERPING1, VCAN and CALCRL) were expressed in both epithelial and stromal cells but were not detected in trophectodermal cells. There were 16 (7.2%) proteins uniquely expressed in epithelial cells and 38 (17.1%) uniquely expressed in stromal cells and 7 (3.2%) proteins were detected in all three cell types.

We further clustered the constructed PPI networks and performed enrichment analyses for each cluster to interpret it and describe the possible role of the predicted interactions. Altogether, 222 interacting proteins were divided into 15 clusters, where the top five clusters included more than 18 proteins (Supplementary Table SVI, Supplementary Fig. S2). The formed clusters were ordered based on

the number of interacting proteins and named alphabetically. In the next section, we will explore the five largest clusters (Clusters A, B, C, D and E) that contained more than half of the interacting proteins.

Cluster A: cell adhesion

The largest cluster (Cluster A, Fig. 3) contained more than a fifth of the interacting proteins (46 proteins, 20.7%) and a tenth of the interactions (85 interactions, 15.2%) and was mainly enriched in proteins known to be involved in cell-adhesion (adj. *P*-value 6.5×10^{-20}). Indeed, 24 (52.2%) of the interacting proteins are transmembrane proteins (Fig. 3). The protein with the highest degree of interactions in this cluster was integrin β1 (ITGB1), a transmembrane protein expressed on both epithelial and trophectodermal cells. Epithelial MET, a proto-oncogene that interacts with trophectodermal ITGB1 (Fig. 3) has been detected as an important endometrial receptivity gene product, physically interacting with foetal interface at implantation (Ntostis *et al.*, 2021). Integrin β1 interaction with basigin (BSG; expressed in stromal and trophectodermal cells (Fig. 3)) has been shown to regulate embryo implantation (Lee *et al.*, 2013) and integrin β1 regulation by all cells expressed galectin-3 (LGALS3) that mediates trophoblast cells invasion (Bojić-Trbojević *et al.*, 2019). The LGALS3 interaction with trophectodermal CD98 (SLC3A2) is, in turn, important for trophoblast cell fusion (Dalton *et al.*, 2007). We also identified the interaction between the epithelial transmembrane protein CD44 and trophectodermal syndecan-1 (SDC1), likely regulating trophoblast invasion (Ibrahim *et al.*, 2017). A previous *in vitro* study showed that functional blocking of epithelial CD44 led to delayed attachment during early stages of implantation (Berneau *et al.*, 2019a), and transcriptome analysis of trophoblast-epithelium in *in vitro* model highlighted CD44 involvement in early trophoblast attachment (Vergaro *et al.*, 2021). Cluster A also contains secreted Insulin-like growth factor-binding protein-2 (IGFBP2), which binds to integrin β1 and this interaction has been shown to regulate cell migration and invasion (Han *et al.*, 2014).

Cluster B: cell adhesion and migration

The second largest cluster was Cluster B (Fig. 4) with 46 interacting proteins (20.7%), including more than quarter of the PPIs (161 interactions, 28.9%). Most of the involved proteins (24 proteins, 52.2%) undergo post-translational protein modifications (adj. *P*-value 9.1×10^{-26}) and could therefore participate in cellular signalling. In fact, ∼60% of the interacting proteins were secreted proteins (Fig. 4). For example, proteolytically cleaved extracellular matrix cytokine osteopontin (*SPP1*) in the epithelium may interact with trophectodermal transmembrane integrin α5β1 (ITGA5) and therefore support cell adhesion and migration (Barry *et al.*, 2000). Epithelially secreted osteopontin interacted with trophectodermal extracellular matrix protein galectin-1 (*LGALS1*), which has been related to blastocyst attachment and trophoblast migration (Barrientos *et al.*, 2014). Versican (VCAN), an extracellular matrix protein, expressed by both endometrial epithelial and stromal cells, and involved in cell adhesion and migration, with a role in implantation (San Martin *et al.*, 2003; Altmäe *et al.*, 2012), was detected as one central molecule from the endometrial interface interacting with the trophectodermal proteins APOE, APOA1, APOA2, LAMB1, LAMC1, LGALS1 and MXRA8 that are all involved in cell adhesion and migration processes.

Cluster C: proteins regulating secretion processes

Proteins in cluster C (Fig. 5) were enriched for proteins regulating the secretion processes (adj. *P*-value 2.4×10^{-7}), key processes in releasing hormones, enzymes and other factors, and intercellular signalling (Trikha *et al.*, 2010). This cluster contained 31 interacting proteins (14.0%) and 32 interactions (5.7%). Almost half of the proteins (45.2%) were secreted proteins, including epithelial cell-specific interferon (IFN)-stimulated gene 15 (ISG15), stromal cell-specific chemokine (C-X-C motif) ligand 13 (CXCL13), epithelial and trophectodermal-specific proteins stratifin (SFN) and granulin (GRN), trophectodermal NPC intracellular cholesterol transporter 2 (NPC2) and gamma-glutamyl hydrolase (GGH), as well as stromal and trophectodermal-specific proteins cathepsin B (CTSB) and fatty acid-binding protein, epidermal (FABP5). We predicted six of these proteins (NPC2, GGH, ISG15, CTSB, FABP5 and GRN) to interact with the extracellular matrix protein annexin A2 (ANXA2), which was expressed by all cell types. This secretion pathway protein is implicated in endometrial epithelial cell migration and trophoblast outgrowth, being therefore essential for embryo adhesiveness to the human endometrium (Garrido-Gómez *et al.*, 2012). Indeed, ANXA2 was recently detected as an important endometrial receptivity gene product that physically interacts with trophectodermal cells at implantation (Ntostis *et al.*, 2021).

