Proteomic insights into endometrial receptivity and embryo-endometrial epithelium interaction for implantation; critical determinants of fertility

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Abstract

In vitro fertilization has overcome infertility issues for many couples. However, achieving implantation of a viable embryo into the maternal endometrium remains a limiting step in optimizing pregnancy success. The molecular mechanisms which characterize the transient state of endometrial receptivity, critical in enabling embryo-endometrial interactions, and proteins which underpin adhesion at the implantation interface, are limited in humans despite these temporally regulated processes fundamental to life. Hence, failure of implantation remains the 'final frontier' in infertility. We utilized a human co-culture model utilizing spheroids of a trophectoderm (trophoblast stem) cell line, derived from pre-implantation human embryos, and primary human endometrial epithelial cells, to functionally identify 'fertile' versus 'infertile' endometrial epithelium based on adhesion between these cell types. Quantitative proteomics identified proteins associated with human endometrial epithelial receptivity ('epithelial receptome') and trophectoderm adhesion ('adhesome'). As validation, key 'epithelial receptome' proteins (MAGT-1/CDA/LGMN/KYNU/PC4) localized to the epithelium of receptive phase (mid-secretory) endometrium obtained from fertile, normally cycling women but were largely absent from non-receptive (proliferative) phase tissues. We demonstrate factors involved in embryo-epithelium interaction in successive temporal stages of endometrial receptivity and implantation and provide potential targets for improving fertility, enhancing potential to become pregnant either naturally or in a clinical setting.

Significance statement

Infertility affects 1:6 couples world-wide and this is increasing. In Vitro Fertilization (IVF) pregnancy success rates are <25% per cycle and couples using such technologies face both financial and emotional hardship. Implantation of a healthy embryo into a receptive endometrium is a critical step in establishment of pregnancy but both receptivity and implantation are considered the 'black box' of reproduction; little is known of the underlying mechanisms or how these are disturbed in infertile women. Importantly, much remains to be discovered about the basic protein interactions that govern trophectoderm–endometrial

epithelium adhesion. Significantly, this study provides a unique functional proteomic strategy to identify the composition of factors involved in embryo-epithelial interactions in the critical stages of receptivity and implantation, which may be targeted to improve fertility without or with existing technologies.

1 Introduction

Establishment of a human pregnancy requires that an embryo (at blastocyst stage), becomes attached to and invades the receptive epithelial surface of the endometrium that lines the uterine cavity. These are the first steps of implantation, which then proceeds by the invasion of the trophectoderm (the outer cellular layer of the blastocyst) through the decidualised endometrial stroma, until it invades and reconstructs the spiral arterioles to fully form the placenta which comprises both maternal and fetal cells. The early processes whereby the endometrium attains receptivity and the trophectoderm first attaches, are little understood. They cannot be studied in vivo in humans, while animal models demonstrate significant differences in basic physiology and there is a paucity of appropriate human models [1].

We recently developed a novel model for human embryo implantation that enables detailed examination of the adhesion of human embryo mimics to endometrial epithelial cells [2]. This utilised a human trophoblast stem cell line [3] developed from donated human embryos, and which had characteristics of trophectodermal cells (TEAD4, CDX2, geminin, HMGA2, LIFR, GDF15 and LGR5 expression). These 'trophectoderm' cells were formed into spheroids, consistently the size of human blastocysts. Their adhesion to primary human endometrial epithelial cells could be manipulated with the hormonal milieu and importantly, these trophectoderm spheroids could discriminate, via adhesion/non-adhesion, between endometrial epithelial cells obtained from fertile versus infertile women respectively.

Using this model, we have here applied a proteomic approach to identify a unique adhesion protein network and define a human embryo implantation 'adhesome' in fertile endometrial epithelial cell-trophectoderm spheroid co-cultures. Using trophectoderm spheroid adhesion to define primary endometrial epithelial cells as 'receptive' or 'non-receptive' to implantation, we also identify a human endometrial epithelial 'receptome'. Interrogation of these protein networks and composition has enabled novel insight into endometrial receptivity and the adhesion stage of human implantation, providing potential novel biomarkers for identification

of endometrial epithelial receptivity and insights into embryo adhesion that will assist in improving outcomes of assisted reproduction.

2 Experimental Procedures

Ethics and tissue collection

Ethical approval for tissue collections; Institutional Ethics Committees at Monash Health and Monash Surgical Private Hospital. Written informed consent was obtained from all subjects prior to tissue collection.

Endometrial Tissue Collection and Patient Details

Endometrial biopsies for culture were collected by curettage from normally cycling women (28-32 day cycles), ≤40 undergoing hysteroscopy and curettage. Women were undergoing investigation as indicated in table 1 and had not used steroid hormone therapy/contraception in preceding 6 months. These women were of proven fertility (≥1 parous pregnancy) and had normal endometrium at hysteroscopy and morphologically normal endometrium as assessed by experienced endometrial histopathologists. Likewise, biopsies were taken from infertile women in an IVF program as indicated in Table 1, with non-endometrial indications (i.e. no diagnosis of fibroids, endometriosis, adenomyosis or other endometrial related disorders) for their infertility, and who were otherwise of similar characteristics. Women were normally cycling and experienced regular menstruation. Women were noted to have normal endometrium at hysteroscopy and patent, unblocked fallopian tubes. As these tissues are collected via altruistic donation from women consented immediately before entry to operating theatre through a private hospital, only limited patient background data is available.

