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MARIANN KOEL

The molecular interactions between trophoblast and endometrial cells in embryo implantation

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The molecular interactions between trophoblast and endometrial cells in embryo implantation

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Institute of Molecular and Cell Biology, University of Tartu, Estonia

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My contributions to the listed publications were as follows:

Ref I: Performed statistical analyses, conducted validation experiments, and participated in writing the manuscript.

Ref II: Developed the protocols and performed TAC-seq experiments, performed TAC-seq data analysis, and participated in writing the manuscript.

Ref III: Participated in the study design, performed the protein-protein network analyses, visualized the results, and participated in writing the manuscript.

Ref IV: Participated in the study design, conducted the trophoblast differentiation experiments, analyzed the RNA-seq data, visualized the results, and participated in writing the manuscript.

ABBREVIATIONS

AMOT	angiomotin
aPKC	atypical protein kinase C
AQP3	aquaporin 3
ART	assisted reproductive technology
BMI	body mass index
BMP4	bone morphogenic protein 4
BV	blood vessel
C4BPA	complement component 4 binding protein alpha
CCDS	Consensus Coding Sequence Set
CD	cluster of differentiation
CDH1	E-cadherin
cfDNA	cell-free DNA
CI	confidence interval
CP	primary chorionic plate
CPM	counts per million
CRYAB	alpha-crystallin B chain
CT	cytotrophoblast
CXCL14	chemokine (C-X-C motif) ligand 14
D	decidua
DF	dominant follicle
DKK1	dickkopf-related protein 1
DNMT3L	DNA methyltransferase 3 like
E	endometrial epithelium
EB	embryoblast
ECM	extracellular matrix
eCTB	endovascular cytotrophoblasts
EG	endometrial gland
EGF	epidermal growth factor
EM	extraembryonic mesoderm
EMT	epithelial-to-mesenchymal transition
EPI	epiblast
ERA	endometrial receptivity array
ET	embryo transfer
EV	extracellular vesicle
EVT	extravillous trophoblast
FC	Fold change
FBS	fetal bovine serum
FDR	false discovery rate
FGF2	fibroblast growth factor 2
FGF4	fibroblast growth factor 4
FOXO1	forkhead box protein O1
FSH	follicle-stimulating hormone

GATA	GATA binding protein
GCM1	glial cells missing transcription factor 1
GE	glandular epithelium
GnRH	gonadotropin-releasing hormone
GPX3	glutathione peroxidase 3
hCG	human chorionic gonadotrophin
hCG-H	hyperglycosylated hCG
hESC	human embryonic stem cell
HGEx-ERdb	Human Gene Expression Endometrial Receptivity database
hPSCs	human pluripotent stem cells
ICM	inner cell mass
ICMART	International Committee for Monitoring Assisted Reproductive Technologies
iCTB	interstitial cytotrophoblasts
IF	immunofluorescence
iFGF2	inhibitor of fibroblast growth factor 2
IFI16	interferon gamma inducible protein 16
IL15	interleukin 15
iPSC	induced pluripotent stem cells
iTGFb	inhibitor of transforming growth factor beta
IVF	in vitro fertilization
IVS	intervillous space
KEGG	Kyoto Encyclopedia of Genes and Genomes
LATS	large tumor suppressor kinase
LE	luminal epithelium
LH	luteinizing hormone
LIF	leukemia inhibitory factor
M	myometrium
miRNA	microRNA
MMP	matrix metalloproteinase
mRNA	messenger RNA
mTSC	mouse trophoblast stem cells
MUC-1	mucin 1
NF	Nitabuch fibrinoid
NF2	neurofibromin 2
NGS	next-generation sequencing
NR2F2	nuclear receptor subfamily 2 group F member 2
PAEP	progestagen associated endometrial protein
PBS	phosphate-buffered saline
pET	personalized embryo transfer
PGF2α	prostaglandin F2α
PGT	preimplantation genetic testing
POU5F1B	POU Class 5 Homeobox 1B
PPI	protein-protein interaction
PrE	primitive endoderm

pTE	polar trophectoderm
RIN	RNA integrity number
RNA-seq	RNA sequencing
rpm	revolutions per minute
RPKM	reads per million mapped reads
RRA	robust rank aggregation
RT-qPCR	reverse transcription quantitative real-time polymerase chain reaction
SA	spinal artery
SART	Society for Reproductive Technology
scRNA-seq	single-cell RNA-sequencing
ST	syncytiotrophoblast
STRT	single-cell tagged reverse transcription
TAC-seq	targeted allele counting by sequencing
TE	trophectoderm
TEAD4	transcriptional enhancer factor TEF-3
TGF-β	transforming growth factor beta
TS	trophoblast stem (cell)
UMI	unique molecular identifier
uNK	uterine natural killer
UV	umbilical vein
VEGF	vascular endothelial growth factor
WOI	window of implantation
YAP1	yes-associated protein 1

INTRODUCTION

Embryo implantation is one of the most fascinating processes in human life. It needs delicately coordinated cooperation between the maternal cells and the developing embryo. Only the attachment to the correct tissue – the endometrium that lines the internal space of the uterus and the perfect timing of the implantation process ensures the successful onset of pregnancy. The endometrial cells undergo monthly differentiation and maturation during the menstrual cycle, undergoing a transformation that renders them receptive to embryo attachment. This occurs within a brief period known as the "window of implantation" during the mid-secretory phase. By this time, the embryo should differentiate to the blastocyst stage, hatch from the protecting extracellular glycoproteinaceous coat called *zona pellucida*, and position itself on the endometrium. Only after a firm attachment, the trophectoderm cells that form the outer layer of the blastocyst cells invade between the endometrial stromal cells and start to differentiate into the functional placental cells that anchor and nurture the developing embryo and fetus.

Although getting pregnant is one of the most natural biological processes in a woman's life, getting a baby poses a problem for many (17.5%) couples (Njagi *et al.*, 2023). "The failure to establish a clinical pregnancy after 12 months of regular, unprotected sexual intercourse" is a condition called infertility (Zegers-Hochschild *et al.*, 2017). While some causes of infertility, like hormonal imbalance, can be treated by medications, assisted reproductive technology (ART) is used for others. Over 8 million babies have been born worldwide with the help of assisted reproductive technology, including *in vitro* fertilization (IVF) (Fauser, 2019). In Estonia, the number of babies born with ART's help was 876 in 2020, constituting 6% of the live births (Ravimiamet 2022).

The probability of live birth after IVF depends on various factors, including embryo quality, endometrial receptivity, and the specific embryo transfer (ET) technique employed (Reshef *et al.*, 2022). Over the past decade, the success rate of IVF has significantly improved thanks to advancements in embryo quality, optimized ovarian stimulation protocols, and the development of preimplantation genetic testing (PGT), which enables the selection of euploid embryos for transfer (Pirtea *et al.*, 2021). However, according to the Society for Reproductive Technology (SART) report, the overall success rate of IVF procedures is approximately 50% for women under 35 and decreases rapidly with increasing maternal age (SART 2023). It has also been observed that high-quality embryos alone do not guarantee successful implantation (Chambers *et al.*, 2014), emphasizing the importance of endometrial quality (Duffy *et al.*, 2021).

Using ART requires a deep understanding of the human embryo implantation process, which is difficult to achieve due to ethical constraints associated with conducting relevant controlled *in vivo* studies. Therefore, an alternative approach is warranted. This Doctoral Thesis addresses these challenges by exploring endometrial receptivity and modeling embryo implantation and early placental cell differentiation. It defines relevant marker genes of endometrial receptivity and

specifies endometrial cell type-specific gene expression during the window of implantation (WOI). Possible protein interaction partners between the receptive endometrium and implantation-competent blastocyst are proposed to model the implantation process. Additionally, a new comprehensive method to detect WOI in endometrial biopsy samples is described, which is faster, cheaper, and more accurate than previous methods. Finally, the thesis presents the effect of different inhibitors on the bone morphogenetic protein 4 (BMP4)-triggered human embryonic stem cell (hESC) differentiation model system to determine the optimal condition for studying trophoblast cell differentiation during early pregnancy. As this thesis investigates various aspects of the communication between the embryo and the endometrium in human embryo implantation, it provides opportunities for further improvements in assisted reproductive technologies.

1. REVIEW OF THE LITERATURE

1.1. Overview of the menstrual cycle

Women have a protracted reproductive lifespan, which commences at an average age of 13 years with menarche, the first menstrual period, and terminates at around 50 years with menopause (Rees, 1995). The menstrual cycle, from the first day of menstrual bleeding until the beginning of the next menstruation, is strictly regulated by endocrine, autocrine, and paracrine factors. Two methods exist to classify the menstrual cycle stages, depending on the endometrial or ovarian/ follicle condition in response to the produced hormones. Based on the endometrial structure, the cycle can be categorized into the proliferative and the secretory phases. Alternatively, based on ovarian follicular development, the follicular and luteal phases of the cycle can be distinguished (Figure 1).

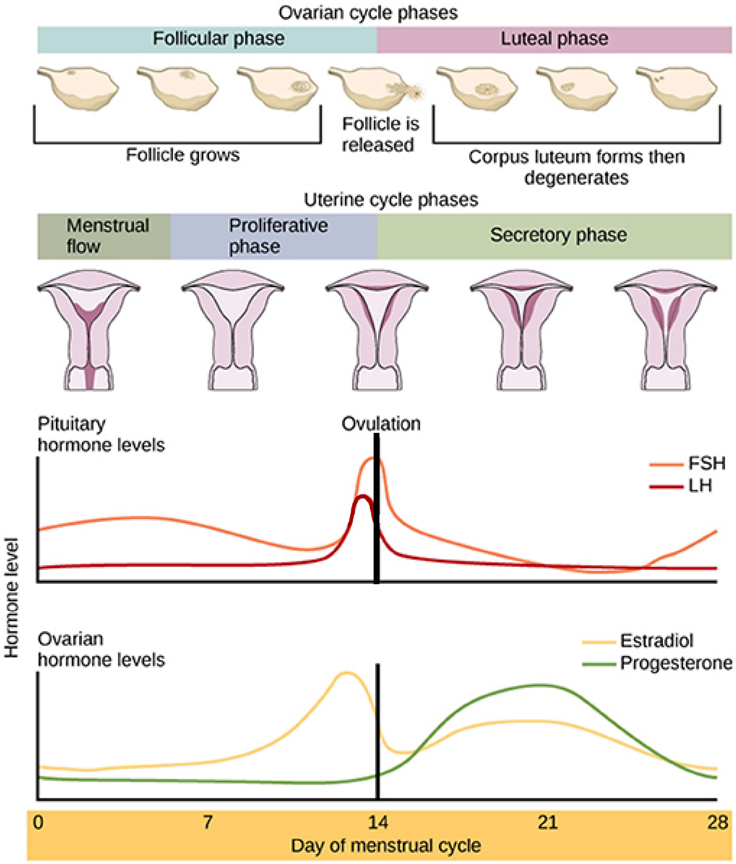

Figure 1. The menstrual cycle is divided into phases based on the uterine or follicular cycle. The ovarian cycle: follicular (blue) and luteal phase (pink). The uterine cycle: menstrual flow (dark green), proliferative phase (violet), secretory phase (green). The hormonal fluctuation through the menstrual cycle is shown with different colored lines. Figure adapted from (Warren *et al.*, 2021).

The endometrium's proliferative phase corresponds to the ovary's follicular phase and lasts until ovulation. Following ovulation, increasing levels of estrogen and progesterone result in the endometrium's secretory phase, which correlates with the ovary's luteal phase (Bates and Bowling, 2013).

The median length of the menstrual cycle in healthy and fertile women between 18 and 45 years of age is typically 28 days (Ecochard and Gougeon, 2000). However, individual cycle lengths are highly variable, ranging from 25 to 34 days on average (Grieger and Norman, 2020). These variations are caused mainly by differences in the follicular phase length, which ends on the day of ovulation. According to data from the Natural Cycles mobile application, which records detailed menstrual cycle information, the mean length of the follicular phase is 16.9 days (95% CI: 10–30), while the mean length of the luteal stage is 12.4 days (95% CI: 7–17) (Bull *et al.*, 2019). The follicular phase length often varies from cycle to cycle in an individual woman. In contrast, the luteal phase has a somewhat more consistent length due to the predictable lifespan of the *corpus luteum* (Fehring *et al.*, 2006). Furthermore, the menstrual cycle length can vary for the same woman during her reproductive years, with a gradual shortening of 3.2 days (95% CI: 3.2–3.3 days) from age 25 to 45, mainly due to a reduction in the mean follicular phase length in the same period, but above 40, the variation increased dramatically (Bull *et al.*, 2019).

1.1.1. Follicle dynamics throughout the menstrual cycle

The development of the ovarian follicular reserve, which provides the source of follicles for maturation throughout a female's life, begins early in fetal life, around the fourth month (Baker, 1963). During embryonic development, primordial germ cells are identifiable on the yolk sac wall from three to four weeks post-conception. These cells migrate through the hindgut, dorsal mesentery, and finally settle in the developing gonads by approximately 6 weeks. Upon reaching the gonads, the primordial germ cells undergo growth and differentiation, transitioning into oogonia. Oogonia undergo mitotic proliferation between the 9th and 22nd week of embryonic development, forming clusters of cells known as germline cysts.

Peripartum, each germ cell cyst regresses and gives rise to a primordial follicle, consisting of a primary oocyte surrounded by a single layer of granulosa cells. The primary oocytes progress through oogenesis, entering meiosis and arresting at meiotic prophase I. During the gonadotropin-independent pre-antral growth phase, these primordial follicles transition into primary follicles through oocyte enlargement and granulosa cell layer proliferation. The formed granulosa cells start expressing follicle-stimulating hormone (FSH) receptors during this phase. Although the growth of these follicles occurs throughout fetal life, infancy, and childhood, the development of antral follicles begins at puberty. The hypothalamic-pituitary-gonadal axis matures and stimulates a pulsatile release of FSH and luteinizing hormone (LH) from the pituitary gland via the gonadotropin-releasing hormone (GnRH). This process culminates in the cyclic development of antral follicles with a diameter of ≥2 mm, onset of ovulation, and menstrual cyclicity until the end of the reproductive lifespan (Cox and Takov, 2023).