Cluster D: cell–cell adhesion

Cluster D is rather small, including 25 (11.2%) of the PPI proteins and 27 (4.6%) of the interactions, and is enriched in cell–cell adhesion molecules (adj. *P*-value 1.4×10^{-7}). Of the interacting proteins in the network 76% were transmembrane proteins and the cluster formed around the transmembrane E-cadherin (CDH1) (Fig. 6), an important molecule in cell–cell adhesion with implications in the implantation process (Haller-Kikkatalo *et al.*, 2014). E-cadherin was expressed in both epithelial and trophectodermal cells, and we predicted it to bind claudins (CLDN3, CLDN4, CLDN7), flotillins (FLOT1, FLOT2), cadherins (CDH3, CDH13), plasminogen activator inhibitor-1 (SERPINE1), alpha-catulin (CTNNAL1) and epithelial cell adhesion molecule (EPCAM). However, it is unclear whether these interactions are heterotypic trans-interactions between the epithelial and the trophectodermal cells or take place on the surface of trophectodermal cells. Interestingly, knock-down of SERPINE1 in the human ECC-1 cell line demonstrated significantly reduced trophectoderm spheroid adhesion (Evans *et al.*, 2020).

Cluster E: extracellular matrix organization

Cluster E was enriched in proteins known to be involved in extracellular matrix organization (adj. *P*-value 1.8×10^{-6}), where seven (40%) of the interacting proteins were extracellular matrix proteins and another seven (40%) were transmembrane proteins (Fig. 7). For example, the secreted protein oestrogen-regulated anterior gradient 2 (AGR2), unique to epithelium/stroma in our study, interacting with Ly6/PLAUR domain-containing protein 3 (LYPD3) and dystroglycan 1 (DAG1), has been shown to increase the aggressiveness of cancer cells through the regulation of receptor adhesion and interaction with the extracellular matrix (Salmans *et al.*, 2013; Arumugam *et al.*, 2015). Trophoblast cell-secreted procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 (PLOD3) could regulate extracellular matrix protein COL12A1 in the stroma to promote cells invasion and migration (Li *et al.*, 2020).

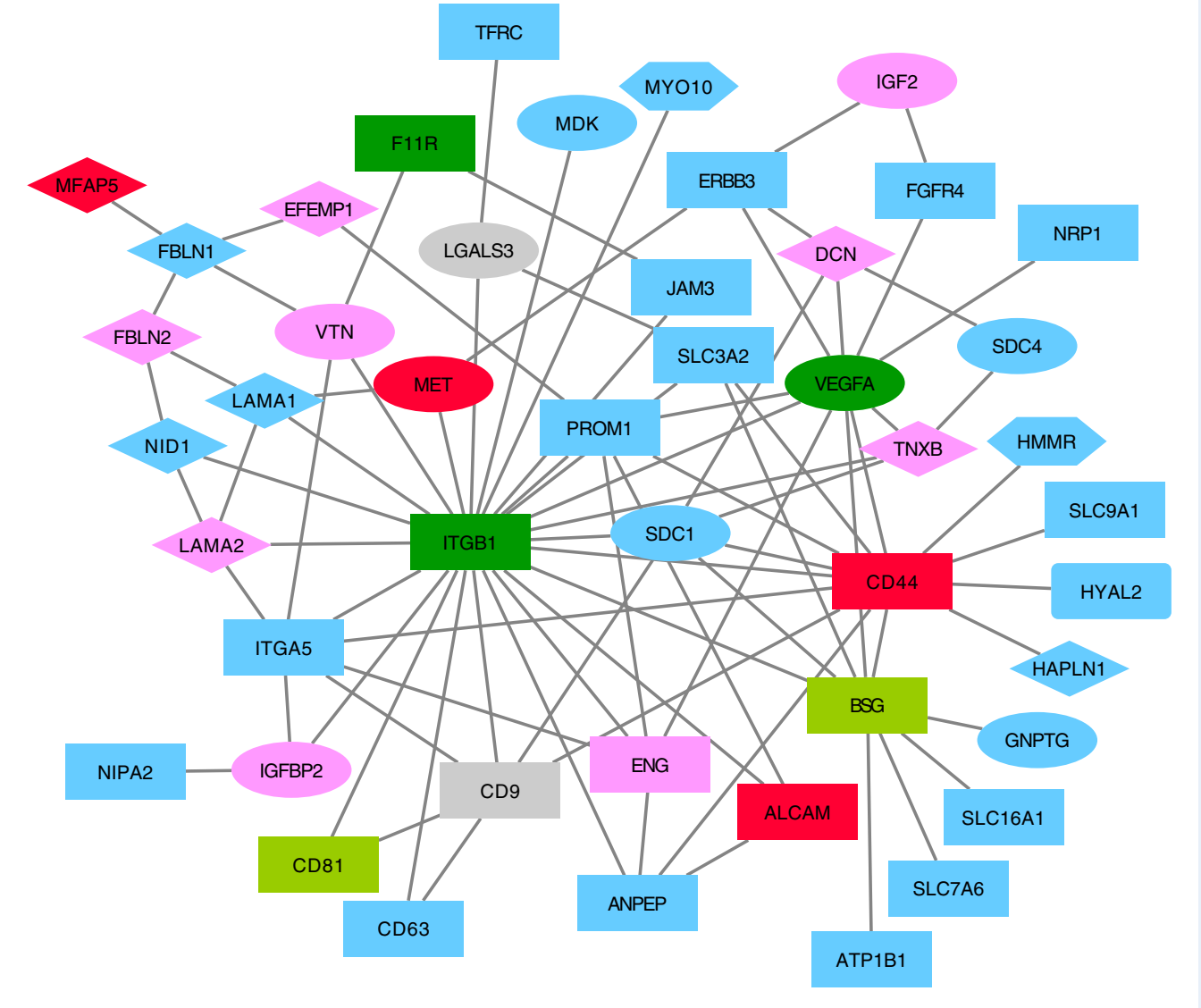

Figure 3. Cluster A. Molecular interactions of polar trophectodermal cells with endometrial epithelial and stromal cells. Pink: stromal proteins, red: epithelial proteins, light blue: trophectodermal proteins, violet: epithelial and stromal proteins, green: epithelial and trophectodermal proteins, light green: stromal and trophectodermal proteins, grey: epithelial, stromal and trophectodermal proteins. Ellipse: secreted proteins, diamond: extracellular matrix proteins, octagon: membrane-bound proteins, rectangle: transmembrane proteins. Interacting proteins and interactions are provided in Supplementary Table SVI.