Epithelial cell isolation from human endometrial tissue

Performed per previous protocols [4]. In brief, within 16 hours of collection, endometrial tissues were washed in phosphate buffered saline (PBS), finely chopped and incubated with 1200U collagenase type III and 100mg/ml DNAse in 2ml of phosphate buffered saline (PBS) for 45 minutes at 37^{o}C with shaking at 130rpm. Digestion was terminated by addition of 4 volumes

of DMEM/F12 containing 1% v/v penicillin/streptomycin (p/s). Digested tissue was passed through a 45μm filter (endometrial stromal cells pass through the filter) and retained epithelial fragments washed off, centrifuged, resuspended in DMEM/F12 containing 10% v/v fetal bovine serum (FBS, Gibco, Invitrogen) and 1% p/s and seeded into 24 well plates ($2cm^2$ surface area). Epithelial fragments were allowed to attach for 48 hours before thorough washing with PBS to remove stromal and other cells. Endometrial epithelial cell preparations were visually assessed for contamination with endometrial stromal fibroblasts and only those with ≥95% epithelial cells used for experimental purposes. Primary human endometrial epithelial cells (pHEEC's) were not passaged and were used at p0 (i.e. at first seeding after isolation) as, in our experience, this reduces the likelihood of stromal cell contamination of the cultures. pHEEC's at p0 were used for experimental purposes within 1 week of isolation. An example of morphologically pure epithelial preparation with characteristic 'rounded' morphology and no contaminating stromal fibroblasts is provided in Figure 1A. This is the typical appearance of epithelial cultures used in the current study.

Cell culture

L2-TSC (trophectodermal) cells are human trophoblast stem cells (kind gift of Prof Susan Fisher, UCSF) [3]; these cells were developed from individual blastomeres of donated human embryos. L2-TSC's have characteristics of trophectodermal cells (TEAD4, CDX2, geminin, HMGA2, LIFR, GDF15 and LGR5 expression) and can be manipulated to differentiate towards a syncytiotrophoblast or cytotrophoblast fate. However, routine maintenance in a 1:1 mix of DMEM:F12 Glutamax (Gibco, Invitrogen) supplemented with 1% p/s and 10% v/v FBS with addition of 10 ng/ml basic fibroblast growth factor (bFGF, 233-FB-025, R&D systems) and 10 μM SB431542 (#1614, Tocris Bioscience) [5] as used herein maintains these cells in their 'stem cell' trophectoderm like state (henceforth termed trophectoderm medium). Cells were grown on flasks coated with 0.5% gelatin (G1393, Sigma Aldrich). Human endometrial adenocarcinoma cells, ECC-1 [6, 7] were used as a model of human endometrial luminal epithelial cells. These were cultured and maintained as previously described [6] in DMEM/F-12 supplemented with 10% FBS, and incubated at 37°C with 5% CO_2 [8]. ECC-1 cells were

validated by Karyotype analysis [9] according to the ATCC guidelines [10], with allele match in STR profile of 100%.

Preparation of trophectodermal spheroids.

Methylcellulose (4000 centipoises, Sigma Aldrich) at 1.5% (w/v), dissolved in DMEM was centrifuged (90 minutes/3500rpm) to remove insoluble methylcellulose. 2500 trophectoderm cells (optimized cell number based on initial studies [2, 5]) in 150μl of 20% methylcellulose/80% trophectoderm medium [11] were seeded into a round bottomed well in which one spheroid formed gradually in each well over 48 hours; each spheroid was approximately the same size as a human blastocyst (0.1-0.2mm). Any mis-formed spheroids (<5%) were discarded. Spheroids were thoroughly washed to remove methylcellulose and trophectoderm media prior to co-culture with endometrial epithelial cell monolayers. In brief, spheroids were collected into 15ml sterile polypropylene tubes using wide bore 1ml tips to prevent disturbing the 3D structure of the spheroids. These spheroids were centrifuged at 800g for 8 min followed by removal of media. Serum free DMEM/F12 media was added to the tubes and the spheroids gently resuspended by flicking the tube with resuspension visually confirmed. The spheroids were again centrifuged, and this process repeated a total of 3 times.

Endometrial epithelial cell-trophectoderm spheroid co-culture and lysate preparation.

We previously demonstrated that spheroids of trophectodermal cells discriminate between endometrial epithelial cells isolated from fertile (Figure 1A, fertile) and idiopathic infertile (Figure 1A, infertile) women based on spheroid adhesion after co-culture with pHEEC's for 6 hours [2]. For the current study, primary human endometrial epithelial cells monolayers in 0.5% charcoal stripped (cs)FBS/DMEM/F12 were sequentially treated with 10^{-8}M 17β-estradiol (estrogen: E) for 24 hours followed by estrogen/10^{-7}M medroxyprogesterone acetate (progestin: MPA) for a further 24 hours to mimic in vivo hormonal regulation during the receptive phase of the menstrual cycle (hormonal priming) [12]. After 48 hours of total hormonal priming, spheroids were resuspended in E/MPA media (concentrations as above) containing 1% FBS and co-cultured with hormonally primed endometrial epithelial cell monolayers for 6 hours (per previously developed protocol, Figure 1Bii and 1Biv). Control

spheroids incubated in absence of endometrial epithelial cells (adherence to plastic only) were also treated with estrogen/progestin media for 6 hours (Figure 1Bv). Spheroid adhesion was determined by a) counting total spheroids present under an inverted light microscope; b) removing medium and gently washing co-cultures with PBS; c) re-counting firmly adhered spheroids. Adhered spheroids were expressed as a % of total spheroids [2]. Endometrial cell monolayers which supported spheroid adhesion were defined as 'adhesive' (Figure 1Bii) while those that did not were defined as 'non-adhesive' (Figure 1Biv). 'Receptive' or 'non-receptive' endometrial monolayers were prepared by hormonal priming as above. These cells were maintained under the same treatment conditions as the endometrial epithelial – trophectoderm spheroid co-cultures but without addition of spheroids. If spheroids adhered to the matched endometrial epithelial monolayers (i.e. cells obtained from the same woman, present on the same culture plate, treated in the same manner) the cells were defined as receptive (Figure 1Bi); if no adhesion exhibited, the cells were defined as non-receptive (Figure 1Biii). All cultures were lysed on ice (15 mins) with 100µl SDS sample buffer (4% (w/v) SDS, 20% (v/v) glycerol, 0.01% (v/v) bromophenol blue, 0.125 M Tris-HCl, pH 6.8). Cell lysates were ultracentrifuged at 435,000g/30 min at 4°C (TLA-100 rotor, Beckman Coulter) [12, 13]. This procedure is outlined in Figure 1C.