The first hormonal event in the menstrual cycle is the rise in FSH level 4 days before the onset of menstruation, caused by the fall of the estradiol and inhibin A at the end of the previous luteal phase (Figure 1). The rise in FSH levels marks the luteal-follicular transition, which recruits a new cohort of antral follicles (Miro and Aspinall, 2005). The oocyte-containing ovarian follicles mature in the early follicular phase during the first half of the menstrual cycle. The increase in FSH concentrations above a critical threshold initiates the major and minor wave patterns required to recruit the dominant follicle (DF) (Baerwald *et al.*, 2003). From the mid-follicular phase, the DF produces estradiol, which provides negative feedback on pituitary FSH secretion, increases inhibin B secretion from follicles, and inhibits the growth of subordinate follicles, ultimately causing their atresia (Groome *et al.*, 1996). In the late follicular phase, the estradiol peak causes a surge of GnRH from the hypothalamus that stimulates the secretion of luteinizing hormone (LH) from the pituitary (Micevych and Sinchak, 2011). The pre-ovulatory follicle, which now has acquired LH receptors, releases the oocyte during the ovulation process on average 8–20 hours after the LH peak (Kerin, 1982), and the remaining DF (granulosa and theca cells) transform into the *corpus luteum*.

At the beginning of the luteal phase, the formed *corpus luteum* is composed of two steroidogenic cell types; small luteal cells of the thecal cell origin, which respond to LH and secrete progesterone, and large luteal cells of the granulosal cell origin, which contain receptors for prostaglandin F2α (PGF2α). If no oocyte fertilization occurs, the *corpus luteum* goes to cellular apoptosis and autophagy triggered by cytokines and PGF2α, causing the rapid withdrawal of estradiol and progesterone and provoking menstruation. However, in case of pregnancy, the invasive trophoblast secretes human chorionic gonadotrophin (hCG), which binds to the luteal LH-receptor and stimulates progesterone, estradiol, and inhibin A production, as well as protects against PGF2α. This rescues the *corpus luteum* from programmed senescence and maintains progesterone production, which is, in turn, crucial for the maintenance of pregnancy (Niswender *et al.*, 2000).

1.1.2. Endometrial remodeling through the menstrual cycle

A complex multicellular tissue layer lines the uterine cavity called the endometrium. The endometrium consists of luminal and glandular epithelial cells, supportive stromal fibroblasts, endothelial cells, and immune cells. The endometrium of a woman of reproductive age is divided into two layers, namely the basal and functional layers (Figure 2). The basal layer, located close to the myometrium, is responsible for tissue regeneration. On the other hand, the functional layer undergoes tissue proliferation, secretion, and degeneration. Blastocyst implantation occurs in this layer 7–10 days after ovulation, provided conception occurs. The proliferation of the endometrium during the ovarian follicular phase, differentiation during the ovarian luteal phase and endometrial secretory phase, and complete shedding during menstruation is regulated by the circulating ovarian sex steroids estradiol and progesterone (Jabbour *et al.*, 2006).

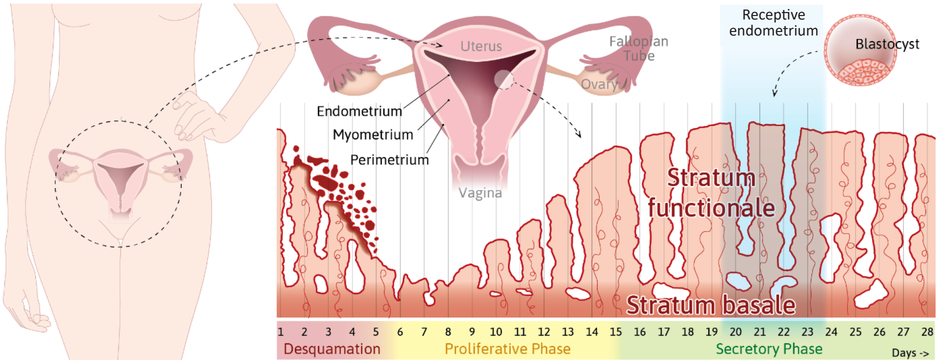

Figure 2. The scheme of the multi-layered wall structure of the uterus and menstrual cycle-dependent endometrial changes. The uterus has three primary layers: the outer connective tissue-rich perimetrium, the smooth muscle-containing contractile myometrium, and the inner endometrium. The endometrium can be further subdivided into the basal (stratum basale) and the functional (stratum functionale) layer, which is shed (desquamation) during menstruation and rebuilt during the proliferative phase, with changes in differentiation during the secretory phase to prepare for blastocyst implantation during the window of implantation. Modified from (Sternberg *et al.*, 2021)

1.1.2.1. Proliferative phase of endometrium

After shedding the functional layer of the endometrium during menstruation, the efficient repair is necessary for successful embryo implantation in the ongoing cycle. Endometrial re-epithelialization starts without ovarian hormones, while the active shedding of the tissue is still ongoing. The fragmentary endometrial loss, where actively shedding areas coexist with regions in the repair process or have already completely resurfaced, ensures that the extent of exposed endometrium is as low as possible at any moment (Garry *et al.*, 2009). It has been suggested that endometrial repair is triggered by progesterone withdrawal, which increases the synthesis of endometrial prostaglandin and therefore regulates vascular endothelial growth factor (VEGF) in human endometrial cells under hypoxic conditions (Maybin *et al.*, 2011). Scanning electron microscopy studies have revealed that the healing endometrial surface first becomes covered with a fibrinous matrix where a smooth surface and low cuboidal shape epithelial cells migrate from the exposed stumps of glands or any remaining intact epithelium in the bordering areas (Ferenczy, 1976; Garry *et al.*, 2009). At the same time, sub-epithelial endometrial arterioles in the stroma are also being repaired. Re-epithelialization is completed by the sixth day of the menstrual cycle when the endometrial surface is covered by a lining surface epithelium (Ludwig and Spornitz, 1991). It is suggested that uterine epithelialization is required before ovarian steroids increase endometrial thickness and cause the regeneration of its structure through massive cellular proliferation in the functional layer. Regeneration requires estrogen, which stimulates different types of somatic stem or progenitor cells in the basal layer and is completed by the time of ovulation (Salamonsen, 2021). It has been shown that rapid endometrial growth begins on day 4 of the menstrual cycle from an approximate thickness of 4.5 mm and increases linearly by 1 mm per day until a plateau of approximately 10 mm is reached by day 9. Endometrial thickness expansion is closely correlated with estradiol levels during this stage, but after reaching the approximate thickness of 10 mm, it stays constant, regardless of the follicular phase length (Bromer *et al.*, 2009).

1.1.2.2. The secretory phase of endometrium

The uterine secretory phase begins after ovulation when progesterone secreted by the *corpus luteum* causes the differentiation of stromal cells into specialized secretory "decidual cells". These morphological changes during decidualization are essential for creating a microenvironment that ensures embryo implantation and further placental development (Gellersen and Brosens, 2014). The endometrium becomes receptive to the embryo during the middle of the secretory phase, within a time frame called the "window of implantation" (WOI) (Navot *et al.*, 1991). During days 16 to 20 of a 28-day menstrual cycle, the epithelial glands show increased secretory activity, subnuclear vacuoles, and a decrease in mitotic activity. Subsequently, on day 21, the stroma abruptly becomes edematous, and the cells transform from an elongated to a rounded morphology while also expressing progesterone-dependent proteins (Noyes *et al.*, 1950). Within the same timeframe, typically between days 19 and 21, under the influence of progesterone, the luminal epithelium develops apical protrusions known as pinopodes (Martel *et al.*, 1991) and loses cellular polarity. These changes weaken the lateral epithelial surface interactions by reducing the expression of luminal adherens junction proteins and disturbing focal adhesions to the basal lamina, which is essential for the embryo implantation (Whitby *et al.*, 2020). Unlike in mice, pinocytotic activity has not been observed in human pinopodes. However, it has been proposed that pinopodes in humans may be involved in exocytosis and there is a hypothesis suggesting that they could release small extracellular vesicles or exosomes (Quinn *et al.*, 2020). Furthermore, during the secretory phase, there is a rapid increase in the number of uterine natural killer (uNK) cells, mediating extensive cross-talk between uterine stromal cells and playing a role in maternal allorecognition of fetal trophoblast and remodeling of spiral arteries before and during trophoblast invasion (Chazara *et al.*, 2011).

1.2. Molecular characteristics of receptive endometrium

The advent of 'omics' technologies has led to a shift in the characterization of the receptive endometrium from histological observations to global gene expression profiling. In the early 2000s, several gene expression analyses were conducted with eutopic endometrial samples using microarray technology, revealing numerous differentially regulated genes between different cycle phases (Carson *et al.*, 2002; Kao *et al.*, 2002; Borthwick *et al.*, 2003; Riesewijk *et al.*, 2003; Horcajadas *et al.*, 2004; Ponnampalam *et al.*, 2004; Mirkin *et al.*, 2005; Talbi *et al.*, 2006; Haouzi *et al.*, 2009; Díaz-Gimeno *et al.*, 2011). However, early gene expression studies were limited by differences in study design, participant selection criteria, conditions of endometrial sampling, expression array/sequencing platforms, bioinformatic pipelines for data processing, and inconsistent standards for data presentation. These studies also had small sample sizes and likely included participants from different ethnicities, which may have affected the data analysis (Bhagwat *et al.*, 2013). To overcome these issues, *in silico* data mining studies have been conducted to identify real receptive endometrial biomarkers. For instance, Tapia et al. intersected the lists of differentially regulated genes from seven microarray studies and identified 40 consistently up-regulated and 21 down-regulated transcripts (Tapia *et al.*, 2011). Zhang et al. analyzed the raw data of 45 endometrial samples from three microarray studies and identified 148 potential biomarkers of receptive endometrium among 1,543 differentially expressed genes (Zhang *et al.*, 2012). In 2013, Bhagwat et al. created the Human Gene Expression Endometrial Receptivity database (HGEx-ERdb), which describes 19,285 genes expressed in human endometrium and identifies 179 receptivity-associated genes (Bhagwat *et al.*, 2013).

Recent advancements in RNA sequencing (RNA-seq) technology, such as next-generation high-throughput RNA sequencing methods, have allowed for detecting rare transcripts and alternative splicing events with greater sensitivity and without dependence on prior knowledge. Hu and colleagues utilized RNA-seq to compare the pre-receptive and receptive phases of natural cycle endometrial biopsies and identified 1099 up-regulated and 1273 down-regulated genes (Hu *et al.*, 2014). Sigurgeirsson and colleagues also used RNA-seq to show that 1648 transcripts have higher expression in the proliferative phase and 1639 have higher expression 7–9 days after ovulation (Sigurgeirsson *et al.*, 2016). More recent studies have revealed that over 30% of the expressed genes exhibit significant variation in mean expression levels throughout the menstrual cycle. The largest differences in gene expression are observed between the proliferative and secretory phases of the menstrual cycle and between the early, mid, and late stages within the secretory phase of the cycle (Fung *et al.*, 2018). In the context of the window of implantation, up-regulated genes are predominantly involved in processes such as cell adhesion, motility and communication, growth factor, cytokine binding and signaling, immune and inflammatory responses, and hormone response. In contrast, most down-regulated genes are related to the cell division (Mortlock *et al.*, 2022).

The molecular characterization of receptive endometrium using endometrial biopsy is challenged by the heterogeneity of the cell types that constitute the tissue and their dynamic nature through a monthly cycle, involving rapid rates of cell cycle entry and exit, remodeling, and differentiation (Wang *et al.*, 2020). Thus, initial cell sorting or novel single-cell RNA-sequencing (scRNA-seq) methods offer a new approach to systematically characterizing human endometrium. Indeed, the single-cell transcriptomic atlas of the human endometrium during the menstrual cycle has been published (Wang *et al.*, 2020), where the transcriptomics profile of seven main endometrial cell types, including stromal fibroblasts, endothelial cells, macrophages, lymphocytes, ciliated and unciliated epithelial cells, and smooth muscle cells with mesenchymal stem cell characteristics, has been described. However, it is important to note that heterogeneity can exist even within the same cell type. Bioinformatic analyses of single-cell sequencing data have revealed multiple stromal populations, indicating the presence of different stromal niches responsible for controlling inflammation and extracellular matrix composition (Queckbörner *et al.*, 2021). These studies have discovered that the human WOI opens when the abrupt and discontinuous transcriptomic activation of genes such as progestagen associated endometrial protein (*PAEP*), glutathione peroxidase 3 (*GPX3*), and chemokine (C-X-C motif) ligand 14 (*CXCL14*) occurs in unciliated epithelial cells. This process is accompanied by widespread decidualization in stromal fibroblasts, up-regulating genes like dickkopf-related protein 1 (*DKK1*), alpha-crystallin B chain (*CRYAB*), forkhead box protein O1 (*FOXO1*), and interleukin 15 (*IL15*). Notably, the WOI closes with a more gradual transition dynamics (Wang *et al.*, 2020).

However, endometrial gene expression is influenced not only by expected hormonal regulation and changes in cellular composition and differentiation during the menstrual cycle but also by several other factors. Epigenetic mechanisms such as DNA methylation, histone post-translational modifications, and regulation by small non-coding RNAs have been shown to play a role in endometrial gene expression (Retis-Resendiz *et al.*, 2021). Genetic variation between individuals has also been found to be a contributing factor (Fung *et al.*, 2017). Therefore, assessing the exact timeframe of endometrial receptivity could be complicated.

1.2.1. Predicting the window of implantation

In IVF, advancements have been made in the quality, screening, and selection of transferable embryos, primarily through increased preimplantation genetic testing for aneuploidy. Although this has led to the elimination of many embryo-related issues and an increase in the chances of successful implantation (Pirtea *et al.*, 2021), the implantation and live birth rates after the first euploid embryo transfer are still less than 70% and 65%, respectively (Reshef *et al.*, 2022). One of the reasons for implantation failure is believed to be suboptimal endometrial receptivity. Therefore, various endometrial preparation protocols have been implemented to support the luteal phase and to transform the endometrium into an optimally receptive state for embryo transfer. However, it is known that each woman has an individual endometrial maturation rate when reaching the WOI (Simón *et al.*, 1997). The WOI can also vary among women due to individual differences, pathology, or hormone treatments during ART procedures (Franasiak *et al.*, 2016). Therefore, endometrial dating methods are essential to improve the efficiency of assisted reproduction.