Discussion

Implantation of a healthy embryo into a receptive endometrium is a critical step in the establishment of pregnancy, but the underlying molecular mechanisms of the first steps of implantation are still not clearly established. Our study reveals *in silico* molecular networks between the polar trophectodermal cells of implantation-competent embryo and the epithelial and stromal cells of receptive endometrium, extending the knowledge of the first steps of embryo implantation processes in humans.

We found that the gene expression profiles are significantly different between the two main cell subtypes in the human endometrium, epithelial and stromal cells, which is in line with the previous single-cell endometrial transcriptome study (Wang *et al.*, 2020; Garcia-Alonso *et al.*, 2021). When analysing the possible epithelial–stromal cellular interplay within the receptive-phase endometrium, we detected a big part of the interactions highlighting the importance of immune responses, wound healing and regulation of cell proliferation, which are important processes in endometrial receptivity (Evans *et al.*, 2016; Altmäe *et al.*, 2017). In fact, a number of common endometrial receptivity biomarkers (Díaz-Gimeno *et al.*, 2011; Altmäe *et al.*, 2017; Enciso *et al.*, 2018; Giacomini *et al.*, 2021) were detected in the endometrial cell subpopulations including *ANXA4*, *ARG2*, *C4BPA*, *CFD*, *CLDN4*, *CP*, *DKK1*, *GPX3*, *IL15*, *MAOA*, *MT1H*, *NNMT*, *PAEP*, *S100P*,

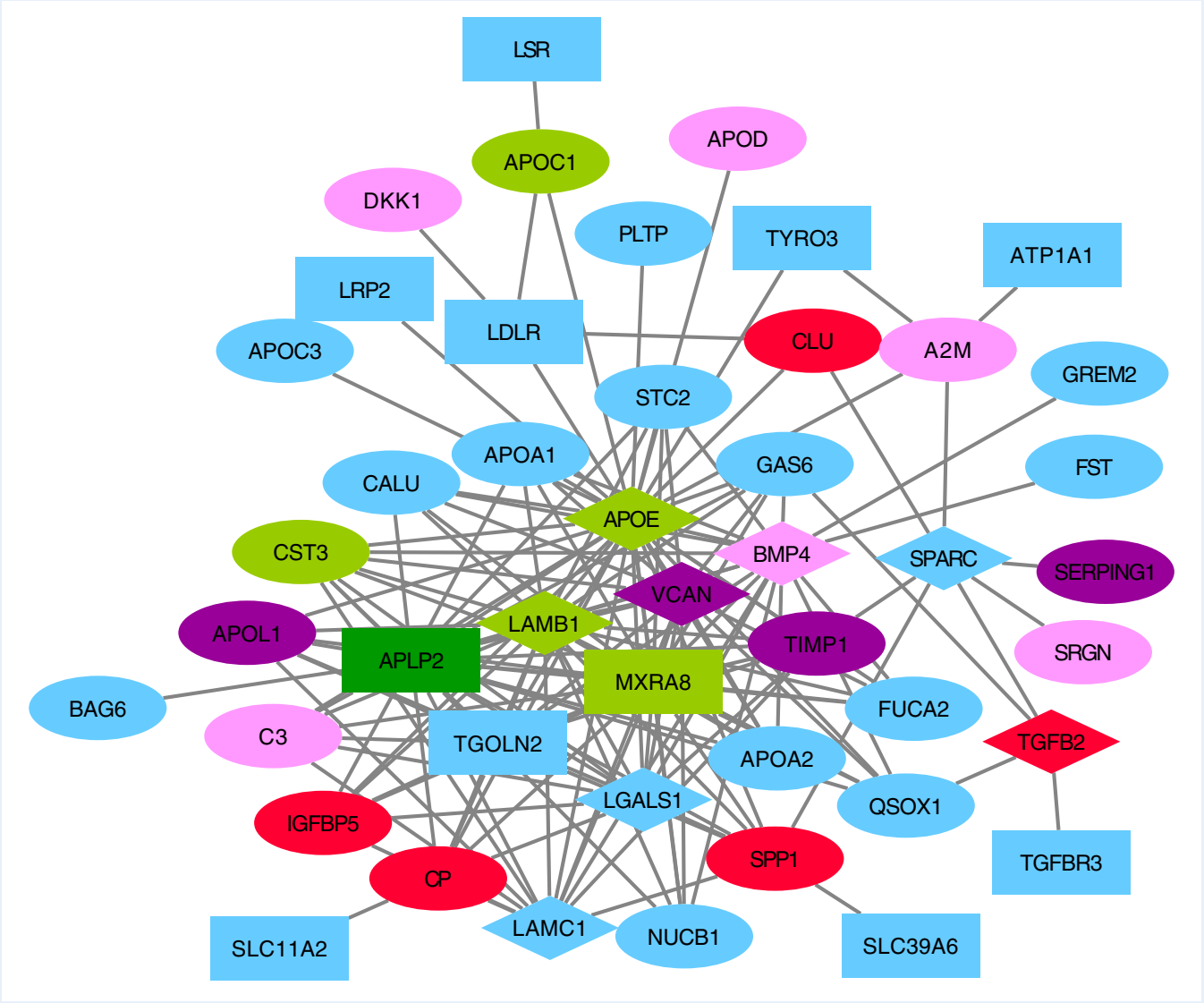

Figure 4. Cluster B. Molecular interactions of polar trophectodermal cells with endometrial epithelial and stromal cells. Pink: stromal proteins, red: epithelial proteins, light blue: trophectodermal proteins, violet: epithelial and stromal proteins, green: epithelial and trophectodermal proteins, light green: stromal and trophectodermal proteins, grey: epithelial, stromal and trophectodermal proteins. Ellipse: secreted proteins, diamond: extracellular matrix proteins, octagon: membrane bound proteins, rectangle: transmembrane proteins. Interacting proteins and interactions are provided in Supplementary Table SVI.