siRNA knockdown: ECC-1 endometrial epithelial cells

ECC-1 cells seeded at 1.5 x 10^5 cells/well in 12 well plates in DMEM/F12 containing 10% v/v FBS without Pen/Strep and allowed to attach & proliferate overnight. 2µl of 10 µM siRNA/scrambled stock (Santa-Cruz Biotechnology) was added then to 100 µl of OptiMEM transfection media (Invitrogen) and, 2 µl of lipofectamine (Life Technologies) was added to 100 µl of OptiMEM, each for 5 min. These solutions were mixed gently and incubated for 30 min before addition of 800 µl of OptiMEM. Cells were washed twice with OptiMEM, then siRNA/scrambled transfection mix added and incubated (8 hrs). Media were replaced with DMEM/F12/FBS media as above, for 48 hrs before cell starvation for 6 hrs. siRNA/scrambled ECC-1 cells were sequentially treated with 10^{-8}M E for 24 hrs followed by E plus 10^{-7}M MPA for a further 24 hrs to mimic the receptive phase of the menstrual cycle [6], and trophectoderm spheroid adhesion assay performed (as above) for 6 hrs.

Protein Quantification

Protein content was determined by microBCA colorimetric protein quantification (Life Technologies, 23235) or quantified by Qubit fluorescence using Qubit 4.0 (Life Technologies, Q33212) as per manufacturer's instructions.

Proteomic sample preparation of endometrial epithelial cell-trophectoderm spheroid co-cultures.

Lysates from cell monolayers (primary endometrium; receptive and non-receptive), co-cultures (primary endometrium with spheroid adhesion assay; adhered and non-adhered) or spheroids alone (20 µg total protein) were solubilised in SDS sample buffer (4% (w/v) SDS, 20% (v/v) glycerol and 0.01% (v/v) bromophenol blue, 0.125 M Tris-HCl, pH 6.8)) with protease inhibitor cocktail (Complete, EDTA-free protease inhibitor cocktail, Roche), lysed at 95°C for 5 min, then fractionated by short-range SDS-PAGE, with fractions (n=2) representing the entire gel excised [13]. Each fraction was destained (50 mM ammonium bicarbonate/50% (v/v) acetonitrile, ACN) for 30 min at 27 °C [14]. Samples were reduced with 2 mM tri (2-carboxyethyl) phosphine hydrochloride (Sigma-Aldrich, C4706) at RT 1 hr on gentle rotation, alkylated by treatment with 25 mM iodoacetamide (Sigma-Aldrich) for 30 min (in the dark), and digested with trypsin (Promega, V5111) for 18 hr at 37 °C. The peptide solutions were acidified to a final concentration of 1% formic acid (FA) and 0.1% triflouroacetic acid (TFA) and desalted with a C18 Sep-Pak column (Waters). Each Sep-Pak column was activated with 100 µL of methanol, washed with 30 µL of 80% acetonitrile, and equilibrated with 3x 30 µL 0.1% TFA. Samples were loaded and each column washed with 2x 20 µL 0.1% TFA. Elution was performed with 2 rounds of 20 µL of 50% acetonitrile. Samples were lyophilised (SpeedVac; Savant, ThermoFisher Scientific) and acidified with 0.1% FA, 2% ACN, and peptide concentrations estimated from A_{280} absorbance (Thermo Scientific Nanodrop 2000).

Mass spectrometry-based proteomics

Proteomic experiments performed in biological triplicate, with technical replicates (n=2), with MIAPE-compliance [5, 15]. MS analyses performed on an Orbitrap LTQ Elite mass spectrometer (ThermoFisher Scientific) with a nanoelectrospray ion source coupled online to a Waters nanoAcquity UPLC. Peptides were loaded (Acclaim PepMap100, 5 mm × 300 µm i.d., µ-Precolumn packed with 5 µm C18 beads, Thermo Fisher Scientific) and separated over a 120-min gradient run using a BioSphere C18 analytical column (1.9 µm 120Å, 360/75 µm × 400 mm, NanoSeparations) at 45 °C. Trapping was for 3 min at 5 µL/min, 98% buffer A (99% water, 0.1% formic acid) and 2% buffer B (0.1% (v/v) FA in 80% (v/v) ACN), before eluting at 2–100% 0.1% FA in acetonitrile (2–40% from 0–100 mins, 40–80% from 100–110 mins (flow rate, 250 nL/min).

The mass spectrometer was operated in data-dependent mode where up to 20 dynamically chosen, most abundant precursor ions in the survey scan (350–1500 Th) were selected for MS/MS fragmentation. Survey scans were acquired at a resolution of 120,000, with MS/MS resolution of 15,000. Unassigned precursor ion charge states and singly charged species were rejected, and peptide match disabled. The isolation window was set to 2.0 Th and selected precursors fragmented by collisional dissociation with normalized collision energies of 35 with a maximum ion injection time of 110 msec. Ion target values were set to 3×10^6 and 1×10^5 for survey and MS/MS scans, respectively. Dynamic exclusion was activated for 90 sec. Samples were run in regional blocks, with sample groups interspersed throughout to allow correction of batch effects. Data was acquired using Xcalibur software v4.0 (Thermo Fisher Scientific). Raw mass spectrometry data deposited in the PeptideAtlas (#PASS01121) and can be accessed at http://www.peptideatlas.org/PASS/PASS01121.