The available methods for evaluating endometrial receptivity and timing are classified into four categories: endometrial biopsy, ultrasonography, hysteroscopy, and endometrial fluid aspirate analysis (Craciunas *et al.*, 2019). The earliest of these was based on the histologic dating of endometrial biopsy. Noyes and colleagues conducted a landmark study investigating histological changes in the endometrium around the time of implantation, describing morphological changes in endometrial biopsy and termed the criteria for endometrial maturing (Noyes *et al.*, 1950). However, many studies have reported poor reproducibility and accuracy of dating due to the variability of the Noyes criteria (Murray *et al.*, 2004). Therefore, morphological characteristics have been added, such as the presence of pinopods on the surface of the endometrium (Usadi *et al.*, 2003), and immunohistochemical biomarkers such as estrogen and progesterone receptors and the proliferation marker Ki-67 (Alfer *et al.*, 2020). Additionally, the presence of molecular markers, such as integrin αvβ3 (Lessey *et al.*, 2000), leukemia inhibitory factor (Serafini *et al.*, 2008), and ligands for L-selectin (Foulk *et al.*, 2007), have been described to improve the accuracy of histopathological dating.

In current clinical practice, two-dimensional and three-dimensional ultrasonography are the most common methods for evaluating endometrial receptivity and timing. These methods measure endometrial thickness, pattern, volume, blood flow, and contractions. Among them, endometrial thickness is the most widely used predictor of receptivity. However, despite the significant association between a thicker endometrium and a higher pregnancy rate, it cannot predict clinical pregnancy (Craciunas *et al.*, 2019). In addition to vaginal ultrasonography, hysteroscopy has been used to examine the endometrial glandular opening and vascular development to assess the quality of the endometrial surface (Inafuku, 1992). However, it is primarily used to exclude physical abnormalities of the uterus and not to determine the optimal time for embryo transfer (Santi *et al.*, 2012).

In contemporary transcriptomics, gene expression changes in the endometrium across the menstrual cycle can be identified and used for transcriptomic endometrial dating. The signature of highly expressed genes during the WOI has led to the development a customized endometrial receptivity array (ERA) (Díaz-Gimeno *et al.*, 2011). The ERA test can diagnose the WOI's temporal shift, allowing for personalized embryo transfer (pET) timing for each patient (Ruiz-Alonso *et al.*, 2013). Using a machine learning algorithm, the ERA test measures the expression of 238 genes from the endometrial biopsy and classifies the sample into a receptive, pre-receptive, or post-receptive stage. The ER Map®/ER Grade® test was developed as the next test, predicting endometrial receptivity status using reverse transcription quantitative real-time PCR (RT-qPCR) with a panel of 40 genes differentially expressed in WOI compared to other states (Enciso *et al.*, 2018).

Nonetheless, the clinical utility of transcriptomic endometrial dating is still controversial. In a 5-year multicenter randomized controlled trial, the developers of the ERA test reported that detecting the window of implantation and utilizing personalized embryo transfer led to higher implantation and live birth rates in the first embryo transfer cycle (Simón *et al.*, 2020). However, several studies have questioned the ERA test's use in patients with a good prognosis, showing no significant improvement or even a negative impact on clinical outcomes (Riestenberg *et al.*, 2021; Cozzolino *et al.*, 2022; Luo *et al.*, 2023). Nevertheless, for poor-prognosis patients with a history of failed implantation of a euploid embryo, transcriptomic endometrial dating and pET may increase the implantation rate (Tan *et al.*, 2018), making it justifiable for a subgroup of patients. Therefore, further high-quality randomized studies are needed to investigate these tests' clinical validity and feasibility, given their relatively high cost and the associated endometrial biopsy-related injury.

Several studies have also explored receptivity markers from endometrial fluid aspirate to develop a non-invasive method to predict endometrial receptivity. One such method is the non-invasive RNA-seq-based endometrial receptivity test, nirsERT, which combines 87 receptivity-related markers and three hub genes (He *et al.*, 2023). While this approach shows promise for detecting the WOI and guiding personalized embryo transfer, it still faces several challenges in confirming test accuracy and convenience in a larger validation cohort before it can be applied clinically.

1.3. Embryo development to blastocyst stage

Once ovulation has occurred and the uterine secretory phase begins to prepare the endometrium for potential implantation, the released oocyte travels to the entrance of the fallopian tube. The period from oocyte fertilization in the fallopian tube to the formation of the implantation-ready blastocyst is called the pre-implantation period.

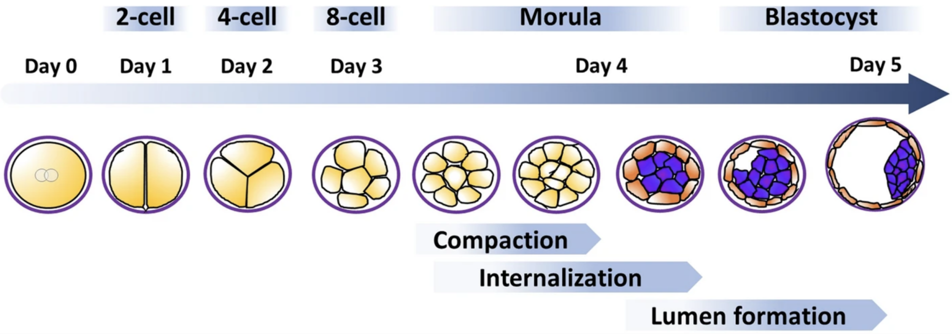

Figure 3. **The process of human blastocyst development and formation.** Starting from Day 0, the zygote of a human embryo undergoes a series of cleavage divisions. On Day 3, around the 4th wave, compaction occurs and is completed on Day 4, forming a morula containing apolar inner cells (purple). During the 5th cleavage, apicobasally polarized outer cells (orange) begin pumping fluid to create a lumen (blastocoele), developing a blastocyst. The violet circle around the embryo represents the *zona pellucida*. Adapted from (Gauster *et al.*, 2022).

In humans, if the secondary oocyte and capacitated sperm meet within the next 12–24 hours in the fallopian tube, fertilization occurs, and the haploid mature egg cell and sperm cell give rise to the diploid embryo. The zygote undergoes three cleavage divisions with no cell growth during interphase, resulting in the formation of blastomeres within 2–3 days after fertilization (Figure 3). Between the 8- and 10-cell stages, around 3–4 days after fertilization, blastomeres undergo rearrangement, forming a compact cluster of cells called a morula (Iwata *et al.*, 2014). During compaction, loosely attached blastomeres broaden their cell-cell contacts, reducing their surface exposure to the outside until the subset of cells becomes surrounded by neighboring cells. The contractile actomyosin machinery and the cell-cell adhesion molecule E-cadherin (CDH1) have been suggested to be relevant for moving blastomeres closer together and forming a tighter structure (Alikani, 2005). This cellular positioning is a prerequisite for the first lineage specification in humans. The non-polarized pluripotent inner cell mass (ICM) or embryoblast gives rise to all embryonic cells and tissues as well as the amnion, yolk sac, and allantois. In contrast, surrounding cells undergo apicobasal polarization and differentiate into trophectoderm (TE) cells that give rise to the trophoblast cells in the placenta (Firmin and Maître, 2021).

Based on the mouse models, it is believed that the Na+/K+-ATPase pumps, enriched at the basolateral membrane of the trophectoderm cells (Hirakawa *et al.*, 2022), help establish an osmotic gradient across the trophectoderm. Afterward, the polarized TE cells initiate fluid transportation through water transporters into numerous microlumens that form across the embryo at cell-cell contacts and multicellular interfaces. However, only one lumen continues expanding and becomes the blastocoel in the process called cavitation. Increasing hydrostatic pressure breaks down cell-cell connections, causes E-cadherin reorganization, and pushes the ICM into one quadrant of the developing blastocyst, five days post-fertilization (Dumortier *et al.*, 2019).

The blastocoel continually takes up fluid, which increases internal hydrostatic pressure, stretching the trophoblast epithelium and thinning the surrounding glycoprotein-rich extracellular matrix called *zona pellucida*. This thinning is a crucial step in the blastocyst hatching process from the *zona pellucida*, facilitated by specialized trophectoderm cells called zona-breaker cells. These cells extend projections that penetrate the *zona pellucida* and release hatching-associated factors such as growth and transcription factors and zona lysing proteases (e.g., cathepsin), which induce focal lysis. Human blastocysts usually hatch on the sixth or seventh day after fertilization *in vivo* and subsequently implant in the uterine endometrium. (Sathananthan *et al.*, 2003; Seshagiri *et al.*, 2009)

Although the primary characteristics of human preimplantation embryos were first described by Arthur Hertig and John Rock already in 1956 (Hertig *et al.*, 1956), the basic understanding of preimplantation development comes from more recent IVF studies, which allowed for the visual examination of embryos developing *in vitro* using time-lapse cameras. However, using human embryos for research is complicated due to ethical considerations and limited availability, as they are solely donated by couples undergoing IVF procedures. Moreover, they are notoriously challenging to work with, and their quality could be poor. To address these challenges, simplified embryo models called blastoids can be generated from human pluripotent stem cells (hPSCs). Blastoids can be easily established from cell lines in the lab, generated in large numbers, and manipulated physically or genetically (Javali, 2021). Despite extensive research on preimplantation embryo development, much of our understanding of blastocyst formation comes from studies on model organisms. Among these, the mouse has been one of the most widely studied. However, recent research has revealed that the timing of key developmental processes differs significantly between mice and humans (Meistermann *et al.*, 2021). These differences are evident in lineage markers' transcriptional and spatiotemporal expression and responses to cell signaling perturbations (Biondic *et al.*, 2023).

In humans, the blastocyst stage initially exhibits early trophectoderm (TE) cells with high *GATA3* (GATA binding protein 3), *GATA2* (GATA binding protein 2), *POU5F1B* (POU Class 5 Homeobox 1B), and *DNMT3L* (DNA methyltransferase 3 like) gene expression. Subsequently, the early TE matures into *GATA3* and *GATA2* positive, *POU5F1B* negative trophectoderm, which differentiates further into polar trophectoderm (pTE) that expresses *GATA3*, *GATA2*, and *NR2F2* (nuclear receptor subfamily 2 group F member 2) (Meistermann *et al.*, 2021). The pTE cells are localized on the embryonic pole, where the inner cell mass is attached to the TE. Recently, Gerri et al. (2020) demonstrated that at the morula stage, outer cells acquire apicobasal cell polarity and express atypical protein kinase C (aPKC), which is allocated by angiomotin (AMOT) to keep the Hippo signaling pathway inactive. In the outer polar cells, where the Hippo signaling is suppressed: dephosphorylated YAP1 (yes-associated protein 1) translocates into the nucleus, interacts with the transcription factor TEAD4, and directly induces TE-specific transcriptional factors, including GATA binding protein 3 (GATA3), which are required to repress pluripotent genes and promote differentiation into TE. However, in the inner apolar cells, AMOT is phosphorylated and distributed in adherens junctions, interacts with NF2 (Neurofibromin 2) and LATS1/2 (large tumor suppressor kinase 1/2) kinases to phosphorylate, and promotes YAP1 in the cytoplasm, thus preventing YAP1 to induce target genes (Kagawa *et al.*, 2022).

Single-cell RNA sequencing has revealed that human ICM cells are transcriptionally distinct from TE cells shortly after cavitation during blastocyst formation. Aquaporin 3 (*AQP3*), a water transporter, is one of the few genes initially expressed in all blastomeres but then restricted to TE (Meistermann *et al.*, 2021). The ICM gives rise to the IFI16 (interferon gamma inducible protein 16) positive pluripotent epiblast (EPI) cells and *GATA4* (GATA binding protein 4) positive primitive endoderm (PrE). However, human embryos lack the distinct ICM molecular signature of EPI and PrE progenitors (unlike to mouse). Therefore, it is likely that PrE specification occurs in the cells that are transcriptionally close to EPI (Meistermann *et al.*, 2021). It has also been shown that signals from the EPI induce pTE maturation (Kagawa *et al.*, 2022) and therefore affect the pTE cells' interaction with the endometrium during the implantation process (Aberkane *et al.*, 2018).

1.4. Embryo implantation

Human embryo implantation occurs during a narrow time frame known as the “window of implantation” in the mid-secretory phase of the menstrual cycle. Successful implantation involves a series of sequential stages, including blastocyst apposition, adhesion, and invasion, to establish attachment with the maternal endometrium (Figure 4). Due to ethical concerns, studying this process *in vivo* is challenging, and thus, most of the knowledge is based on *in vitro* models. These models involve attaching embryos or trophoblastic spheroids generated from cell lines, primary tissue, or embryonic stem cells to either primary tissue or cell lines of endometrial epithelium in 2D or 3D cultures (Ojosnegros *et al.*, 2021). Successful implantation involves numerous mediators, including cytokines, lipids, adhesion molecules, and growth factors. Crosstalk between the receptive endometrium and the blastocyst-stage embryo is necessary, and this communication is also mediated by embryo-derived extracellular vesicles (EVs) and microRNAs (miRNAs). EVs are small membrane-derived structures that carry biomolecules such as lipids, proteins, DNA, messenger RNA (mRNA), and miRNA. EVs and secreted miRNAs have been isolated from cultured trophoblasts and embryo-conditioned media. Research has shown that endometrial EV uptake by trophoblast cells enhances their adhesive capacity and increases their ability to attach to the endometrium (Greening *et al.*, 2016).

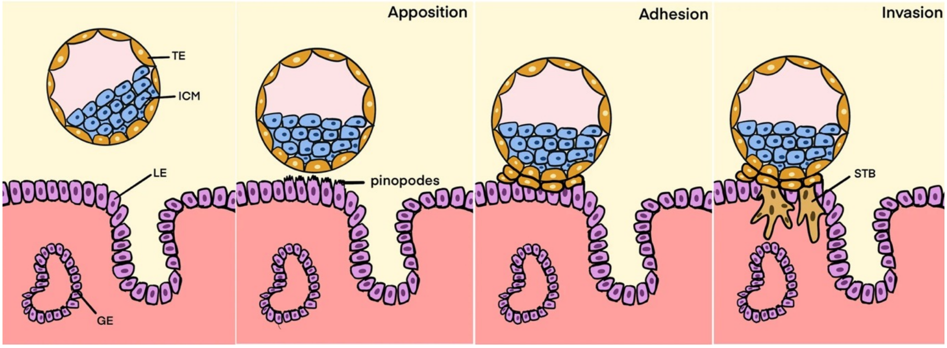

Figure 4. **The series of events involved in human embryo implantation.** The hatched blastocyst undergoes sequential apposition, adhesion, and invasion stages to attach to the maternal endometrium. ICM: inner cell mass, TE: trophectoderm, LE: luminal epithelium, GE: glandular epithelium, ST: syncytiotrophoblast. Modified from (Li *et al.*, 2022)

1.4.1. Apposition

Human implantation occurs 2–4 days after the blastocyst enters the uterine cavity and freely rolls over the endometrium. The rolling process is necessary to orient the inner cell mass and polar trophectodermal cells towards the receptive endometrium. The initial loose connection or contact between the blastocyst and the endometrium is called apposition (Figure 4). At this stage, critical interactions occur between receptors and ligands and adhesion molecules such as L-selectin (Genbacev *et al.*, 2003), facilitating the preliminary interaction between the blastocyst and the luminal epithelium of the endometrium. The L-selectin ligands are primarily located on pinopodes, where blastocyst adhesion is initiated (Nejatbakhsh *et al.*, 2012). In contrast, the anti-adhesion molecule MUC-1 covers the surrounding area where implantation is less likely to occur, preventing the blastocyst from attaching to the wrong location (Horne *et al.*, 2002).