SERPING, *SPP1*, *SOD2* and *TCN1* among other genes. Considering the feasibility of the whole-tissue biopsies versus isolation of cell subpopulations within the sample, we propose that the differentially regulated genes *C4BPA*, *GPX3*, *MT1H* and *S100P* that were identified in both endometrial cell types, and highlighted by previous studies, could serve as high-confidence candidates for endometrial receptivity biomarkers.

A few studies have investigated the possible human embryo–endometrium interactions where whole tissue samples have been analysed (Altmäe *et al.*, 2012; Haouzi *et al.*, 2012; Vilella *et al.*, 2015). Here, we focussed on the specific cell subpopulation transcriptome profiles of freshly isolated non-cultured endometrial epithelial and stromal cells, and model the initial molecular embryo–endometrium dialogue together with the potential paracrine interactions (Fig. 8). While we confirmed the presence of several previously detected proteins in the crosstalk (FBLN1—MFAP5, NID1—FBLN2, LAMA1—FBLN2, DAG1—LAMA2, VEGFA—ADAMTS, SPP1—ITGAV, PDGFA—PDGFRA) (Altmäe *et al.*, 2012), our focus on embryo–endometrium interactions using confirmed PPI based on cell-type RNAseq data provided a model with increased specificity. Indeed, the top molecules detected in the endometrium (Fig. 8) have been identified on the single-cell RNA-seq resolution in the luminal epithelium and stroma, respectively (www.reproductivecellatlas.org, Garcia-Alonso *et al.*, 2021). With the highly curated protein–protein network, we highlight the involvement of cell adhesion, post-translational protein