Data analysis

Peptide identification and quantification were performed using MaxQuant (v1. 6.0.1) with its built-in search engine Andromeda [16] . Tandem mass spectra were searched against a human reference proteome (71,798 entries, downloaded 10-2018) supplemented with common contaminants. Search parameters included carbamidomethylated cysteine as fixed modification and oxidation of methionine and N-terminal protein acetylation as

variable modifications. Data was processed using either trypsin/P as the proteolytic enzyme with up to 2 missed cleavage sites allowed. Where possible, peptide identification information was matched between runs of the fractionated samples within MaxQuant. Precursor tolerance was set to ±4.5 ppm, and fragment ion tolerance to ±10 ppm. Results were adjusted to 1 % false discovery rate (FDR) on peptide spectrum match (PSM) level employing a target-decoy approach at the peptide and protein levels. In cases of redundancy, shared peptides were assigned to the protein sequence with the most matching peptides, thus adhering to principles of parsimony. The label free quantification (LFQ) algorithm [17] in MaxQuant was used to obtain quantification intensity values. Perseus (v1.6.0.7) was further used to process data, where resulting p-values were adjusted by the Benjamini-Hochberg multi-test adjustment method for a high number of comparisons [18] and statistics performed as previously described [19]. For pathway analyses, Kyoto Encyclopedia of Genes and Genomes (KEGG) and NIH Database for Annotation, Visualization and Integrated Discovery Bioinformatics Resources 6.7 (DAVID) resources were utilised using recommended analytical parameters [20]. For gene ontology enrichment and network analyses UniProt (www.uniprot.org) database resource (biological process, molecular function), Ingenuity Pathway Analysis, and Reactome knowledgebase were utilized. Clustering of samples was performed by principal component analysis (PCA) and visualized using ggplot2 [21] and ggfortify (https://cran.r-project.org/web/packages/ggfortify/index.html). The heat map of proteins used gplots (https://cran.r-project.org/web/packages/gplots/index.html).

Immunohistochemistry

For immunohistochemistry [22] proliferative (non-receptive) and secretory (receptive) phase endometrial tissues were incubated with antibodies directed against MAGT1, KYNU, CDA, LGMN or PC4 (all Santa-Cruz Biotechnology) overnight at 4˚C or isotype matched IgG negative controls. Biotin-labelled secondary antibodies were applied followed by avidin-HRP before colorimetric development with diaminobenzidine and counterstaining with hematoxylin.

Statistics

GraphPad Prism v7.0 was used with all data pre-tested for normality. If the data was non-parametric, a Kruskal-Wallis with a Tukey's post-hoc test or Mann-Whitney U analysis was performed and if parametric, one-way ANOVA with a Tukey's post-hoc test or unpaired t-test was applied. All data presented as mean plus/minus standard error of the mean (mean±SEM). Statistical testing of proteomic data used Poisson distribution with EdgeR software (v3.2). Student's t-tests used GraphPad Prism v7.0). In all analyses, *p<0.05 considered statistically significant.

3 Results

Implantation of a healthy embryo into a receptive endometrium is a critical step in establishment of pregnancy but this is considered the 'black box' of infertility [23]; little is known of the underlying mechanisms of endometrial receptivity, the adhesion stage of implantation or how these are disturbed in infertile women. To gain critical insights into this interaction, we performed functional integration of human endometrial epithelial cell adhesion (from patients with proven fertility, and in patients with infertility) to trophectoderm spheroids. Here, we demonstrate for the first time, proteins and their associated networks that can be identified as associated with trophectoderm–epithelium adhesion in an unbiased fashion (**Figure 1**). Significantly, this study provides a unique insight into the composition of factors involved in embryo-endometrial epithelium interaction in temporal stages of receptivity and implantation, and key changes in these networks associated with endometrial-associated infertility.

Functional adhesion of endometrial epithelial cells from fertile and non-fertile women

We previously demonstrated that our trophectoderm–epithelium co-culture system clearly differentiates between endometrial epithelial cells derived from fertile women or those with idiopathic fertility [24]. Indeed, trophectoderm spheroids adhered to 85% of fertile tissues assessed (11/13) but only to 11% of infertile tissues (2/18, p<0.001) despite appropriate *in vitro* hormonal (estrogen/progestin) priming. The present study design, described above, and in Figure 1, enabled complex analyses between receptive and non-receptive endometrial

epithelium (the receptome) and attachment between epithelium and spheroids in co-culture (the adhesome). Characteristics of women used in these co-culture studies is presented in Table 1. No significant differences were found between the groups.

Proteome Analysis of Human Trophectoderm–Epithelium Adhesion

Proteomic profiling was performed on adhesive/non-adhesive co-cultures, receptive versus non-receptive primary cell monolayers and on spheroids alone (with control for spheroid protein expression upon adhesion to plastic and spheroid quality) (v, **Figure 1B**). Endometrial epithelial monolayers and co-cultures (per Figure 1B) were terminated after 6 hours of co-culture/media change (for monolayers) and cell lysates processed for analysis by nanoLC-MS/MS data-dependent acquisition. Samples were analysed in biological triplicate, with technical replicates, and a stringent metric for protein and peptide identification. A total 3,760 proteins were identified in the global proteomics analysis (**Suppl. Figure 1, Suppl. Table 1**) representing the largest human embryo mimetic (trophectoderm) protein data set yet reported. The protein expression heatmap of endometrial monolayers and trophectoderm co-culture analyses is shown (**Figure 2A**). The proteins identified in this study represent significantly low abundant factors as judged from normalised LFQ values (**Figure 2B**), indicating an increased sampling depth collectively spanning over five orders of magnitude.