1.4.2. Adhesion and attachment

During the adhesion phase of embryonic implantation (Figure 4), the positioned blastocyst induces the cleavage of mucin-1 (MUC-1) at the implantation site to promote a successful attachment (Meseguer *et al.*, 2001). Stable, shear stress-resistant blastocyst adhesion depends on several cellular adhesion molecules, including heparin-binding EGF-like growth factor (HBEGF), trophinin, and integrins. The transmembrane HBEGF on the endometrial epithelium binds to heparan sulfate proteoglycan and EGF receptor family members ERBB1 and ERBB4 on the trophectoderm of the hatched blastocyst (Chobotova *et al.*, 2002). ERBB4 tyrosine phosphorylation is necessary for trophectoderm differentiation into an invasive trophoblast phenotype, which is inhibited by the presence of the transmembrane protein trophinin and its associated cytoplasmic proteins bystin and tastin. If the homophilic binding of trophinin between trophectoderm and endometrial epithelial cells is established, bystin dissociates to enable ERBB4 autophosphorylation in the trophectoderm (Sugihara *et al.*, 2007). Additionally, trophinin and the Fas/Fas-L death system are involved in the trophectoderm contact-induced apoptosis of endometrial epithelial cells (Galán *et al.*, 2000; Tamura *et al.*, 2011).

In the adhesion phase, human trophoblasts penetrate a specific region of the luminal epithelium and then expand underneath the resealed epithelium (Ye, 2020), thereby exposing the extracellular matrix (ECM) components of the basement membrane below, to which the trophoblasts reinforce adhesion. Integrins play a predominant role in mediating attachment in this step. For instance, in humans, the αvβ3 dimer is present on the endometrial epithelium and the blastocyst, allowing the attaching embryo to bind to ECM ligands such as fibronectin, laminin, vitronectin, tenascin, and thrombospondin (Kimber and Spanswick, 2000). Studies have demonstrated that integrin β1 and integrin β3 bind to extracellular galectin 3 proteins, regulating endometrial cell proliferation and adhesion (Lei *et al.*, 2009; Yang *et al.*, 2011). Moreover, the binding of integrin αVβ3 as well as integrin α5β1 to the extracellular matrix protein osteopontin triggers downstream signaling pathways involved in cellular adhesion and cytoskeletal remodeling, thereby regulating the migration of trophectoderm cells (Kim *et al.*, 2010). The abnormal expression of the αVβ3 heterodimer has been associated with recurrent pregnancy loss and infertility (Lessey *et al.*, 1995). Furthermore, up-regulation of β3 integrins in cultured human endometrial epithelial cells is partially mediated by the embryonic interleukin-1 system, indicating paracrine cross-talk between the blastocyst and endometrial epithelium during embryonic implantation (Simón *et al.*, 1997).

1.4.3. Invasion and trophectoderm differentiation

During the invasion stage, trophoblast cells from the blastocyst penetrate the endometrial epithelium (Figure 4), invading the underlying endometrial stroma to reach maternal blood vessels. It has been suggested that once the blastocyst has properly adhered and apposed, the trophectoderm cells at the embryonic pole that make direct contact with uterine epithelial cells undergo proliferation and differentiation. The daughter cells, called cytotrophoblasts, initiate cell-cell fusions, forming a multinucleated cell, primitive syncytiotrophoblast, which has lost its proliferative capacity. Only this multinucleated layer is believed to penetrate the uterine epithelium, enabling the embryo to implant into the uterine stroma (Gauster *et al.*, 2022). The process of trophectoderm penetration through the uterine epithelium has not been observed *in vivo* in humans due to ethical considerations. A unique collection of images, assembled from Professor Allen Enders' (https://www.trophoblast.cam.ac.uk/Resources/enders) and the Carnegie Collection (Hertig *et al.*, 1956), depicts human implantation stages beginning one day after implantation. The images reveal that in humans, the embedding of the blastocyst within the stroma is completed 7–8 days after ovulation when the invasive primitive syncytiotrophoblast takes over most of the contact area between embryonic and maternal tissues.

During implantation, the extracellular matrix undergoes remodeling via the action of matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs). Successful cytotrophoblast invasion is mainly mediated by MMP-2 and MMP-9, which degrade the basement membrane composed primarily of type IV collagen and disrupt intercellular junctions. Protease activity is regulated by TIMP-1 and TIMP-2, facilitating the anchoring of trophectoderm cells to the implantation site (Seval *et al.*, 2004). The process is also regulated by various chemokines and cytokines that attract the blastocyst to the implantation site. One of the most well-studied cytokines in this context is leukemia inhibitory factor (LIF), which protein expression increases in the luminal and glandular epithelium during the mid-secretory to late-secretory phase (Aghajanova *et al.*, 2003). LIF has also been shown to stimulate the differentiation of extravillous trophoblast (EVT) cells toward the invasive phenotype by regulating cell adhesion to ECM components (Tapia *et al.*, 2008).

In addition to cytokines, the blastocyst elicits maternal-fetal signaling through human chorionic gonadotropin (hCG) secretion. hCG emerges as one of the earliest blastocyst-derived products, with RNA expression detectable as early as the 8-cell stage, and its presence can be identified in the maternal circulation approximately 7 days after fertilization, coinciding with implantation (Bonduelle *et al.*, 1988). Both the blastocyst and later the placental hCG function as luteotrophic factors, ensuring sustained progesterone production by "rescuing" the corpus luteum through activation of the LHCG receptor during the initial 3–4 weeks of pregnancy (Cole, 2012). Additionally, investigations have confirmed that hCG enhances the expression of pro-implantation factors, including leukemia inhibitory factor (LIF), vascular endothelial growth factor (VEGF), and matrix metallopeptidase 9 (MMP9) (Licht *et al.*, 2007). Distinct from the conventional hCG, early invasive cytotrophoblast cells generate a more heavily glycosylated and acidic form of hCG initially referred to as invasive trophoblast antigen but now denoted as hyperglycosylated hCG (hCG-H). This form of hCG functions in an autocrine manner similar to cytokines and promotes its own invasion (Evans, 2016). Furthermore, hCG-H is thought to augment the expression and activation of protease enzymes, thus facilitating the process of invasion. It is hypothesized that hCG-H adopts a unique conformation compared to hCG, thereby exposing a cysteine knot structure that enables its binding to the TGF-β receptor. This interaction counteracts the effects of TGF-β, resulting in diminished trophoblast apoptosis and heightened metalloproteinase activity (Cole, 2012). An imbalance in hCG-H is postulated to contribute to pregnancy failures, given that inadequate implantation accounts for a significant proportion of pregnancy losses (Sasaki *et al.*, 2008).

1.5. Trophoblast cells differentiation and early placenta formation

After implantation (Figure 5A), when the ICM becomes surrounded by a spherical structure, the early phase of placental development, known as the prelacunar phase, begins. During this phase, the ICM undergoes differentiation, giving rise to two distinct layers: the hypoblast (or primitive endoderm), which faces the blastocoel cavity, and the epiblast (or primitive ectoderm), situated towards the embryonic pole and adjacent to the future amniotic cavity. These two cell layers form the bilaminar embryonic disk (Figure 5B). Subsequently, the cytotrophoblast secretes an acellular substance called the extraembryonic reticulum, which forms a spongy layer. As migratory cells originating from the epiblast invade this layer, the extraembryonic mesoderm is formed. Concurrently, fluid-filled spaces known as lacunae begin to develop within the syncytial mass. Over time, these lacunae gradually enlarge and merge, leading to the development of a lacunar system. As a result of the erosion of the endothelial lining of maternal capillaries, the lacunae become filled with maternal blood, leading to the primitive filling of the lacunae with maternal blood (Figure 5C). (Silini *et al.*, 2020).

The early stage of villous formation in humans begins 12 to 15 days post-conception (Figure 5D). The layer of proliferating villous cytotrophoblasts appears under the villous syncytiotrophoblast, forming finger-like protrusions called primary villi. Two days later, extra-embryonic mesoderm cells originating from the embryo invade beneath the cytotrophoblast inside the primary villi, transforming them into secondary villi (Figure 5E). At 18–20 days post-conception, the *de novo* formation of blood vessels within the mesenchymal stromal cells commences, leading to the formation of tertiary villi (Figure 5F) (Castellucci, 2000).

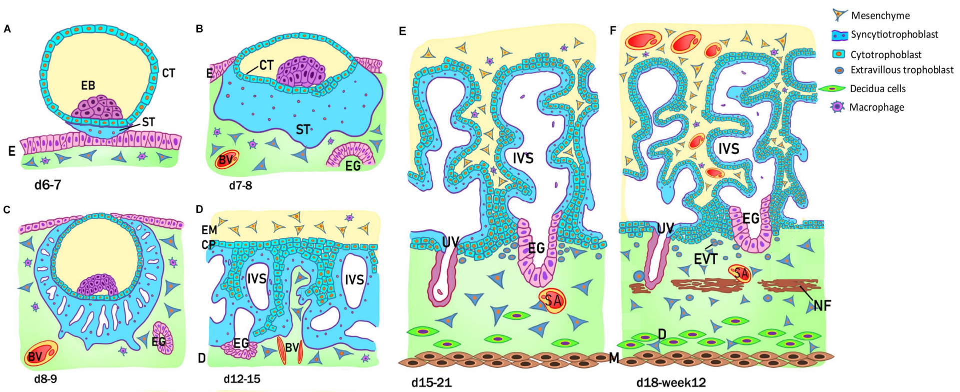

Figure 5. Stages of chorionic villus formation: From embryo attachment to anchoring villus. (A) Implantation at 6 to 7 days (d) after conception; (B) prelacunar period (7 to 8 days); (C) beginning of lacunar period (8 to 9 days); (D) transition from lacunar period to primary villus stage (12 to 15 days); (E) secondary villus stage (15 to 21 days); and (F) tertiary villus stage (18 days to week 12). BV, blood vessel; CP, primary chorionic plate; CT, cytotrophoblast; D, decidua; E, endometrial epithelium; EB, embryoblast; EG, endometrial gland; EM, extraembryonic mesoderm; EVT, extravillous trophoblast; IVS, intervillous space; M, myometrium; NF, Nitabuch fibrinoid; SA, spinal artery; ST, syncytiotrophoblast; UV, umbilical vein. Modified from (Silini *et al.*, 2020).

Simultaneously, during the formation of primitive villous trees, cytotrophoblast cells migrate from the tips of the placental villi, proliferate, and differentiate to form a trophoblast cell column (Figure 5E), which is the progenitor cell population for the differentiated extravillous trophoblast (EVT) cells. EVT from the distal part of the cell column detaches from the villi and invades the interstitial compartments of the maternal uterine wall, anchoring the fetus. EVT from the proximal portion of the cell column, which is attached to the anchoring villus, exhibits similar characteristics to proliferative villous cytotrophoblasts and expresses the typical epithelial integrin types α3β1 and α6β4. However, EVT on the distal part within the uterine stroma acquires an invasive phenotype. During EVT differentiation, there is a switch from proliferative to invasive integrin types (α5β1, αvβ3/5), a change in cell morphology to spindle-shaped, an increase in intercellular space, and the expression of marker genes such as *HLA-G*, *ERBB2*, and *NOTCH2* (Gauster *et al.*, 2022).

During EVT differentiation, the cells secrete metalloproteinases, down-regulate genes that express junction proteins, and up-regulate genes associated with epithelial-to-mesenchymal transition (EMT). It is suggested that partial EVT differentiation is related to the induction of key transcription factors of EMT, such as SNAIL, and controlled by upstream signaling cascades, including FGF, TGF-β, and WNT However, the epithelial marker keratin 7 is still detectable, contrary to the mesenchymal marker vimentin in EVT cells, indicating that the transition is not fully established (E. Davies *et al.*, 2016). Depending on their location and function, EVT can be divided into interstitial cytotrophoblasts (iCTBs) that invade the decidual stroma and endovascular cytotrophoblasts (eCTBs) that colonize the maternal spiral arteries (Knöfler *et al.*, 2019). Different subtypes of trophoblasts are also distinguished, such as endoarterial-, endovenous-, endolymphatic-, and endoglandular trophoblasts (Gauster *et al.*, 2022).

1.5.1. Methods to study trophoblast lineage specification

Deviations in trophoblast differentiation during early gestation are considered a fundamental cause of various placenta-associated pregnancy complications, including miscarriage, preeclampsia, and intra-uterine growth restriction (Norwitz, 2006). However, due to ethical limitations that restrict the use of human embryos for research purposes beyond 14 days post-fertilization, very few documents are available on the first few weeks of human postimplantation development (Cavaliere, 2017). Consequently, understanding early human development heavily relies on *in vitro* (cell culture) and animal studies. Among the animal models used to study placental development, mice are the primary model. However, there are notable differences in trophoblast cell types, placental organization, and gene regulation between mice and humans (Soncin *et al.*, 2018).