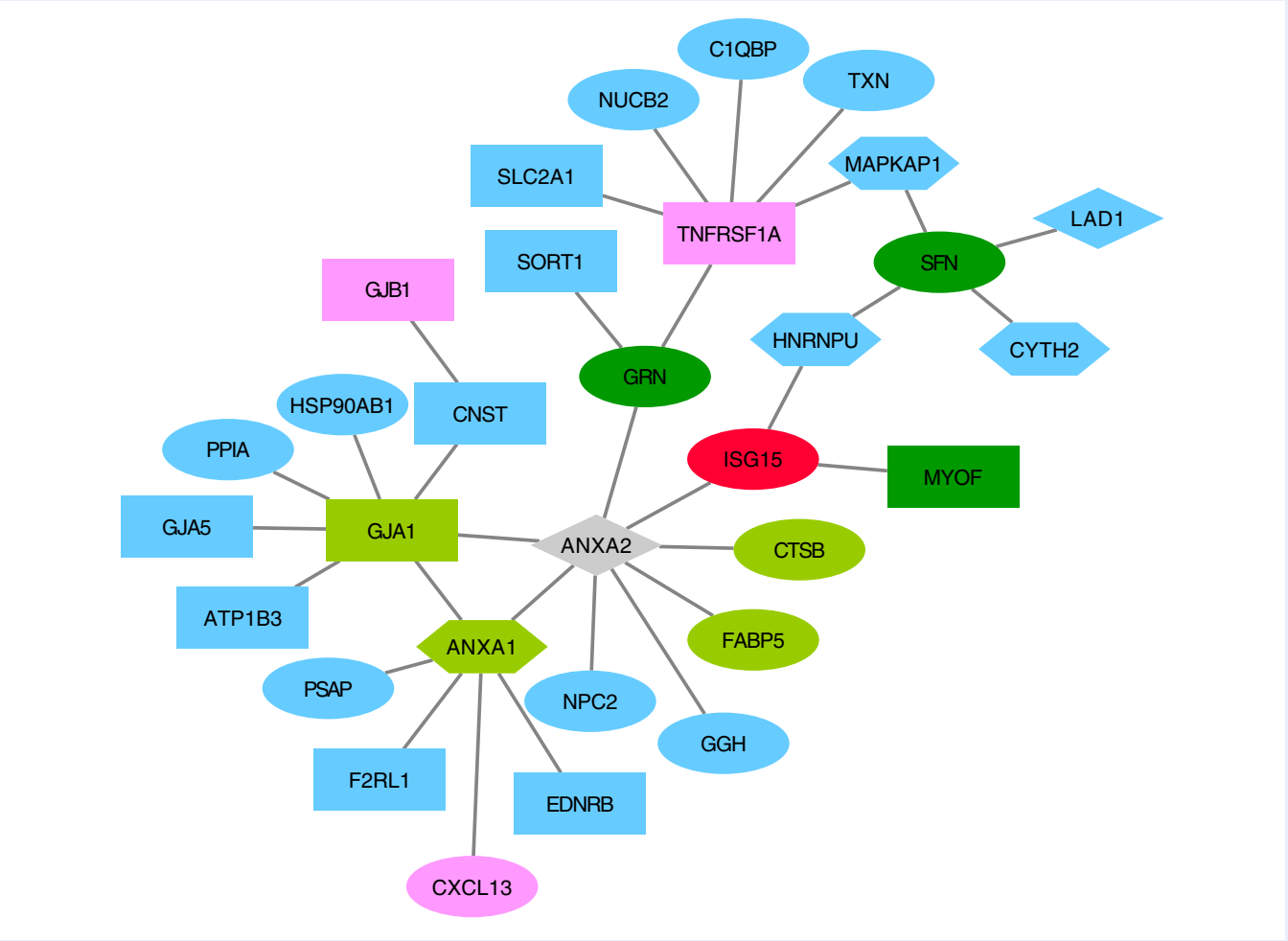

Figure 5. Cluster C. Molecular interactions of polar trophectodermal cells with endometrial epithelial and stromal cells. Pink: stromal proteins, red: epithelial proteins, light blue: trophectodermal proteins, violet: epithelial and stromal proteins, green: epithelial and trophectodermal proteins, light green: stromal and trophectodermal proteins, grey: epithelial, stromal and trophectodermal proteins. Ellipse: secreted proteins, diamond: extracellular matrix proteins, octagon: membrane bound proteins, rectangle: transmembrane proteins. Interacting proteins and interactions are provided in Supplementary Table SVI.

modifications, regulation of secretion processes and extracellular matrix organization processes in the early implantation events such as apposition, attachment and initiation of invasion. A previous human proteome study on cultured cells detected cellular protein network changes between monolayer and spheroid trophectoderm models with regard to their adhesive protein landscape (Evans *et al.*, 2020). Furthermore, a previously published modelling of embryos breaching the endometrial epithelium using human blastocysts or trophoblast stem cell spheroids cultured with endometrial epithelial cells predicted a set of genes in embryo–endometrium networks providing new knowledge to understand the embryo implantation process (Ruane *et al.*, 2020). A number of the interacting proteins were also detected in the current study of freshly separated non-cultured endometrial epithelial and stromal subpopulations, such as CP, VCAN, SERPING1, ANXA2, DCN and APOD, and on the trophectodermal side, HSP90AB1, FURIN, HAPLIN1, CD55 and CTSB.

Embryo implantation is a complex process, likely to require a cascade of interacting proteins. A previous extensive endometrial proteome study identified 188 proteins crucial in the acquisition of endometrial receptivity (Pérez-Debén *et al.*, 2019), out of which we identified 41 (22%) proteins among the endometrial cell subpopulations, where 8 were interacting as stroma-specific secreted proteins (C3, A2M, NAMPT, CFB, VTN, CST3, APOD, CST3) and CP was interacting as an epithelial-cell-specific secreted protein in the modelled embryo–endometrium crosstalk. The *NAMPT* gene product in the receptive phase endometrium has also been detected as physically interacting with trophectodermal cells (Ntostis *et al.*, 2021).

One of the hub molecules in our PPI networks was the transmembrane protein integrin β1, an important component of the surface of the trophectodermal and receptive-phase endometrial epithelial cells. A previous clinical trial showed that integrin β1 is a promising biomarker for evaluating uterine receptivity and determining the optimal