Defining the human endometrial 'epithelial receptome'

Proteomic comparison of adhesive endometrial monolayers from fertile women (**Figure 1B**; i) with non-adhesive monolayers from infertile women (**Figure 1B**; iii, both estrogen/progesterone treated) led to identification of an 'epithelial receptome' (**Figure 3A**). Of the 2,048 proteins identified, 137 were unique to receptive fertile group, while 134 proteins were identified in the infertile, non-receptive group (**Figure 3A**; **Suppl. Table 2**). Protein functional annotation and pathway analysis of unique protein subset associated with receptivity in the fertile group revealed protein categories associated with cell-cell adhesion (1.40E-03), cell-cell adherens junctions (1.60E-04), type I interferon signaling pathway (7.04E-02), antigen processing and presentation (6.81E-03), cell projection (4.32E-03), and vascular endothelial growth factor receptor signaling pathway (7.22E-02). For unique proteins

from non-receptive endometrium, functional annotation and pathway analysis revealed proteins associated with focal adhesion (4.95E-04), regulation of membrane permeability (1.81E-02), lipid metabolism (8.25E-03), fatty acid metabolism (4.29E-03), protein transport (1.03E-02), mitochondrion (4.80E-02), and mitochondrial respiratory chain complex I (3.36E-02) (**Figure 3A**). Taking these findings together, this analysis shows that the receptive endometrial proteome is tailored to cell adhesion and cellular attachment, interaction/projection, while in contrast metabolic regulation (including lipid and mitochondrial function), and membrane permeability and attachment are prominently represented in the non-receptive, infertile endometrium.

For proteins differentially expressed, label-free quantitation (LFQ, precursor ion intensity, normalised by maxLFQ [25]) demonstrated significant differential expression of 296 proteins (i, F mono in comparison to iii, IF mono); 136 proteins up-regulated and 132 proteins down-regulated (ratio fold change ≥2, p≤0.05) in the hormonally-primed adhesive monolayer vs hormonally-primed non-adhesive monolayer (**Figure 3B**, **Suppl. Table 1** [total proteome]; **Suppl. Table 3** [epithelial receptome only]). Of the 136 up-regulated proteins, 50 have previously been associated with endometrial receptivity (**Suppl. Table 4, Suppl. Information 1**), 4 included within Endometrial Receptivity Array [26]), yielding 86 potential new protein markers for receptivity. Functions/biological pathways significantly (p≤0.05) enriched in the 'epithelial receptome' included components associated with membrane (cell membrane, membrane raft assembly), translation (translation initiation), mitochondria/membrane, and cellular adhesion changes (cell adhesion, focal adhesion, cell-cell adherens junctions, cadherin binding involved in cell-cell adhesion) (**Figure 3C**, **Suppl. Table 5**). This was confirmed by enrichment analyses using orthogonal approaches including STRING [27] where the 'epithelial receptome' showed significant (p≤0.05) enrichment for translational initiation, translational elongation, and membrane organisation. Down-regulated processes during receptivity included endoplasmic reticulum functions, focal adhesion and response to oxidative stress/oxygen species (**Figure 3D**).

Definition of a human embryo implantation 'adhesome'

To understand embryo-endometrium interactions at the time of implantation, we first compared proteins identified in adhesive endometrium (adhesome), in comparison to non-adhesive, and subsequently examined whether this adhesome network was also involved in the trophectoderm-endometrial epithelium interaction (**Figure 4**). For the 'adhesome', the proteomes of receptive endometrial monolayer [Figure 1Bi] and non-adhesive endometrial epithelial cells [Figure 1 Biii &Biv] were compared to adhesive co-culture [Figure 1 Bii]), 143 components were significantly upregulated (≥2 ratio, $p \leq 0.05$), and 143 components significantly downregulated (≤-2 ratio, $p \leq 0.05$) in expression (**Supp Table 6**). Of the up-regulated proteins, 42 have previously been associated with endometrial receptivity/embryo implantation (**Supp Table 6, Suppl. Information 2**) yielding 100 novel proteins for further investigation. Networks associated with membrane, calcium ion binding, cell proliferation, translation, cell-cell adhesion, cytoskeletal/cell projection, and lamellipodia were identified (**Figure 4A**), along with establishment of protein localization to membrane, and membrane organization determined by STRING network enrichment analysis. Down-regulated processes (**Figure 4B**) included cell-cell adhesion and specific molecules involved in this adhesion which may reflect alterations in the epithelial cell monolayer to promote implantation.

To determine whether adhesome proteins were specific to trophectoderm-endometrial epithelium interaction or simply regulated upon trophectoderm spheroid adhesion to a non-specific substrate (plastic, as used in other 'implantation' studies [28]), the adhesome and spheroid-only proteomes were compared (**Figure 4C**). 44 of the 143 up-regulated adhesome proteins were also expressed upon adhesion of trophectoderm spheroids alone to the plastic substrate, with 31/143 proteins expressed in ≥50% of the plastic adhered trophectoderm spheroid samples examined. Importantly, 78/143 proteins (54.5%) were exclusive to the adhesome proteome (**Suppl. Table 7**); these proteins are exclusively up-regulated upon trophectoderm-endometrial epithelial adhesion. Uniquely identified adhesome proteins included CNIH4 and SDHC (previously implicated in receptivity/implantation) and DYNLRB1 and LIMS3, neither of which have previously associated with receptivity/implantation. Cross-referencing of the adhesome list with: i) proteins not previously implicated in endometrial receptivity/embryo implantation; implicated in receptivity/embryo implantation, (**Suppl. Table**

6) and; ii) those exclusive to trophectoderm-endometrial epithelial adhesome (**Suppl. Table 7**) revealed 55 unique proteins which together provide a valuable resource for future investigation of embryo implantation (**Suppl. Table 7**).