Although the first attempts to use model systems for trophoblast cell differentiation were made 60 years ago, recent technological advancements have provided new opportunities to explore the field in greater depth (Morey *et al.*, 2022). The first *in vitro* model of human trophoblast was a cell line called BeWo, which was established from an isolated choriocarcinoma tissue (Pattillo and Gey, 1968). Subsequently, a Percoll gradient-based purification protocol allowed for the isolation of functional cytotrophoblasts from human term placentae and the fusion of these cells in culture to form functional syncytiotrophoblasts (Kliman *et al.*, 1986). The first immortalized primary trophoblast cell line, HTR-8/SVneo, was developed to maintain stable long-term cultures (Graham *et al.*, 1993). However, choriocarcinoma cell lines and primary cytotrophoblasts are already differentiated, so the lineage decisions toward the trophectoderm cannot be studied. The next breakthrough in trophoblast studies was the derivation of mouse trophoblast stem cells (mTSC) from mouse blastocysts or early postimplantation trophoblasts, which differentiated into other trophoblast subtypes and proliferated in the presence of fibroblast growth factor 4 (FGF4) (Tanaka *et al.*, 1998). Nevertheless, FGF4 could not establish stable human trophoblast stem cell lines from peri-implantation stage embryos (Kunath *et al.*, 2014), so alternative methods were proposed.

In 2002, the focus of human trophoblast cell studies shifted to differentiating trophoblast-like cells from human embryonic stem cells or induced pluripotent stem cells (iPSC). Xu et al. (2002) was the first to report that bone morphogenic protein 4 (BMP4), a member of the transforming growth factor-β (TGF-β) superfamily, mediates hESC differentiation to extra-embryonic trophoblast cells. However, it was soon discovered that fibroblast growth factor (FGF) signaling switches BMP4-induced human ESC differentiation outcome towards mesendoderm (Bernardo *et al.*, 2011; Yu *et al.*, 2011). Therefore, over the following two decades, the differentiation protocol was optimized by adding inhibitors to the FGF receptor, TGF-β (Amita *et al.*, 2013), or WNT (Kurek *et al.*, 2014) pathway to achieve a pure trophoblast identity. Nevertheless, several research groups have questioned whether BMP4 treatment alone or with added inhibitors (one by one or in combination) truly resembles human trophoblast subtypes or rather shows some features similar to first-trimester trophoblast (Lee *et al.*, 2016; Roberts *et al.*, 2014).

In 2018, a new approach to studying trophoblast differentiation emerged when Okae and colleagues analyzed the transcriptomes of primary trophoblast cells and developed a cell culture condition that contained epidermal growth factor (EGF), a WNT activator, and inhibitors for GSK-3 (CHIR99021), TGF-β (A83-01 and SB431542), histone deacetylase (valproic acid, VPA), and Rho-associated protein kinase (Y27632) to derive human trophoblast stem (TS) cells from cytotrophoblast cells and blastocysts (Okae *et al.*, 2018).

In 2021, two groups developed protocols to induce trophectodermal cells from naïve human pluripotent stem cells. Guo et al. showed that MEK/ERK inhibition (by PD0325901) is necessary and sufficient for trophectoderm specification, and NODAL inhibition (by A83-01) promotes lineage entry (Guo *et al.*, 2021). In addition to inhibitors for ALK4/5/7 (A83-01) and MEK (PD0325901), Io *et al.* used the inhibitor for Janus kinase 1 and induction with BMP4 (Io *et al.*, 2021). Both groups could also mature converted trophectodermal cells into cytotrophoblast cells using Okae trophoblast stem cell medium with modifications. Moreover, the forskolin-containing medium could force the cytotrophoblast cells to differentiate into multinucleated syncytia. In contrast, the medium containing ALK4/5/7 inhibitor (A83-01), Neuregulin-1, and Geltrex could lead to the formation of spindle-shaped EVT-like cells (Io *et al.*, 2021). At the same time, it was discovered that human pluripotent stem cells retain greater plasticity than previously thought. Therefore, primed pluripotent stem cells could convert to human trophoblast stem cells by continuous culturing in an human trophoblast stem cell medium and the presence of endogenous BMP4 (Wei *et al.*, 2021; Soncin *et al.*, 2022). Nonetheless, despite the active and rapid development of the field of trophoblast differentiation, many questions remain about selecting the most relevant model system for placental disease modeling.

2. AIMS OF THE STUDY

The present doctoral thesis aims to acquire knowledge on the biological processes that lead up to and happen during human embryo implantation to improve human *in vitro* fertilization success. This study concentrates on endometrial maturation into the embryo-receptive tissue, the critical factors regulating trophoblast differentiation, and the first molecular crosstalk between the endometrium and trophoblast at the beginning of embryo implantation.

The specific aims of the present doctoral theses were accordingly:

1) Identify a consensus meta-signature of endometrial receptivity transcriptomic biomarkers and specify their expression in main endometrial cell types.
2) Design a sensitive, robust, and cost-efficient method for transcriptome assay to analyze endometrial lining biomarkers for embryo implantation compatibility.
3) Identify endometrial cell-type specific receptivity genes and therefore predict the protein interactome of the embryo-maternal communication at the implantation site.
4) Investigate the BMP4-induced trophoblast cell differentiation and highlight the effects and potential co-action between FGF2 and TGF-β/activin/nodal pathway inhibition to understand the mechanisms that regulate early trophoblast differentiation.

3. METHODS

3.1. Overview of the data used in this thesis

Data from multiple sources were collected and analyzed to achieve the thesis objectives (Figure 6). The investigation of endometrial receptivity involved the utilization of previously published original experimental studies on the endometrial transcriptome in healthy women during the mid-secretory phase, comparing it to the "pre-receptive" phase, which encompasses the proliferative and early secretory phases (Carson *et al.*, 2002; Kao *et al.*, 2002; Borthwick *et al.*, 2003; Riesewijk *et al.*, 2003; Mirkin *et al.*, 2005; Talbi *et al.*, 2006; Díaz-Gimeno *et al.*, 2011; Altmäe *et al.*, 2012; Hu *et al.*, 2014). Furthermore, data from two independent sample sets obtained from the NOTED project (EU-FP7 Eurostars Programme, EU41564) and the SARM project (EU-FP7, IAPP, EU324509) were included to analyze the gene expression between the pre-receptive and receptive phase endometrium (Figure 7).

Previously published transcriptomic data of embryonal trophectodermal cells, derived from 17 human blastocyst-stage embryos on days six and seven, focused on the expression of transcripts in the polar trophectoderm, were incorporated into the study (Petropoulos *et al.*, 2016). Additionally, the H9 human embryonic stem cell line was cultured under various conditions to assess the influence of inhibitors on BMP4-mediated differentiation into trophoblast-like cells. Furthermore, a comparison was made between differentiated human embryonic stem cells (hESC) and full-term placental tissue samples to evaluate their similarities (Figure 7).

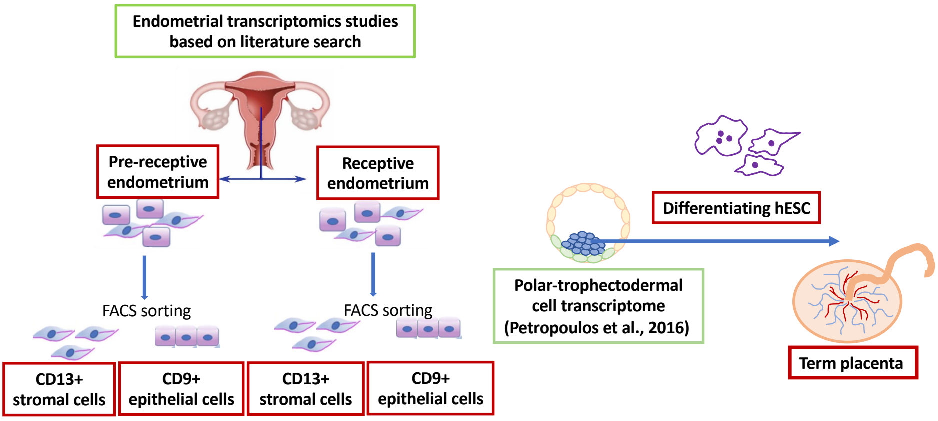

Figure 6. **Summary of the used samples**: transcriptome datasets generated in this study are indicated in the red boxes, while datasets acquired from public sources are represented in the green boxes. The pre-receptive and receptive endometrium samples were FACS sorted based on the detection of cluster of differentiation (CD) 13 antigen on endometrial stromal cells and CD9 antigen on endometrial epithelial cells, respectively.

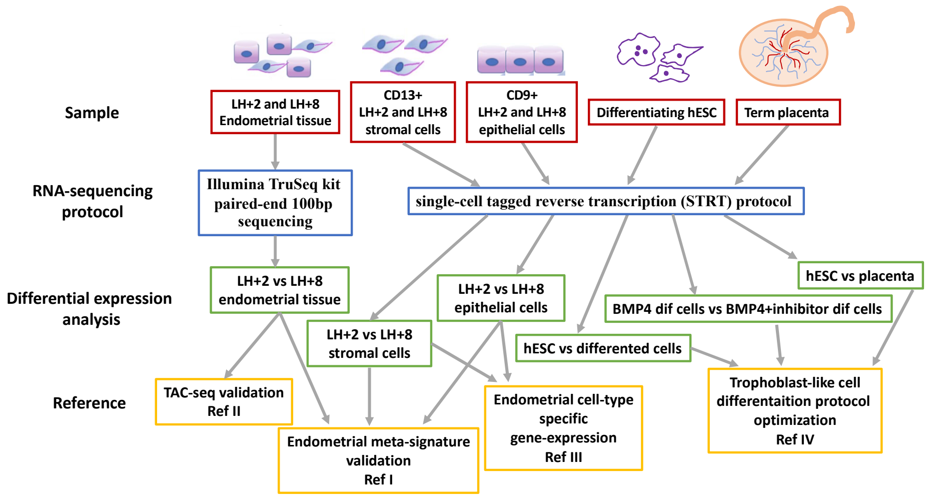

Figure 7. Overview of gene expression profiling and differential expression analysis employed in the thesis.

3.2. Meta-signature of endometrial receptivity-associated genes (Ref I)

3.2.1. A systematic search of the literature on endometrial transcriptomics studies

A systematic literature review was conducted in PubMed and Scopus up to June 2016 to perform an endometrial meta-signature analysis. The search targeted original experimental studies published in English, focusing on the endometrial transcriptome in healthy women during the mid-secretory phase compared to the pre-receptive phase, which includes the proliferative and early secretory phases. Nine eligible publications were identified, resulting in a compilation of 164 endometrial samples for final analysis. This dataset consisted of 76 pre-receptive endometrium samples (proliferative or early secretory phase) and 88 receptive endometrium samples (mid-secretory phase) (Carson *et al.*, 2002; Kao *et al.*, 2002; Borthwick *et al.*, 2003; Riesewijk *et al.*, 2003; Mirkin *et al.*, 2005; Talbi *et al.*, 2006; Díaz-Gimeno *et al.*, 2011; Altmäe *et al.*, 2012; Hu *et al.*, 2014). All probe names and transcripts were standardized to ENTREZ IDs. For one study, the differentially expressed probe lists were acquired by reanalyzing the data stored in the Gene Expression Omnibus under accession number GSE6364, using the GEO2R web tool with default options for differential analysis and gene list acquisition a false discovery rate (FDR) threshold of <0.05 and fold change (FC) >2. Probes not annotated by ENTREZ IDs were removed from subsequent analyses, and gene fold changes were ranked based on the up-and-down-regulated genes reported in the original paper.

3.2.2. Meta-analysis

The RobustRankAggreg package (RRA package v.1.1) (Kolde *et al.*, 2012) was employed to calculate and normalize the rank matrices to identify a consensus list of differentially expressed genes between the receptive and pre-receptive groups. To account for multiple testing, a stringent Bonferroni threshold was applied by multiplying all p-values by the maximal number of elements in all input lists (39,030). All lists used in the analysis represented the expression in the mid-secretory group compared with another group (proliferative or early-secretory phase).

3.2.3. Whole endometrial samples RNA-sequencing

Endometrial biopsies were collected from a total of 20 healthy volunteers within the fertile age range (≤35 years) and with a normal BMI (range: 19–25) participating in the NOTED and SARM projects. The selected women had regular menstruation, no hormonal aberrations upon clinical examination, and no uterine pathologies, endometriosis, or polycystic ovary syndrome. They were non-smokers, had not received hormonal treatments for three months before the study, had no previous history of infertility, and had at least one live-born child. The endometrial biopsies were collected using a Pipelle catheter (Laboratoire CCD, Paris, France) on days 2 and 8 after the luteinizing hormone (LH) surge (LH+2 and LH+8 respectively) within the same natural cycle. Menstrual cycle dating was confirmed using LH testing (estimated by the BabyTime hLH urine cassette, Pharmanova), vaginal ultrasound, and histological biopsy evaluation based on the Noyes' criteria to determine the receptive phase of endometrial maturation. The endometrial tissue was frozen after biopsy at –80 °C in RNAlater (Ambion Inc., Austin, TX) for subsequent analysis.

Total RNA was extracted from up to 30 mg of endometrial tissue for the whole endometrium. The biopsy was homogenized in the presence of QIAzol reagent (Qiagen, Venlo, Netherlands) and processed using the miRNeasy Mini kit following the manufacturer's protocol (Qiagen).

The Agilent 2100 Bioanalyzer and RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA) were utilized for evaluating the RNA integrity number (RIN) and concentration of total RNA samples. For endometrial samples, a RIN value greater than 7 was considered acceptable. Additionally, RNA concentrations were assessed using Qubit (Invitrogen) fluorometric quantitation.

For endometrial samples, cDNA libraries were generated from approximately 1 μg of endometrial total RNA using the Illumina TruSeq kit (Illumina, San Diego, CA, USA). The resulting paired-end 100 bp sequencing data from the Illumina HiSeq2500 instrument were subjected to bioinformatical analysis. Before preprocessing, the raw read quality of the RNA was assessed using FastQC v.0.11.3. Preprocessing steps included adapter removal and trimming using Trimmomatic tool v.0.32, as well as low-quality read filtering with fastq_quality_filter from the fastx toolkit v.0.0.13. High-quality reads were aligned to the human reference genome hg19 using the TopHat v.2.0.11 aligning tool. Read counts were determined using the HTSeq package htseq-count v.0.6.1, with the gtf Ensembl v.72 annotation file serving as the genomic feature file and the aligned reads as the input.

For whole endometrial samples, the Bioconductor package edgeR v.3.6.2 was used for paired differential analysis, with the raw read counts used as input. Transcripts with counts per million (CPM) >2 in at least 15 of the 40 samples were included in the analysis. The p-values from multiple testing were adjusted using Benjamini-Hochberg's approach to control the false discovery rate (FDR). Transcripts with an FDR <0.05 were identified as differentially expressed.