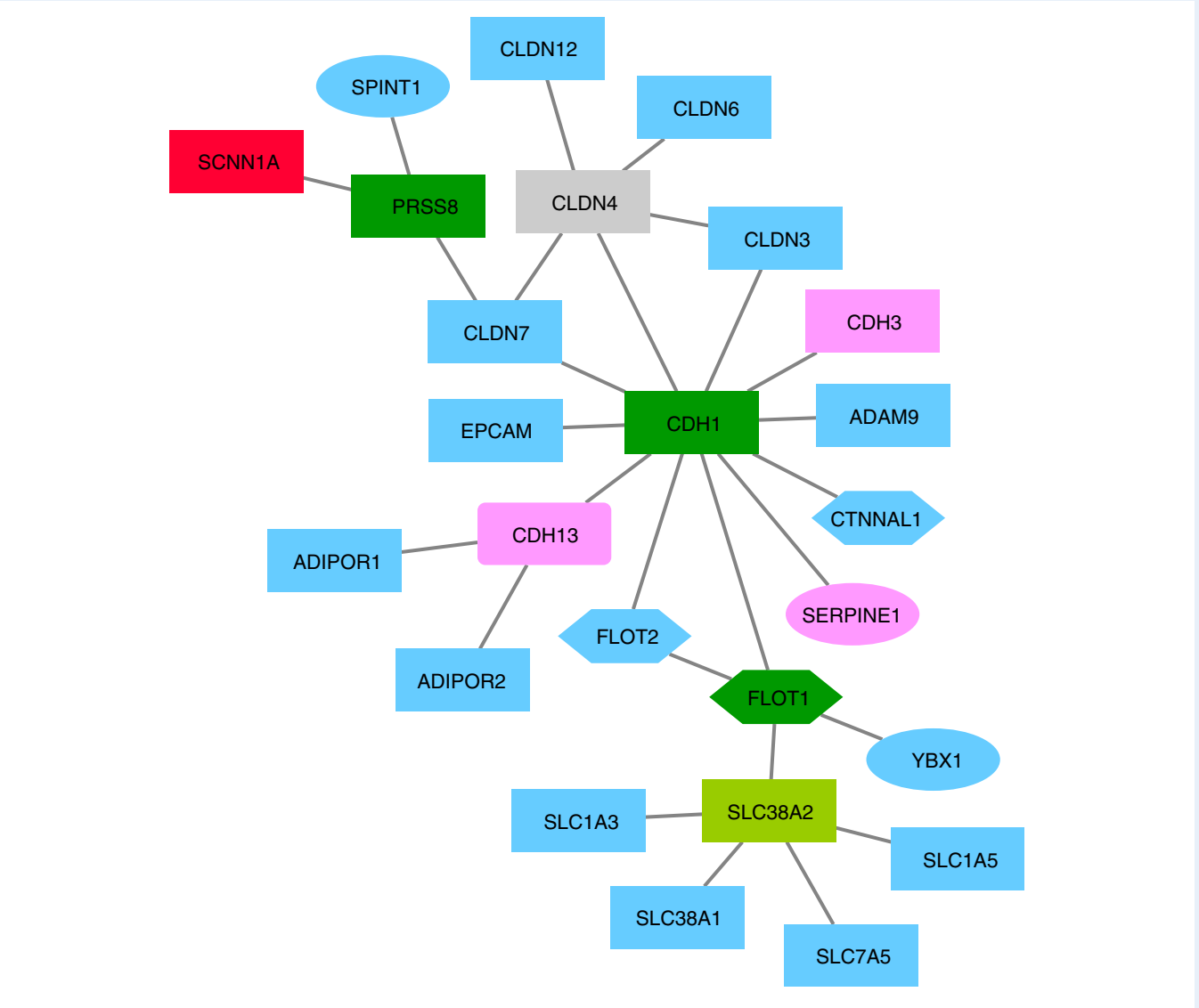

Figure 6. Cluster D. Molecular interactions of polar trophectodermal cells with endometrial epithelial and stromal cells. Pink: stromal proteins, red: epithelial proteins, light blue: trophectodermal proteins, violet: epithelial and stromal proteins, green: epithelial and trophectodermal proteins, light green: stromal and trophectodermal proteins, grey: epithelial, stromal and trophectodermal proteins. Ellipse: secreted proteins, diamond: extracellular matrix proteins, octagon: membrane-bound proteins, rectangle: transmembrane proteins. Interacting proteins and interactions are provided in Supplementary Table SVI.

time for embryo transfer (Chen *et al.*, 2016). Interestingly, mouse blastocysts that lack the integrin β1 subunit fail to implant, which is due to the inability to adhere to or invade the subepithelial stroma (Brakebusch *et al.*, 1997). Although there are differences between the initial implantation processes between human and mice (i.e. in mice, epithelial cells are lost by entosis and the trophectoderm interacts directly with the stroma (Ye, 2020)), animal models provide additional information of the process. In our study setting, ITGB1 seems to mediate a variety of interactions, which likely regulate embryo implantation. Integrin β1 levels on the surface of a trophectodermal cell are regulated by stromal/epithelial cell-secreted galectin-3, which therefore controls trophoblast cell migration (Bojić-Trbojević *et al.*, 2019). Galectin-3 expression on the uterine epithelia of pregnant mice was found immediately after implantation, but not during the pre-implantation stage (Phillips *et al.*, 1996). We also predicted integrin β1 to interact with the transmembrane glycoprotein BSG on the surface of trophectodermal and stromal cells. It is known that female mice lacking the BSG gene are infertile due to implantation failure (Kuno *et al.*, 1998). Another galectin family member detected in our PPI network is galectin-1 (LGALS1), expressed by trophectoderm cells. Although mice embryos lacking galectin-1 implant normally (Colnot *et al.*, 1998), galectin-1 expression in the trophectoderm of blastocysts has been suggested to play an important role in embryo implantation, by improving embryo adhesion or regulating maternal immunity