Further, of the 2,212 proteins identified across adhesive and non-adhesive endometrium (**Figure 4**), 116 were unique to the fertile implantation/adhesion group (ii), while 74 proteins were identified in the infertile non-implantation/non-adhesion group (**Figure 4D**; **Suppl. Table 8**). Protein functional annotation and pathway analysis of unique protein subset associated with implantation (adhesome) in the fertile group revealed protein categories associated with cell-cell adhesion (1.20E-02), cell-cell adherens junctions (1.84E-02), endocytosis (1.51E-02), protein biosynthesis (2.75E-02), cell projection (4.32E-03), and microtubule cytoskeleton organization (4.92E-02). For unique proteins from infertile non-implantation/non-adhesion endometrium (not identified in fertile implantation/adhesion endometrium or spheroid alone) (**Figure 4D**), functional annotation and pathway analysis revealed proteins associated with protein binding (7.61E-03), ubiquitin activating enzyme activity (1.62E-02), antigen processing and presentation (1.75E-02), cell-cell adherens junction (1.83E-02), glutathione biosynthetic process (5.0E-02), and membrane (3.72E-02). Taking these findings together, this analysis shows that the fertile implantation/adhesion endometrial proteome (as distinct from infertile non-implantation/non-adhesion endometrium, and trophectoderm alone; i.e., adhesome) is tailored to cell adhesion and cellular attachment, and organisation of the membrane and cytoskeletal network, while in contrast immune regulation (antigen presentation), cell-cell adhesion, glutathione and ubiquitin activities are prominently represented in the infertile endometrium which cannot support implantation.

'Epithelial receptome' versus 'adhesome': commonalities, differences and unique protein signature

A subset of proteins were commonly expressed between 'receptome' and 'adhesome' (33 up-regulated proteins, 40 down-regulated proteins, (**Suppl. Table 9**) suggestive of roles in both processes. Proteins exclusive to the 'receptome' or 'adhesome' may be considered to define more accurately, epithelial receptivity and embryo adhesion respectively. 139 proteins were

exclusive to 'receptivity', 78 up-regulated, 61 down-regulated (**Suppl. Table 10**). 156 proteins were exclusively associated with adhesion, 78 up-regulated, 78 down-regulated (**Suppl. Table 7**).

Protein landscape of trophectoderm co-culture with adherent and non-adherent endometrium

To directly compare the protein landscape of trophectoderm between adherent and non-adherent monolayer and spheroid models we performed cell morphology-based proteomic profiling (**Figure 5A**) (**Suppl. Table 11**). Proteins differentially (≥2 ratio) expressed between adherent and non-adherent monolayer and spheroid models revealed 114 proteins were selectively upregulated (**Figure 5B**), associated with membrane, mitochondria, cell adhesion (focal adhesion), protein transport, response to oxidative stress, and gluconeogenesis; 358 proteins were downregulated in this comparison (**Figure 5C**). Functional enrichment analyses of spheroid growth condition revealed networks associated with cell adhesion (cadherin binding, cell-cell adherens junction, cell-cell adhesion, focal adhesion) and vascular endothelial growth factor receptor signalling pathway. This comparison reveals important cellular protein network changes between monolayer and spheroid trophectoderm models with regards to their adhesive protein landscape.

Tissue expression of endometrium validates receptome protein expression

Immunostaining for KYNU, LGMN, PC4, CDA and MAGT1 (**Figure 6A-E respectively**) was evident within receptive phase (mid-secretory) endometrium, mainly localized to epithelial cells, with minimal/no immunostaining within non-receptive (proliferative) endometrium (**Figure 6F-J**) confirming the validity of our proteomic approach (**Figure 6K**) in identification of potential receptivity proteins. PC4 clearly demonstrates some degree of staining within the proliferative phase endometrium (**Figure 6H**). Importantly, this endometrial tissue expression approach validates receptome protein expression of several protein targets identified by proteomic profiling, but does not distinguish between stromal and epithelial components of the endometrium.

Validation of adhesome function: perturbing the interaction between trophectoderm and endometrium

Given that the ultimate aim of this study was to examine and understand 'embryo' (trophectoderm spheroid) adhesion to the endometrial epithelium in a human model, validation in mouse knockout models was inappropriate due to the different modes of implantation between species. We targeted proteins identified in the proteomic profile of fertile implantation/adhesion endometrium (adhesome; ii), in comparison to infertile non-receptive endometrium (iii) and infertile non-implantation/non-adhesion endometrium (iv) (**Figure 6L**). Knockdown of LGMN, SERPINE1, and PTGS2 in the human ECC-1 cell line followed by E/MPA treatment of the knockdown/scrambled construct cells demonstrated significantly reduced trophectoderm spheroid adhesion associated with LGMN ($p<0.01$) and SERPINE1 ($p<0.05$), and reduced adhesion associated with PTGS2 (ns) knockdown versus scrambled construct (sc siRNA; control, **Figure 6M**). This further validates our functional proteomic strategy and confirms the functional involvement of these proteins in adhesion between trophectoderm and endometrium.

4 Discussion

This work interrogates and validates the cellular proteomes of the endometrial epithelium at the human implantation site, in terms of the 'receptome' and the adhesome. The 'receptome' data defines the state of the epithelium for that very short period of time in each menstrual cycle when the endometrium is appropriately differentiated to enable embryo implantation, while the 'adhesome' represents receptive endometrial epithelium that has additionally been influenced by the presence of a blastocyst. The study utilizes a recently developed co- culture model of implantation, using true trophectodermal cells and primary in vivo-derived receptive and non-receptive endometrial cells, and offers promise for identifying and improving endometrial receptivity in women [3]. Importantly, the data provides uniquely regulated

proteins of importance for both receptivity and adhesion, critical for establishing pregnancy in women.