3.2.4. Sorted endometrial cells RNA-sequencing

The endometrial biopsies were collected from 16 healthy volunteers using the same criteria described in Chapter 3.2.3. The endometrial biopsy was dissociated in 5 ml DMEM medium containing 0.5% collagenase (Sigma-Aldrich, St. Louis, USA) using a shaking incubator rotating at 110 rpm at 37 °C until the biopsy was digested in less than 20 minutes. Ice-cold FBS (500 µl) and 45 ml of ACK lysing buffer (Life Technologies, Carlsbad, CS, USA) were added, and the suspension was centrifuged at 205 × g at 4 °C for 6 minutes. The cells were resuspended in 4 ml ice-cold phosphate-buffered saline (PBS) containing 5% fetal bovine serum (FBS) solution. The suspension was filtered twice through 50 µm and 35 µm Falcon Tubes with Cell Strainer Caps (BD Biosciences, San Jose, CA, USA) to separate single cells from undigested endometrial tissue fragments. The filtrate was centrifuged at 210 × g at 4 °C for 6 minutes to collect cells, which were then resuspended in 200 µl of PBS/FBS solution. Endometrial stromal cells were stained with fluorochrome-conjugated mouse anti-human CD13 monoclonal antibody (1:20 dilution, clone TÜK1, R-Phycoerythrin) (Thermo Fisher Scientific), and epithelial cells were simultaneously stained with fluorochrome-conjugated mouse anti-human CD9 monoclonal antibody (1:5 dilution, clone MEM-61, FITC) (Novus Biologicals, Littleton, CO, USA) on ice for at least 15 minutes. After incubation, 1.8 ml ice-cold PBS/FBS solution was added, and the mixture was centrifuged at 210 × g at 4 °C for 5 minutes. The cells were suspended in 300 µl of PBS/FBS solution and filtered using a 35 µm Falcon Tube with Cell Strainer Cap (Thermo Fisher Scientific). The filtered cells were stained with DAPI (1 mg/ml, 1:2000 dilution) (Thermo Fisher Scientific) to exclude dead cells. Cell suspensions were kept at 4°C until flow cytometric analysis and cell sorting. CD9 or CD13 positive and DAPI negative (alive) cells were sorted into QIAzol Lysis Reagent, and RNA was extracted immediately after cell lysis.

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 required for bulk RNA analysis. Samples of 10 ng of high-quality RNA extracted from enriched epithelial and stromal cell populations were converted into cDNA and amplified using 25 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, align to the hg19 genome, and quantitate the expression levels. The significance of fluctuation in gene expression was tested by comparison with the fluctuation of spike-in levels as described before (Krjutškov *et al.*, 2016).

Spike-in-based normalization and differential expression analysis for sorted cells were conducted using the R package edgeR (v.3.12.0), with a FDR threshold of <0.05 for determining differential expression. The gene expression was compared between early (LH+2) and mid-secretory (LH+8) phases for CD9-positive epithelial cells and CD13-positive stromal cells.

3.2.5. Enrichment analyses

Enrichment analyses were performed using the g:Profiler software (Reimand *et al.*, 2016) to interpret the biological functions.

3.2.6. Association between meta-signature genes and exosomal transport

The proteins encoded by the meta-signature genes were compared with the human exosome database to determine if endometrial receptivity-related genes could participate in exosome communication. Among the 57 endometrial receptivity-associated genes, 28 were experimentally identified in human exosomes according to the ExoCarta database. Statistical analysis using Fisher's Exact Test showed that the proteins translated by meta-signature genes had a 2.13 times higher probability ($p = 0.0059$) of being present in exosomes than the rest of the protein-coding genes in the human genome.

3.3. TAC-seq – a novel method for absolute molecule counting (Ref II)

3.3.1. Biomarker selection and TAC-seq probe design

The selection of biomarkers for endometrial receptivity was based on a meta-signature study (Ref I). For each targeted gene, a pair of TAC-seq detector DNA oligonucleotide probes were designed using specialized TAC-seq probe design software (http://nipt.ut.ee/design/). Each detector pair included a specific sequence (27-bp), a unique molecular identifier (UMI) (4-bp), and a left or right universal sequence. The targeted coding sequences were derived from the Consensus Coding Sequence Set (CCDS), and for genes without CCDS, the most likely transcript was manually selected from the Ensembl 87 database. Routine genetic testing detectors were preferentially designed near the 3'-end of the transcripts instead of using poly-A at the mRNA 3'-end for cDNA priming to minimize potential RNA degradation effects. GC content filtering was applied to ensure optimal melting temperature, with the overall GC content of a probe ranging between 40–60% and the GC content of adjacent ends (4-bp) up to 50%. Detector oligonucleotides with inter- or intra-complementarity issues were excluded from the selection. In addition, specific detectors for the ERCC spike-in sequences were designed following similar principles and located near their poly-A tails.

3.3.2. Endometrial samples mRNA library preparation

Endometrial biopsies were obtained, and total RNA extraction was performed following the procedures outlined in Chapter 3.2.3. The total RNA samples with RIN values above 7.7, quantified using Qubit (Invitrogen), were diluted to a 90 ng/µl concentration. For library preparation, 1 µl of the diluted RNA was used.

The RT master mix included 1 µl of a 1:50,000 dilution of the ERCC RNA Spike-In Mix 1 (Life Technologies) for technical normalization. A 64-plex TAC-seq probe set, consisting of 57 biomarker genes and seven ERCC spike-ins, was employed to generate a high-coverage library for analysis. Additionally, a low-coverage analysis was performed using a 70-plex probe set comprising 57 biomarker genes, five ERCC spike-ins, and eight housekeeping genes (ACTB, GAPDH, YWHAZ, PPIA, CYC1, HMBS, TBP, and SDHA).

Following cDNA synthesis, 1 µl of a 5 µM TAC-seq detector mixture was added to the RT mixture (Figure 8). The cDNA was denatured at 98 °C for 1 minute, followed by incubation at 60 °C for 1 hour to allow specific cDNA and TAC-seq probe hybridization. After hybridization, 5 µl of a thermostable Taq DNA ligase master mix (containing 2× Taq DNA ligation buffer and 1 U Taq DNA ligase) was added.

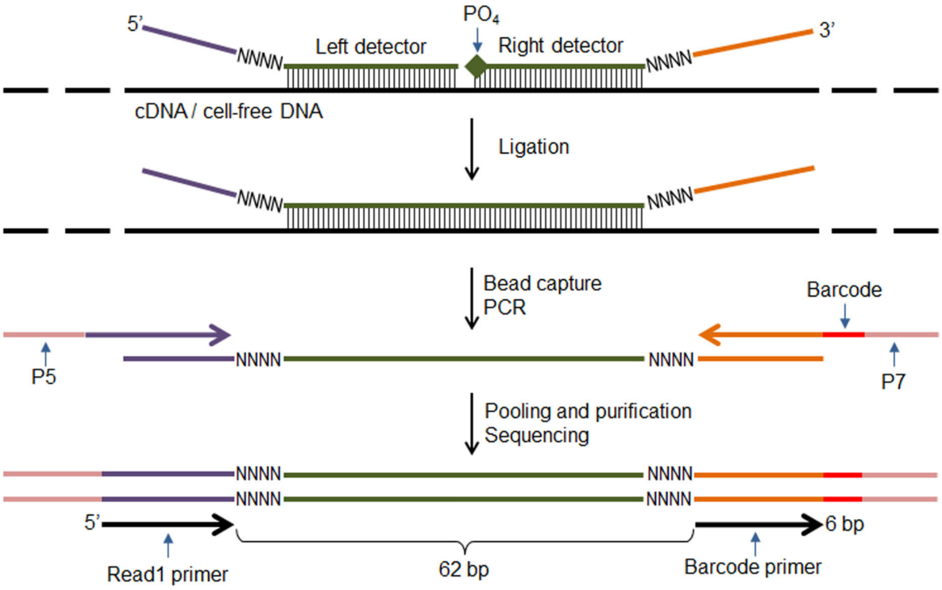

Figure 8. Schematic diagram of the assay designed to detect specific mRNA or cell-free DNA. Under stringent conditions, the assay involves hybridizing target-specific DNA oligonucleotide detector probes to the studied cDNA or cell-free DNA (cfDNA). Each detector oligonucleotide is composed of a specific 27-bp region (green), a 4-bp unique molecular identifier (UMI) motif (NNNN), and universal sequences (purple and orange). The right detector oligonucleotide has a 5′ phosphorylated group. Following strict hybridization, the detector probes are ligated using a thermostable ligase under stringent conditions. The ligated detectors bound to the target region are then captured with magnetic beads and PCR amplified. Sample-specific barcodes and other common motifs necessary for single-read NGS are introduced during PCR amplification. Adapted from (Teder *et al.*, 2018).

The ligation reaction was stopped after 20 minutes by placing the reaction tubes on ice. A mixture of Dynabeads MyOne Carboxylic Acid beads and capture buffer was added to the ligated sample. The mixture was mixed and subjected to capture for 10 minutes at room temperature. After incubating the tubes on a magnet, the supernatant was removed, and the beads were washed once with fresh 80% ethanol. The ethanol was removed, and the clean pellet was dried. Once dry, PCR master mix (containing 1× proofreading HOT FIREPol Blend Master Mix and 250 nM TAC-seq left primer) was added directly to the beads. PCR was performed with 12 cycles, following the 2+10 principle, for both high- and low-coverage libraries.

The PCR products were pooled and purified using DNA Clean & Concentrator-5 columns (Zymo Research). The eluted library was size-selected using AMPure XP beads to achieve the size of 180 bp. The size-selected library was visualized and quantified and then subjected to sequencing using the Illumina NextSeq 500 platform with a 90-bp single-read protocol.

3.3.3. Whole biopsy endometrial cells RNA-sequencing

The whole biopsy endometrial cells RNA-sequencing was conducted as referred in Chapter 3.2.3.

3.3.4. Data visualizing

The heatmaps depicting the results were created using the 'pheatmap' package in R. To facilitate the graphical representation of the data, log transformation was applied to the CPM values obtained from edgeR. The transformation used was log(CPM+1).

3.4. Predicted protein-protein interactions between trophectoderm and endometrial cell types (Ref III).

3.4.1. Sorted endometrial cells RNA-sequencing

The sorted endometrial cells RNA-sequencing was conducted as referred in Chapter 3.2.4. For this study, spike-in-based normalization and differential expression analysis for sorted cells were conducted using SAMstrt, a method based on Wilcoxon statistics with multiple Poisson resamplings, specifically developed for analyzing STRT protocol-based RNA-sequencing libraries. Significantly differentially regulated genes were defined based on a p-value <0.05 for fluctuation and a q-value <0.05 for differential expression. To focus on potential endometrial cell type biomarkers and mitigate the effect of individual differences, an additional filtration step was applied to remove endometrial genes detected in less than 75% of the receptive samples.

3.4.2. Polar trophectoderm transcriptome

The transcriptome of the polar trophectoderm of blastocyst-stage embryos was obtained from a previously published human embryo transcriptional mapping study. This study utilized RNA-seq analysis of 466 single embryonal cells from 17 human blastocyst-stage embryos collected on days 6 and 7 (Petropoulos *et al.*, 2016). Gene expression levels were estimated as reads per million mapped reads (RPKM). Genes expressed in the polar trophectoderm cells with a mean expression of >10 RPKM were selected.

3.4.3. Construction of protein-protein interaction networks

The proteome encyclopedia compiled by Nusinow et al. (2020) was utilized to prioritize transcript interactions based on gene-protein correlation. Specifically, genes exhibiting a positive correlation (Spearman's $R > 0$) with the protein expression profile were considered. Further filtering was performed based on the UniProt database, focusing on genes encoding proteins localized at the cell surface, extracellular matrix, or secreted by the cells. This selection aimed to explore potential interactions between the receptive endometrium and embryo. Subsequently, these proteins served as anchors for the assembly of protein-protein interaction (PPI) networks.

Two highly reliable and extensively validated databases were employed to construct the PPI networks. The first was the UniHI version 7.1 database (http://www.unihi.org) developed by Kalathur *et al.* (2014), which provides high-confidence human protein-protein interactions. The second was the STRING 11.0 database (http://version11.string-db.org/) introduced by Szklarczyk *et al.* (2015). Both databases offer a wealth of experimentally verified protein-protein interaction data. Only the protein-protein interactions between different cell types (epithelium, stroma, trophectoderm) were described in the study. To identify the most significant PPIs for implantation processes, the predicted interactions were clustered using the Cytoscape software with AutoAnnotate (http://apps.cytoscape.org/apps/autoannotate) using default settings, as previously described (Kucera *et al.*, 2016).

3.4.4. Enrichment analyses

The proteins in the PPI network were subjected to enrichment analyses using g:Profiler (Reimand *et al.*, 2016). A hypergeometric test was used for the analysis, and the resulting P-values for enrichment were adjusted for multiple testing using the g:SCS (Set Counts and Sizes) method, addressing the non-independent structure of tested GO (Gene Ontology) terms (Raudvere *et al.*, 2019).

3.5. Optimizing BMP4-mediated differentiation of human embryonic stem cells into trophoblast-like cells. (Ref IV)

3.5.1. The pluripotent hESC line H9 differentiation

The pluripotent hESC line H9 (WiCell Research Institute, Madison, WI, USA) was cultured on a growth factor-reduced Matrigel® coating (BD Biosciences, Bedford, UK) in StemPro medium (Life Technologies, Carlsbad, CA, USA). For trophoblast-directed differentiation, hESCs were split at a 1:5 ratio using 0.02% EDTA (Sigma-Aldrich, St. Louis, MO, USA) and mechanical scraping. After splitting, cells were cultured in StemPro medium for 24 hours to allow attachment to the plate. On day 0, StemPro was replaced with N2B27 medium after gentle washing twice with Dulbecco's phosphate-buffered saline (DPBS; Life Technologies). N2B27 medium consists of DMEM/F12 (1:1) supplemented with 1× N2 supplement, 1× B27 supplement, 0.1 mmol/l β-mercaptoethanol, 1% non-essential amino acids solution (NEAA), and 0.5 mg/ml bovine serum albumin (all from Life Technologies).