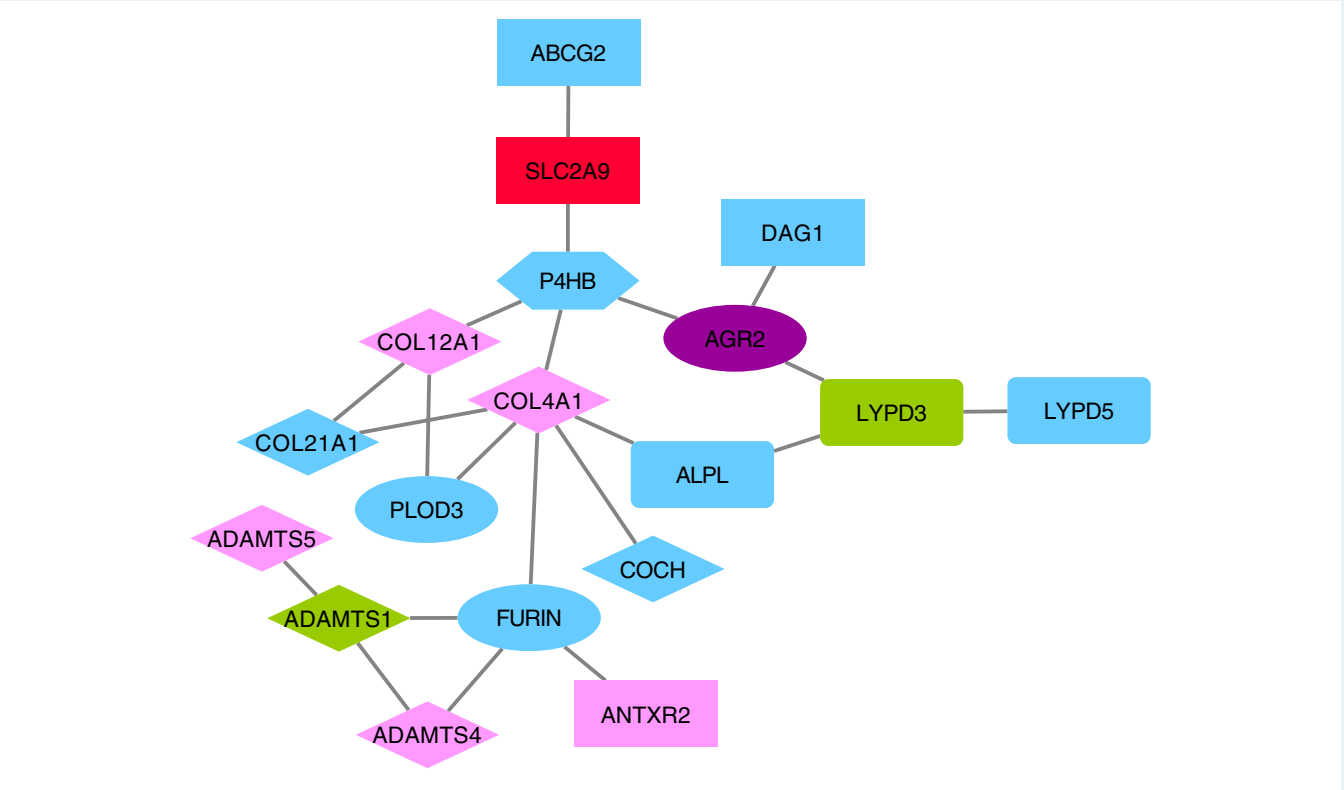

Figure 7. Cluster E. Molecular interactions of polar trophectodermal cells with endometrial epithelial and stromal cells. Pink: stromal proteins, red: epithelial proteins, light blue: trophectodermal proteins, violet: epithelial and stromal proteins, green: epithelial and trophectodermal proteins, light green: stromal and trophectodermal proteins and grey: epithelial, stromal and trophectodermal proteins. Ellipse: secreted proteins, diamond: extracellular matrix proteins, octagon membrane-bound proteins, rectangle: transmembrane proteins. Interacting proteins and interactions are provided in Supplementary Table SVI.

(Tirado-Gonzalez *et al.*, 2013). One of the interacting molecules with trophectodermal galectin-1 is epithelial cell secreted osteopontin. Osteopontin (SPP1) is one of the few genes repeatedly identified in transcriptome studies as endometrial receptivity-specific genes (Altmäe *et al.*, 2017; Rekker *et al.*, 2018; Wang and Yu, 2018), and it has been shown to function in cell-to-cell adhesion during the early stages of implantation in different *in vitro* models (Kang *et al.*, 2014; Berneau *et al.*, 2019b; Vergaro *et al.*, 2021). Furthermore, like integrin β1, SPP1 has previously been implicated in the embryo implantation process in other species. For example, experiments on ovine luminal epithelial and trophectodermal cells demonstrated that SPP1 binding into the integrin receptor leads to cytoplasmic reorganization and induction of focal adhesions (Johnson *et al.*, 2001), possibly triggering embryo attachment. Epithelial cells also uniquely secrete the IFN-stimulated gene 15 (ISG15), shown to be expressed as an early endometrial response to pregnancy in human as well as bovine, ovine, mice and other mammals, strongly suggesting that the ISG15 might be critical for pregnancy maintenance (Henkes *et al.*, 2015).

The penetration of the embryo between the endometrial stromal cells is mainly regulated by the cellular signalling and extracellular matrix restructuring. One of the matrix proteins, expressed by the stromal cells, is collagen Type XII alpha 1 chain (COL12A1), whose remodelling is induced by the trophectodermal protein PLOD3. Stromal cells also secrete several proteins, being involved in trophoblast cell invasion and migration, such as the epithelial and stromal cells secreted Anterior gradient protein 2 homolog (AGR2) interaction with trophectodermal membrane proteins Dystroglycan 1 (DAG1) and Ly6/PLAUR domain-containing protein 3 (LYPD3) (Salmans *et al.*, 2013; Arumugam *et al.*, 2015; Li *et al.*, 2020) or stromal cell-secreted Insulin Like Growth Factor Binding Protein 2 (IGFBP2) regulation through integrin β1 (Han *et al.*, 2014). The stromal secreted protein cathepsin B (CTSB) in mouse has been shown to be necessary for normal embryo development and uterine decidualization (Afonso *et al.*, 1997) and the secreted protein CXCL13 has been related to successfully implanted blastocysts (Dominguez *et al.*, 2008).

Our study has its limitations, such as providing a static snap-shot analysis of a dynamic process and the nature of prediction analysis, which is limited to known interactions available in databases. Our approach also does not account for *in vivo* cell-to-cell influences, e.g. seminal plasma effects on endometrial gene expression (Chen *et al.*, 2014) or interactions between different cell types within endometrium (Hantak *et al.*, 2014). Due to the ethical and technical considerations, we could only study embryo–endometrial dialogue using biopsies taken from non-pregnant women, meaning that stromal cells were not decidualized as would be likely by the time the trophectoderm has penetrated the luminal epithelium in parallel with trophectodermal