It is clear from the proteomic data, that a differential response to a high quality human embryo may be mounted by the endometrial epithelial cells depending on the fertility status of the woman [29] as highlighted by our embryo mimic. These essential changes within the luminal epithelium in the initial stages of implantation are not replicated in some infertile women. Indeed, in such women, the barrier function of the epithelium cannot be appropriately modulated to enable trophectoderm attachment, thus resulting in implantation failure [30].

A global understanding of human endometrial receptivity remains elusive, most likely due to the plethora of cell types included in analyses and the variability between women. Furthermore, endometrial receptivity may be pathological, display altered timing or be a combination of pathological and altered in timing [31]. Such differentiation and determination of fertility issues may be aided by the current model. Since the first contact between the embryo and endometrium is the epithelium which lines the uterine cavity, the current study specifically examines the functional status of endometrial epithelium and associated changes in its proteome. Its strength lies in the unique power of the trophectoderm spheroid model to discriminate between ‘fertile’ and ‘infertile’ endometrium rather than reliance on menstrual cycle day or apparent/previous fertility status, both of which are unreliable ‘fertility determinants’. Proteomic analysis of appropriately hormone-primed (estrogen/progesterone) endometrial epithelial cells, defined as ‘receptive’ or ‘non-receptive’ based on trophectoderm spheroid adhesion, has revealed a protein signature encompassing a large number of proteins not previously investigated in this role. Our ‘epithelial receptome’ analysis has a concordance of 36.7% with previous studies investigating endometrial receptivity (Supplementary references: epithelial receptome). This is encouraging, particularly considering the overlap of a number of the proteins identified with genes utilized by the Endometrial Receptivity Array to predict whether the endometrium is capable of supporting a pregnancy [26], and that our proteome is specific to epithelium. However, it is not surprising that the concordance rate is relatively low given the lack of agreement between the many

existing genomic and proteomic studies of endometrial receptivity [32] and that the only previous study globally analysing epithelium alone, found distinct mRNA signatures for epithelium and stroma [33].

As anticipated, many cellular adhesion proteins were up-regulated within receptive endometrial epithelial cells preparing for embryo adhesion, together with cell membrane alterations supporting a 'plasma membrane transformation' [34]. Focal adhesion proteins were down-regulated, also encompassing aspects of the plasma membrane transformation whereby the cells become less adherent to each other and to their underlying basal lamina. Independent immunohistochemical validation of 5 receptome proteins localized them primarily to endometrial epithelial cells of receptive (secretory) endometrium. Collectively, these findings provide validity to our analyses, which are a unique resource for studies of essential epithelial-specific changes and for diagnosis of receptivity.

Identifying mechanisms that regulate/characterize the adhesion phase of embryo implantation, is important if we are to improve establishment of pregnancy [23]. The 'adhesome' at the trophectoderm-epithelial interface, includes many previously unidentified proteins in this setting. Differentially expressed cell-surface ligands, cell-cell and cell-matrix adhesion and receptors identified herein, need defining both in normal implantation and when adhesion, cellular reprogramming, and specific cell interactions are disturbed with infertility and complications of early pregnancy. Important up-regulated adhesome proteins included PTGS2 (previously implicated in implantation [35]), LGMN and SERPINE1, for which mutations are associated with recurrent pregnancy loss [36]. Importantly, knockdown of 3 of these proteins in epithelial cells functionally reduced their adhesive capacity. Knockdown of PTGS2 did not significantly reduce adhesion, potentially suggesting this protein may be of lesser importance in the adhesive process. The proteins identified display a 30% concordance with previous studies investigating receptivity and implantation (Supplementary references; adhesome) (annotated, **Table 2**). This relatively low concordance likely reflects the human focus of the current model as opposed to previous studies conducted in mouse models.

570 **Summary**

571

572 Identification of the proteome of receptive endometrium represents a key step towards
573 alleviating some infertility and provides potential targets for inhibition of receptivity as a
574 contraceptive strategy. Furthermore, this first classification of the human adhesome, provides
575 strong new targets for further investigation of the basic mechanisms underpinning the critical
576 first step in implantation. Both the epithelial receptome and the adhesome provide a valuable
577 resource for future studies focussed on improving embryo implantation by
578 endogenous/exogenous interventions. In addition, our proteomic strategy is broadly
579 applicable to other cell surface, developmental and stem cell systems. Future work to
580 enhance our endometrial/embryo proteomic resource could include alternative methodologies
581 to enrich membrane and cell surface subsets, or related modifications associated with these
582 cell subsets. In addition, human extracellular protein and RNA cellular data sets should be
583 integrated to generate detailed knowledge of the intra- and extra-cellular signalling pathways
584 (i.e., mediated through exosomes [5] and soluble mediators) that regulate receptivity and
585 implantation.

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Author Contributions

J.E. conceived and designed the experiments. J.E, and D.G. carried out the majority of experiments. JH performed experimental work. DG performed proteomic analysis, bioinformatics. LAS helped develop project, wrote and edited manuscript and provided critical insight. J.E. L.A. and D.G wrote, reviewed and edited the manuscript. All authors approved the final manuscript.

Conflicts of interest: The authors declare no competing interests.

Data and Software Availability: The accession number for the mass spectrometry data reported in this paper is PeptideAtlas Consortium via the PeptideAtlas proteomics repository: PASS01121.