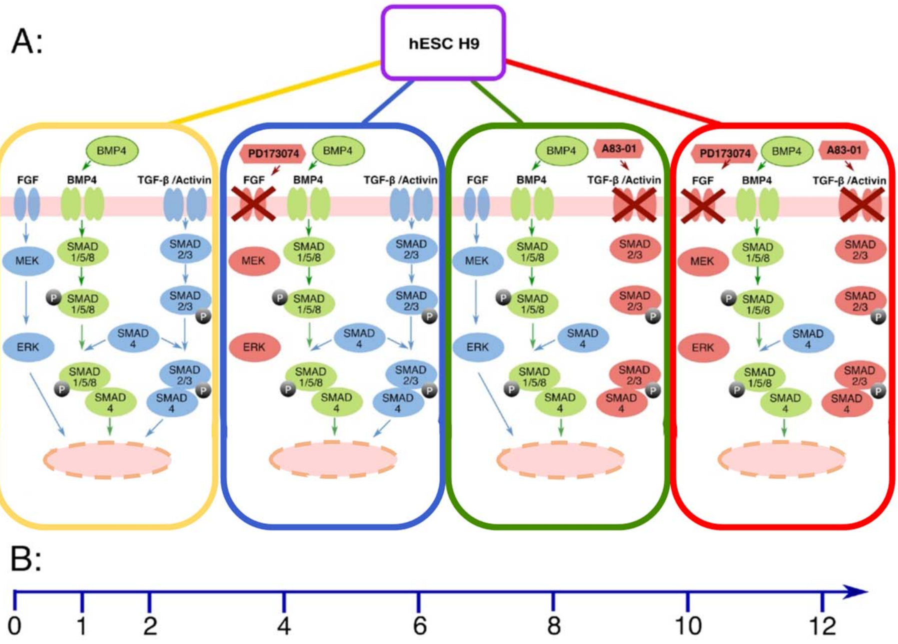

Figure 9: Experimental design of hESC line H9 differentiation. The activated BMP4 pathway is shown in green, and inhibited pathways and their inhibitors are shown in red. The unmanipulated pathways are in blue. (A) Colored outlines of the figure represent the four conditions used: yellow, BMP4; blue, 'iFGF2 + BMP4'; green, 'iTGFb + BMP4' and red, 'iFGF2 + iTGFb + BMP4'. (B) Timeline of experimental design. Numbers show days when RNA samples were collected. Two replicas for every condition at every time point were used. Modified from (Koel *et al.*, 2017).

For trophoblast differentiation, four different conditions were used (Figure 9A): (i) BMP4 induction by 10 ng/ml BMP4 (PeproTech, NJ, USA) without inhibitors (marked in yellow); (ii) 'iFGF2 + BMP4' induction by 10 ng/ml BMP4 and inhibition by 0.1 µmol/l fibroblast growth factor and vascular endothelial growth factor (FGF/VEGF) receptor tyrosine kinase inhibitor PD173074 (Santa Cruz Biotechnology, CA, USA) (marked in blue); (iii) 'iTGFβ + BMP4' induction by 10 ng/ml BMP4 and inhibition by 1 µmol/l TGF-β type I receptors (ALK4/5/7) inhibitor A83–01 (Abcam, UK) (marked in green); and (iv) 'iFGF2 + iTGFβ + BMP4' induction by 10 ng/ml BMP4 and co-inhibition by 0.1 µmol/l FGF/VEGF receptor inhibitor PD173074 and 1 µmol/l ALK4/5/7 inhibitor A83–01 (marked in red). Untreated hESCs were marked in violet. Media and supplements were replaced every 24 hours. Every other day, for 12 days (Figure 9B), two 35-mm plates of cells were dissociated in TRIzol Reagent (Thermo Fisher Scientific), and the cell culture medium was collected and stored at –80 °C.

3.5.2. Differentiated human embryonic stem cells RNA-sequencing

For the differentiated hESC, the total RNA of treated cells was isolated using TRIzol Reagent following phenol/chloroform extraction and isopropanol precipitation. According to the manufacturer's instructions, DNA contamination was removed from RNA samples with the DNA-freeTM Kit (Thermo Fisher Scientific).

The Agilent 2100 Bioanalyzer and RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA) were utilized for evaluating the RNA integrity number (RIN) and concentration of total RNA samples. In the case of differentiated hESC, a RIN value greater than 9 was considered acceptable. Additionally, RNA concentrations were assessed using Qubit (Invitrogen) fluorometric quantitation.

Bulk-RNA full transcriptome analysis of cultured hESC and placental tissue was performed using ten nanograms of high-quality input RNA, which was converted to cDNA and amplified to form an Illumina-compatible library. In total, 25 PCR cycles were used, but as four base-pair unique molecular identifiers were applied, only the absolute number of unique reads was calculated per analyzed sample. The libraries were sequenced by Illumina HiSeq2000 and processed by Casava 1.8.2 (both Illumina, San Diego, CA, USA). Quality control was performed by STRTprep (https://github.com/shka/STRTprep, commit number 81a41adc7be5d388709b7f50504dc9a79ba72c25). Reads were aligned against the human hg19 genome and synthetic ERCC92 spike-in sequences using TopHat software ver. 2.0.10 (Kim *et al.*, 2013). Reads aligning to spike-ins and 5' UTR regions were quantified by Htseq (ver. 0.6.1) (Anders *et al.*, 2015).

For differentiated cells, spike-in-based normalization and differential expression analyses were performed using the R package edgeR (ver. 3.12.0) (Robinson and Oshlack, 2010), with an FDR <0.01 and FC > 2 used as thresholds for determining differential expression.

3.5.3. Placental samples RNA-sequencing

Full-thickness placental blocks, approximately 2–3 cm in size, were obtained from two full-term healthy pregnancies and stored at –80 °C in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) within 1 hour after normal delivery.

For the placenta, total placental RNA was extracted from frozen tissue with the mirVanaTM miRNA Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions without isolating small RNA. Extractions were performed from three samples collected from three locations of the same placenta, and all RNA samples from one placenta were pooled together.

The Agilent 2100 Bioanalyzer and RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA) were utilized for evaluating the RNA integrity number (RIN) and concentration of total RNA samples. In the case of differentiated hESC, a RIN value greater than 9 was considered acceptable. For placenta samples, a RIN value greater than 5.7 was considered acceptable. Additionally, RNA concentrations were assessed using Qubit (Invitrogen) fluorometric quantitation.

The placenta samples were RNA-sequenced and analyzed in the same way as differentiated hESC (chapter 3.5.2).

3.5.4. Enrichment analyses

The involvement of genes in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was predicted by GeneNetwork (http://genenetwork.nl/GeneNetwork/ (Fehrmann et al., 2015)).

3.5.5. Quantitative real-time PCR

Dnase-treated RNA, obtained using the TURBO DNA-freeTM kit (Ambion Inc., Austin, Texas, USA), was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., MA, USA). Quantitative reverse transcription-PCR (qRT-PCR) was performed with 1 × HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Estonia) following the manufacturer's instructions. Comparison with the exogenous spike-in (pSPIKE1) (Thermo Fisher Scientific) was performed for differentiated hESC. The relative expression levels were calculated using the 2-ΔΔCt method. The concordance between RNA-seq and qRT-PCR was evaluated using an association test for Pearson correlation.

3.5.6. Measurement of secreted total hCG and hyperglycosylated hCG (hCG-H)

To analyze the secretion of trophoblast cells' specific hormone, hESC differentiating culture supernatants were collected and stored at −80°C, and the levels of total hCG and hCG-H were quantified by the time-resolved immunofluorometric assay as described previously (Alfthan *et al.*, 1992; Stenman *et al.*, 2011).

3.5.7. Cytokeratin 7 immunostaining

To describe the pan-trophoblast marker protein cytokeratin 7 presence, cells differentiated until day 5 were fixed with ice-cold 100% methanol for 15 min at −20 °C and washed three times with PBS. Fixed cells were incubated with UltraVision Protein Block (Thermo Fisher Scientific, LabVision Inc., Fremont, CA, USA) for 5 min, treated with primary antibody against cytokeratin 7 – KRT7 (Antibody Registry: AB_2265604, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and visualized with Alexa Fluor 488-conjugated donkey anti-mouse secondary antibody (Life Technologies, Eugene, OR, USA). The primary antibody was omitted in the negative control. Cells were mounted with Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories Inc., Burlingame, CA, USA) for nuclear staining. Stained cells were imaged using an EVOS FL Cell Imaging System (Thermo Fisher Scientific).

4. RESULTS AND DISCUSSION

4.1. Meta-signature of endometrial receptivity-associated genes (Ref I)

One of the main goals of assisted reproduction technologies is to assess endometrial receptivity for successful embryo implantation. As a result, numerous studies have been conducted to identify the most suitable markers of endometrial receptivity using transcriptomic analyses. However, these studies have been limited by small sample sizes, variations in research design, participant selection criteria, endometrial sampling techniques, expression array/sequencing platforms, computational pipelines for data processing, and inconsistent standards for data presentation. This has led to inconsistent biological conclusions and difficulties integrating raw data in meta-analyses. To overcome these limitations, a robust rank aggregation algorithm (RRA) has been applied to compare gene lists and identify commonly overlapping genes across multiple transcriptomic studies involving diverse ethnic groups. This approach enables the identification of a meta-signature or consensus signature of highly putative biomarkers of endometrial receptivity-associated genes, which can be used to define the most relevant marker genes of endometrial receptivity. The analysis revealed a statistically significant meta-signature of 52 up-regulated and five down-regulated genes in the receptive endometrium compared to the pre-receptive endometrium (Figure 10; Ref I, Table 2).

4.1.1. The role of endometrial meta-signature genes in the immune response during embryo implantation

Enrichment analyses were performed using the g:Profiler software (Reimand *et al.*, 2016) to interpret the biological functions of the meta-signature genes. The study revealed that many of these genes are involved in immune responses, including the inflammatory response, immunoglobulin-mediated immune responses, and the complement and coagulation cascade pathway in the receptive-phase endometrium. The meta-signature includes five genes involved in the complement system in mid-secretory endometrium, namely *C1R*, *SERPING1*, *CD55*, *C4BPA*, and *CFD*, as well as the complement regulatory protein coding gene *CD55* (Figure 10). CD55 is suggested to protect the embryo from maternal complement-mediated attack and prevent epithelial destruction from increased complement expression at the implantation (Franchi *et al.*, 2008). A similar protective role has been suggested for complement component 4 binding protein alpha (C4BPA), where increased expression of this inhibitor of complement system activation could reduce the possibility of an uncontrolled complement attack on the embryo (Tapia *et al.*, 2011). It is known that a balance between maternal immune tolerance towards the semi-allogeneic implanting embryo and protective anti-infectious mechanisms in the receptive-phase uterus is necessary for successful embryo implantation (Haller-Kikkatalo *et al.*, 2014). Therefore, the meta-signature genes highlight the importance of immune responses, specifically the complement cascade pathway in mid-secretory endometrial functions.

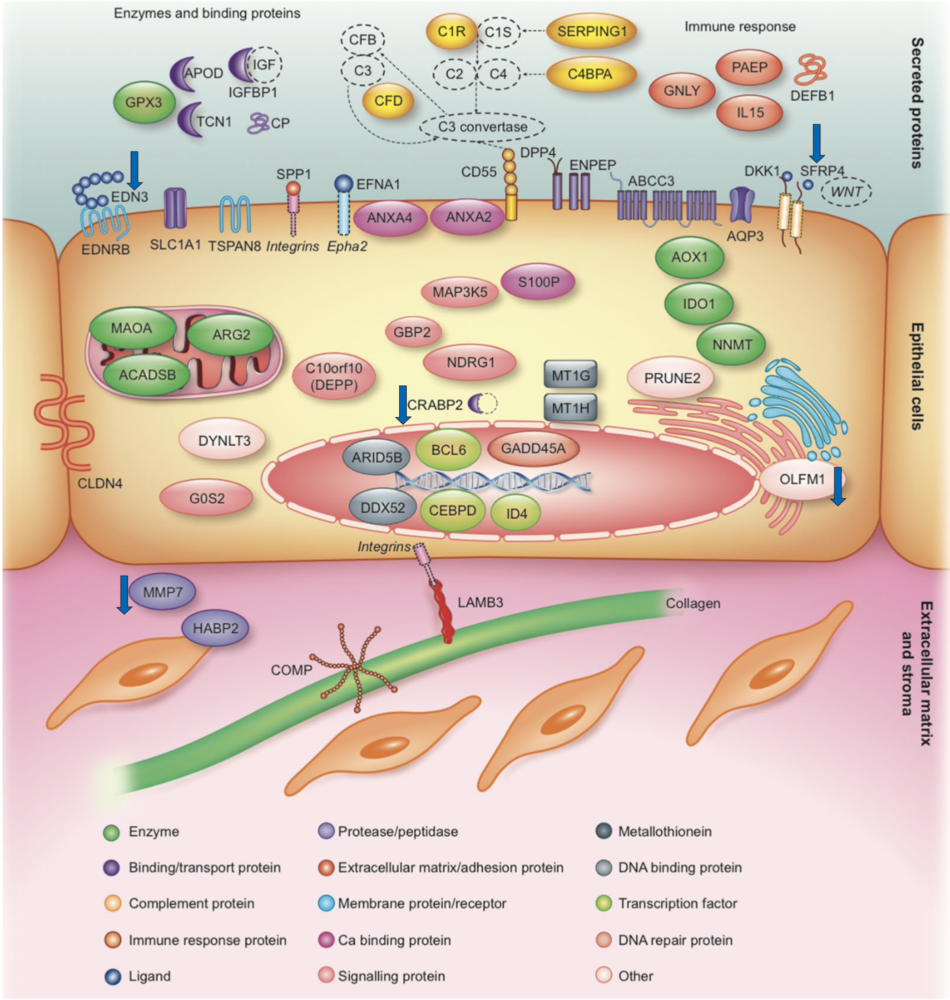

Figure 10. Schematic overview of the 57 proteins, coded from the meta-signature genes, their literature-based localization, and involvement in the receptive endometrium. The five proteins (SFRP4, EDN3, OLFM1, CRABP2, MMP7), encoded by down-regulated transcripts are indicated by blue down-pointing arrows. Modified from (Altmäe *et al.*, 2017).