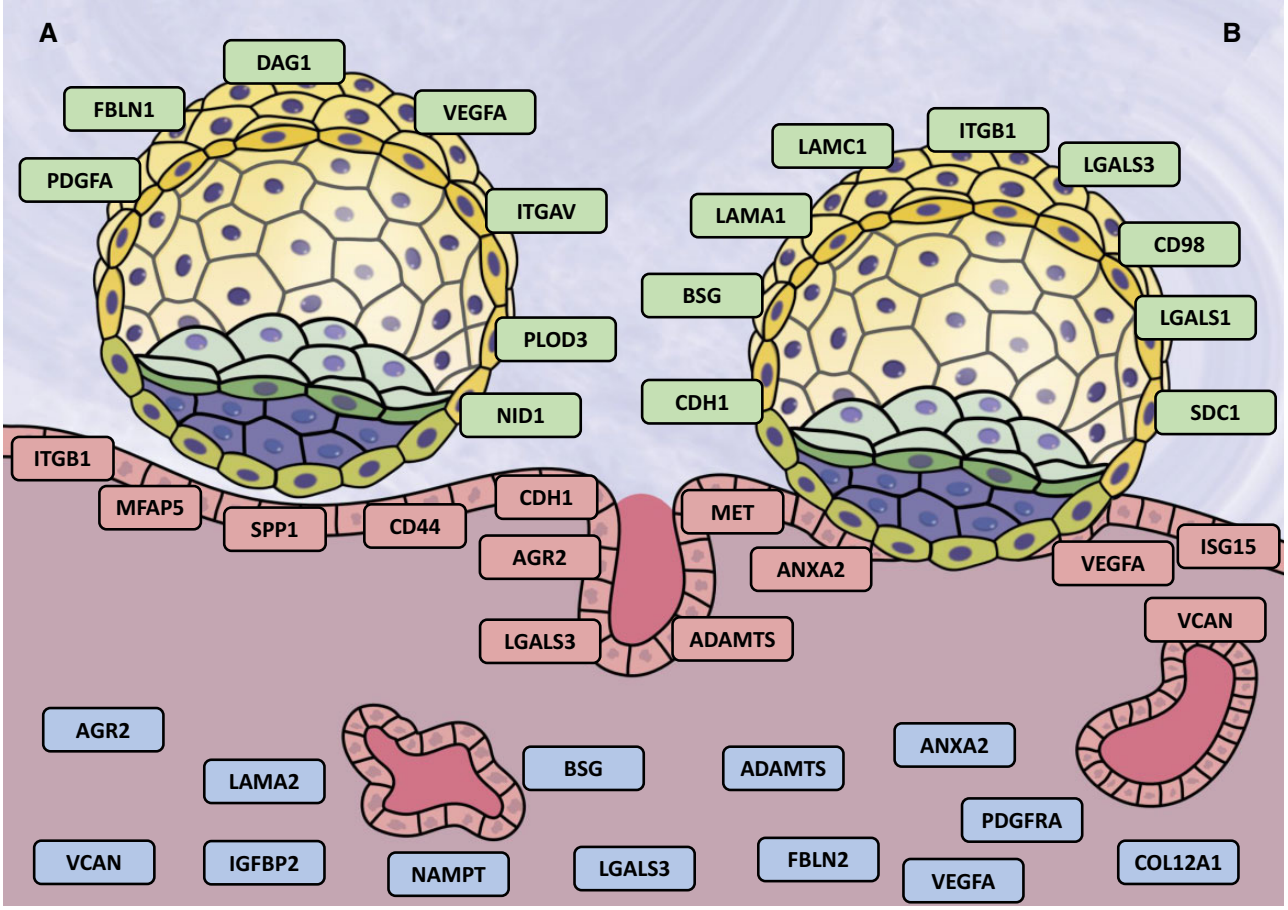

Figure 8. Molecular crosstalk between the trophectodermal cells and the endometrial epithelial and stromal cells in implantation. The initial stages of the process: apposition-attachment (**A**) and the initiation of invasion (**B**). The molecules involved in the embryo–endometrium dialogue are highlighted; in green: trophectodermal proteins, pink: endometrial epithelial proteins, blue: stromal proteins. All proteins involved in the interactions are provided in Supplementary Table SVI.

changes, and we could only use spare IVF embryos, likely missing some of the critical interactions taking place in case of natural conception. Furthermore, the effect of individual cells such as uterine natural killer cells and macrophages together with factors released from extracellular vesicles known to have role in the embryo–endometrium interplay (Altmäe *et al.*, 2017; Ye, 2020; Díaz-Hernández *et al.*, 2021) could not be assessed in the current study setting. Moreover, the cell sorting technique used separated enriched epithelial cells from stromal cells but did not separate luminal from glandular epithelium, which is shown to differ (Evans *et al.*, 2012, 2014). Nonetheless, our study has several strengths to be highlighted, such as the analysis of specific types of cells (avoiding thus the cellular heterogeneity associated with whole-tissue biopsies (Suhorutshenko *et al.*, 2018)), the use of a comprehensive RNA sequencing platform, and most importantly, the study of *ex vivo* rather than cultured endometrial cells. The latter seems to be critical for getting more interpretable results from gene expression profiling, as we have seen in our previous study that culturing of endometrial cells rapidly alters their transcriptome (Krjutškov *et al.*, 2016).

In conclusion, our results provide a unique insight into the molecular mechanisms of endometrial receptivity by highlighting the relevant molecules involved in embryo apposition, attachment and initiation of invasion events in the initial steps of implantation. Our study identifies putative molecular networks between the polar trophectodermal cells of implantation-competent blastocyst and the epithelial and stromal cells of the receptive endometrium. This new knowledge of the endometrial cell-type-specific molecules that coordinate successful implantation is vital for understanding human reproduction and the underlying causes of implantation failure and infertility. Our study results provide a useful resource for future reproductive research, allowing the exploration of unknown mechanisms of implantation. We envision that those studies will help to improve the understanding of the complex embryo implantation process in humans, and hopefully generate new prognostic and diagnostic biomarkers and therapeutic approaches to target both infertility and fertility, in the form of new contraceptives.

Supplementary data

Supplementary data are available at *Human Reproduction Open* online.

Data availability

Data are available at www.ncbi.nlm.nih.gov/geo under accession number GSE97929 and also at our previous publication (doi.org/10.1016/j.cell.2016.03.023).

Authors' roles

Conceptualization: M.K., K.K., S.A., J.K. and A.S.; validation: M.S.; formal analysis: M.K.; investigation: K.S., D.L. and M.S.; resources, P.G.L., K.G.-D., D.B. and C.S.; data curation: S.K., E.E., E.V. and F.L.; original draft preparation: M.K., S.A., M.S., K.K.; review and editing: M.K., S.A., M.S., A.S.-L., K.K., J.K. and A.S.; visualization: K.K., M.K., E.V. and A.S.-L.; supervision: K.K., J.K., S.A. and A.S; project administration: K.K. and S.A.; funding acquisition: A.S., J.K. and S.A.

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Conflict of interest

K.G.-D. has received consulting fees and/or honoraria from RemovAid AS, Norway Bayer, MSD, Gedeon Richter, Mithra, Exeltis, MedinCell, Natural cycles, Exelgyn, Vifor, Organon, Campus Pharma and HRA-Pharma and NIH support to the institution; D.B. is an employee of IGENOMIX. The rest of the authors declare no conflict of interest.

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