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Figure legends:

Figure 1: **Experimental design to understand human trophectoderm-endometrial adhesion during fertility**. (A) Representative image of morphologically normal primary human endometrial epithelial cells at >95% purity. (B) To understand the adhesion network between trophectoderm spheroids (outer layer of embryo) and endometrium during fertility, we employed primary human endometrial epithelial cells from women with proven fertility (F, ✔, morphologically normal endometrium), and from infertile women in an IVF program (IF, ✖, with non-endometrial indications for infertility). (C) Cells from each women were independently grown in monolayer culture in the presence of estrogen/progestin, either alone or in the presence of trophectoderm spheroids. They were then defined as 'receptive' (i) or 'non-receptive' (iii) depending on whether trophectoderm spheroids attached (adhesion (ii): non-adhesion (iv)) to matched endometrial monolayers. Spheroids were also maintained in isolation (v). (D) Experimental design of endometrial cells hormonally primed with estrogen/progestin, and where applicable, spheroid co-culture and attachment, before imaging and outgrowth quantification, and cell lysis performed.

Figure 2: Protein landscape of endometrial receptome and adhesome. (A) Protein expression heatmap of proteins identified in receptive (i, F) and non-receptive (iii, IF) epithelium, depending whether matched monolayers demonstrated adhesion (ii, F) or non-adhesion (iv, IF) with trophectodermal spheroids (monitored alone, v). Scale represents label-free quantitation intensity. (B) Estimated abundance by LFQ intensity (log10) of proteins identified in endometrial receptome and adhesome, indicating the sampling depth of each dataset spanning over five orders of magnitude.

Figure 3: Protein landscape of endometrial receptome. (A) Venn diagram of proteins identified in receptive (i, F) and non-receptive (iii, IF) epithelium, with functional annotation of each unique subset. (B) Protein expression heatmap of proteins identified in receptive (i) and non-receptive epithelium (iii). Scale represents label-free quantitation intensity. (C/D) Protein functional annotation and pathway analysis for significantly ($p \leq 0.05$) enriched (C) and down-

743 regulated (D) components between i vs iii, ‘epithelial receptome’ using Gene Ontology,
744 STRING and Reactome.
745
746 **Figure 4: Protein landscape of human embryo implantation ‘adhesome’**. (A/B)
747 Functional metrics of differentially (p≤0.05) enriched (A and down-regulated (B) proteins
748 between adherent (ii) and non-adherent (iv) co-culture, based on enrichment analysis (Gene
749 Ontology, STRING and Reactome) and analyzed by hierarchical clustering. (C) Protein
750 expression heatmap of proteins identified in implantation ‘adhesome’ for fertile (ii) and infertile
751 (iv) co-culture, relative to trophectodermal spheroids (v). Scale represents label-free
752 quantitation intensity. (D) Venn diagram of proteins identified in fertile adhesome (ii) and
753 infertile (non-adherent) endometrium co-culture (iv), with trophectodermal spheroids alone (v).
754
755 **Figure 5: Endometrial adhesion to trophectoderm influences proteome.** (A) Venn
756 diagram of proteins identified in fertile adhesome (ii) and infertile (non-adherent) endometrium
757 co-culture (iv), indicating differences in cellular proteome based on co-culture. (B) Functional
758 metrics of differentially (p≤0.05) enriched proteins between adherent and non-adherent
759 endometrium to trophectoderm spheroids, performed based on enrichment analysis using
760 (Gene Ontology, STRING and Reactome) and. Data were analyzed by hierarchical clustering.
761 (C) Significantly (p≤0.05) down-regulated proteins (based on enrichment analysis as above)
762 using Gene Ontology, STRING and Reactome were analyzed by hierarchical clustering.
763
764 **Figure 6: Protein expression validation of receptome markers in human receptive**
765 **endometrial tissue**. Immunohistochemistry of receptome proteins KYNU (A), LGMN (B),
766 PC4 (C), CDA (D) and MAGT1 (E) positively immunostain endometrial epithelial cells within
767 receptive (mid-secretory phase) human endometrium with minimal/no staining within
768 endometrial epithelial cells of proliferative phase endometrium for KYNU (F), LGMN (G), PC4
769 (H), CDA (I) or MAGT1 (J). Closed arrowheads indicate endometrial glandular epithelium, *
770 indicates endometrial glands invaginating from luminal epithelium, # indicates endometrial
771 stromal localization. (K) Protein abundance by LFQ intensity of proteins identified in receptive
772 (i) and non-receptive (iii) endometrium. (L) Protein abundance by LFQ intensity of LGMN,

773 SERPINE1, and PTGS2 identified in adherent endometrium co-culture (ii, F), non-adherent
774 endometrium monolayer (iii), and non-adherent endometrium co-culture (iv, IF). Knockdown
775 of LGMN (■, $p<0.01$), SERPINE1 (■, $p<0.05$) and PTGS2 (■) within endometrial epithelial
776 cells reduced trophectoderm adhesion versus scrambled control (■, M).
777

Table 1: Characteristics of women used in co-culture studies

Fertile						
	Endometrial findings	**Parity**	**Age**	**BMI**	**Reason for hysperoscopy/D&C**	**Fertility status**
P1	Normal	P3	**39**	31.2	Mirena insertion	Fertile
P2	Normal	P2	**40**	22.8	Benign ovarian cyst assessment	Fertile
P3	Polyps	P1	**30**	17.6	Polypectomy	Fertile
Infertile						
P4	Normal	P1 (IVF)	**39**	20.8	Pain	Secondary infertility
P5	Normal		**28**	27.5	Tubal assessment: patent, unblocked tubes present	Secondary infertility
P6	Normal		**36**	30.9	Tubal assessment: patent, unblocked tubes present	Primary infertility