4.1.2. The possible role of endometrial meta-signature genes in endometrial-based communication.

The enrichment analyses of meta-signature genes also revealed that many genes were related to the extracellular region and exosomes. The secretion of small vesicles, which can transfer biomolecules from recipient cells to target cells and modulate normal physiological and abnormal pathological processes, is one mechanism for cellular communication. For example exosomes and their cargo are involved in the progression of different reproductive disorders, including polycystic ovary syndrome, premature ovarian failure, Asherman syndrome, endometriosis, endometrial cancer, cervical cancer, ovarian cancer, and pre-eclampsia (Esfandyari *et al.*, 2021). These vesicles can carry molecular cargo such as proteins, microRNAs, or transferred mRNAs, and their contents can influence target cell activity. Exosomes, which are small nanovesicles (30–150 nm) secreted by most cell types, have been extensively studied, and their components are listed in the exosome database ExoCarta (www.exocarta.org) (Keerthikumar *et al.*, 2016). Proteins encoded by the meta-signature genes were overlapped with the human exosome database to investigate whether endometrial receptivity-related genes could contribute to exosome communication. Twenty-eight out of 57 endometrial receptivity-associated genes were experimentally detected in human exosomes based on the ExoCarta database (Figure 11). Fisher's Exact Test revealed that proteins translated by meta-signature genes had a 2.13 times higher probability (p= 0.0059) of being present in exosomes than the rest of the protein-coding genes in the human genome. Also, Giacomini and colleagues have demonstrated that 38 transcripts out of the 57 receptivity markers were identified in higher concentrations in EVs derived from the uterine fluid of fertile women during the WOI compared to those extracted from the pre-receptive stage (Giacomini *et al.*, 2021). Therefore, these EV proteins could play a role in embryo-endometrium molecular crosstalk.

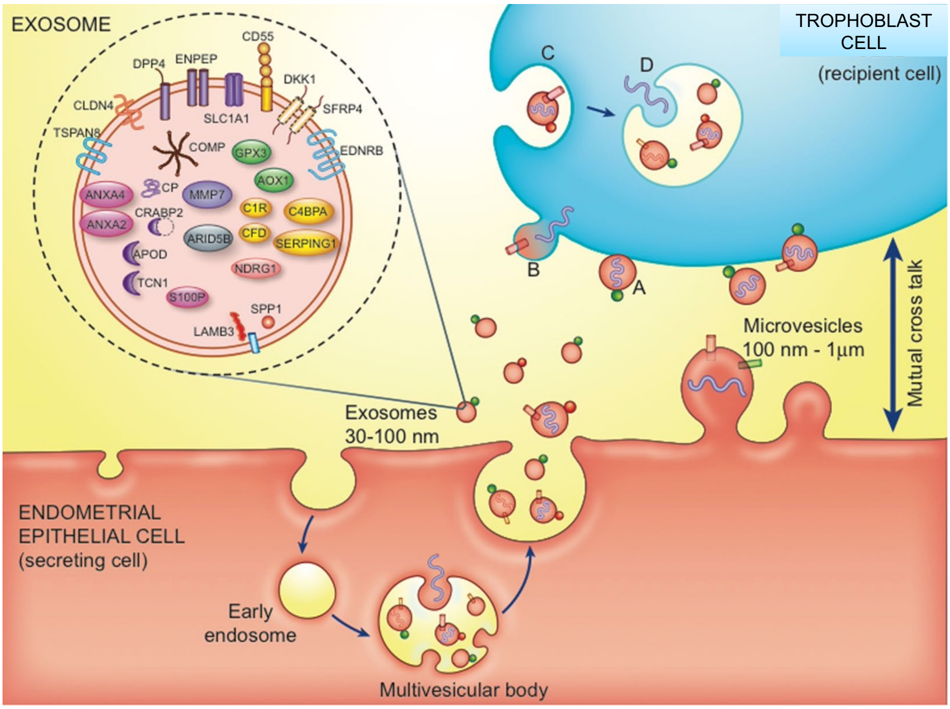

Figure 11. The 28 identified proteins from the meta-signature gene list are in exosomes based on the ExoCarta database. Modified from (Altmäe *et al.*, 2017).

4.1.3. The validation of meta-signature genes in the independent whole endometrial tissue and cell-specific endometrial cells

To check the robustness of the meta-signature, we used two independent RNA-sequencing sample sets to analyze the expression levels of 57 endometrial receptivity-related genes. The first set comprised whole endometrial tissue biopsies from 20 women in the early secretory (2 days after the luteinizing hormone peak. LH+2) and mid-secretory (8 days after the luteinizing hormone peak, LH+8) phases. The second set of flow cytometry separated CD13-positive endometrial stromal cells and CD9-positive endometrial epithelial cells from 16 women during the same phases. Fifty-two transcripts in the whole endometrium were identified as differentially expressed between the pre-receptive and receptive stages, with FDR < 0.05. The direction of the effect agreed with the meta-signature for all the genes. Therefore, the differential expression of the consensus list of 39 endometrial meta-signature genes was confirmed in independent sample sets (Figure 12).

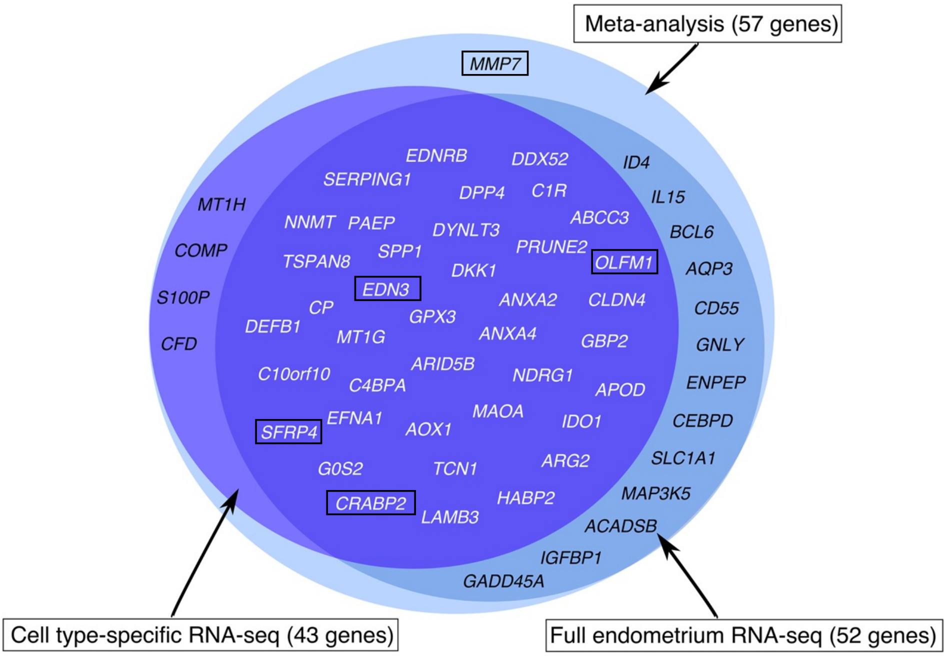

Figure 12. Comparison of the meta-signature genes with independent full endometrium RNA-seq and cell type-specific RNA-seq. The black box indicates the five proteins (SFRP4, EDN3, OLFM1, CRABP2, MMP7) encoded by down-regulated transcripts. Modified from (Altmäe *et al.*, 2017).

In our meta-analysis, only one of the meta-signature genes, secreted phosphoprotein 1/osteopontin coding gene *SPP1*, was consistently reported as differentially expressed in all nine gene expression studies. The inconsistency in published results highlights the significant problem of heterogeneous mRNA expression profiles, particularly in the endometrial data, where sample sizes are relatively small. Therefore, this meta-signature allows the identification of a consensus set of genes whose expression changes in a similar direction during endometrial maturation to the receptive state.

4.1.4. Endometrial epithelial and stromal cells have distinct transcriptomes

The analysis of endometrial receptivity-related genes in epithelial and stromal cells has demonstrated that transcript regulation is largely cell-type specific. In these cell populations, a total of thirty-nine meta-signature genes were significantly up-regulated, and four were down-regulated (*CRABP2*, *EDN3*, *OLFM1*, *SFRP4*) when comparing the pre-receptive and receptive phase (with a fold change of ≥2). Out of the 39 up-regulated genes (Figure 12), 16 (41%), including *ANXA2*, *COMP*, *CP*, *DDX52*, *DPP4*, *DYNLT3*, *EDNRB*, *EFNA1*, *G0S2*, *HABP2*, *LAMB3*, *MAOA*, *NDRG1*, *PRUNE2*, *SPP1*, and *TSPAN8*, were significantly up-regulated only in epithelial cells. In contrast, four genes (10.2%), including *APOD*, *CFD*, *C1R*, and *DKK1*, were only up-regulated in stromal cells (Figure 13). Furthermore, it is worth noting that although 19 genes (48.8%) were up-regulated in both cell types, their baseline expression levels were still higher in epithelial cells (as depicted in Figure 13).

In the meta-analysis study, five genes were found to be significantly down-regulated. *SFRP4*, *EDN3*, and *CRABP2* were down-regulated in both studied endometrial cell types. *OLFM1* was specifically down-regulated in stromal cells, while the *MMP7* transcript was not differentially expressed in either epithelial or stromal cells (Figures 12 and 13). These findings suggest that the number of cells in the endometrial biopsy may strongly influence the observed gene expression patterns. The differences in endometrial tissue used in different transcriptomics studies may obscure the detection of important genes related to endometrial receptivity and lead to the observed small overlaps in the reported gene lists. Therefore, in studies involving whole endometrial tissue, it is crucial to consider the proportions of different cell types in the biopsy material; however, it is often very challenging.

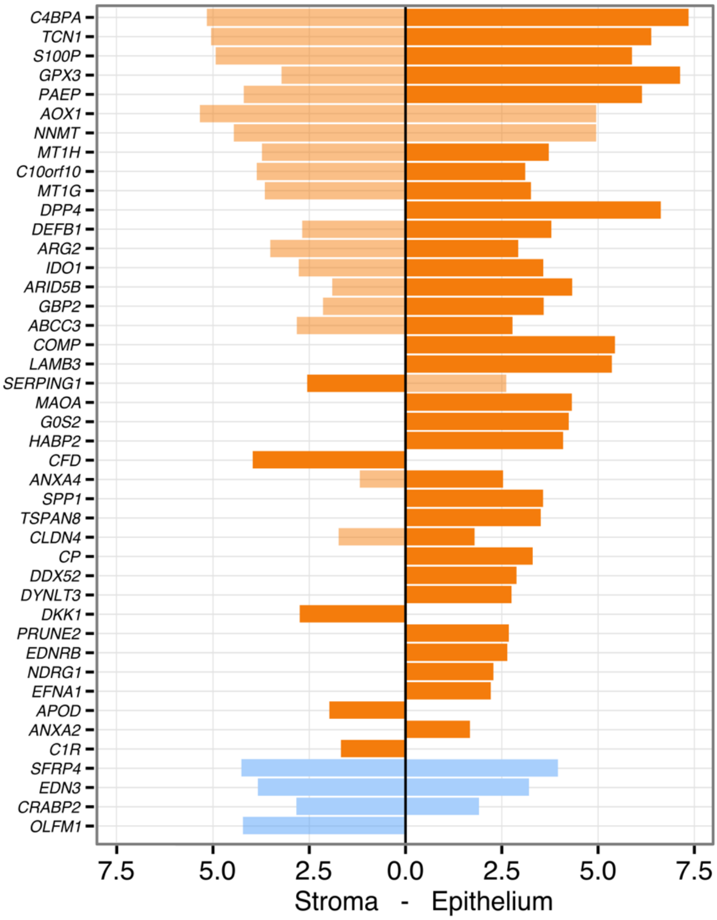

Figure 13. Meta-signature genes regulation on cell type-specific RNA-seq data. Significantly up-regulated (orange) and down-regulated (blue) genes in the endometrium's FACS-sorted stromal and epithelial cells. The color intensity represents the gene expression values (higher expression highlighted as darker orange) between epithelial vs. stromal cells in the mid-secretory phase endometrium (LH+8). The x-scale represents log2(FC) between LH+8 vs. LH+2 comparisons in stromal and epithelial cells. All reported results are significant at FDR < 0.05. Adapted from (Altmäe *et al.*, 2017).

4.2. TAC-seq – a novel method for absolute molecule counting (Ref II)

Tissue transcriptional profiling is typically accomplished using either next-generation sequencing (NGS) or quantitative or digital PCR methods. However, both methods have limitations. NGS offers high sensitivity, high throughput, and flexibility but can be relatively costly. On the other hand, quantitative PCR and digital PCR are cost-effective and straightforward but do not enable the high multiplexing required for more comprehensive healthcare applications. Although NGS sensitivity has been combined with the high specificity of ligation-PCR to extend the available precise target quantification methods, these applications still suffer from the random ligation at low target nucleic acid levels and polymerase-induced errors in both PCR and sequencing steps, leading to an overestimation of the original number of studied molecules.

In the case of endometrial receptivity, where some of the most relevant transcripts are expressed or up-regulated only in a small timeframe with a low copy number, PCR amplification bias can cause significant misinterpretation and lead to false results. Therefore, unique molecular identifiers (UMIs) are applied to overcome amplification bias and maximize nucleic acid detection sensitivity. The specific UMI sequence is enclosed into the original target molecule through oligonucleotides used in library preparation before amplification. Later, the identical UMI clones are eliminated as PCR duplicates, allowing the detection of the actual number of biomolecules. Based on the combination of PCR and NGS with UMI usage, a novel ligation- and NGS-based method, targeted allele counting by sequencing (TAC-seq), was developed.

4.2.1. Absolute molecule counting of endometrial receptivity biomarkers

The TAC-seq method was developed to target 57 mRNA transcripts identified as potential biomarkers of endometrial receptivity in the meta-signature study (Ref I). The mRNA transcripts were uniquely identified using a 54-bp-long target complementary sequence detected by two side-by-side located TAC-seq probes. The detector probes are hybridized to the target cDNA molecule and then ligated together by a thermostable ligase. Therefore, the ligated detector-target complexes are captured by magnetic beads, amplified by PCR, and identified by sequencing (Figure 8).

To experimentally evaluate the TAC-seq method, ten endometrial biopsy samples were analyzed using high-coverage (TAC-seq high) and low-coverage (TAC-seq low) sequencing and whole transcriptome RNA sequencing to compare the effectiveness of the TAC-seq assay. While all methods grouped samples similarly on principle component analyses and hierarchical clustering (Figure 14), the high-coverage sequencing using eight nucleotide-long UMIs caused technical limitations in molecule detection, resulting in the six most highly expressed genes being subject to UMI-related saturation and not precisely quantified. Therefore, low-coverage sequencing is the preferable assay for measuring endometrial receptivity genes.

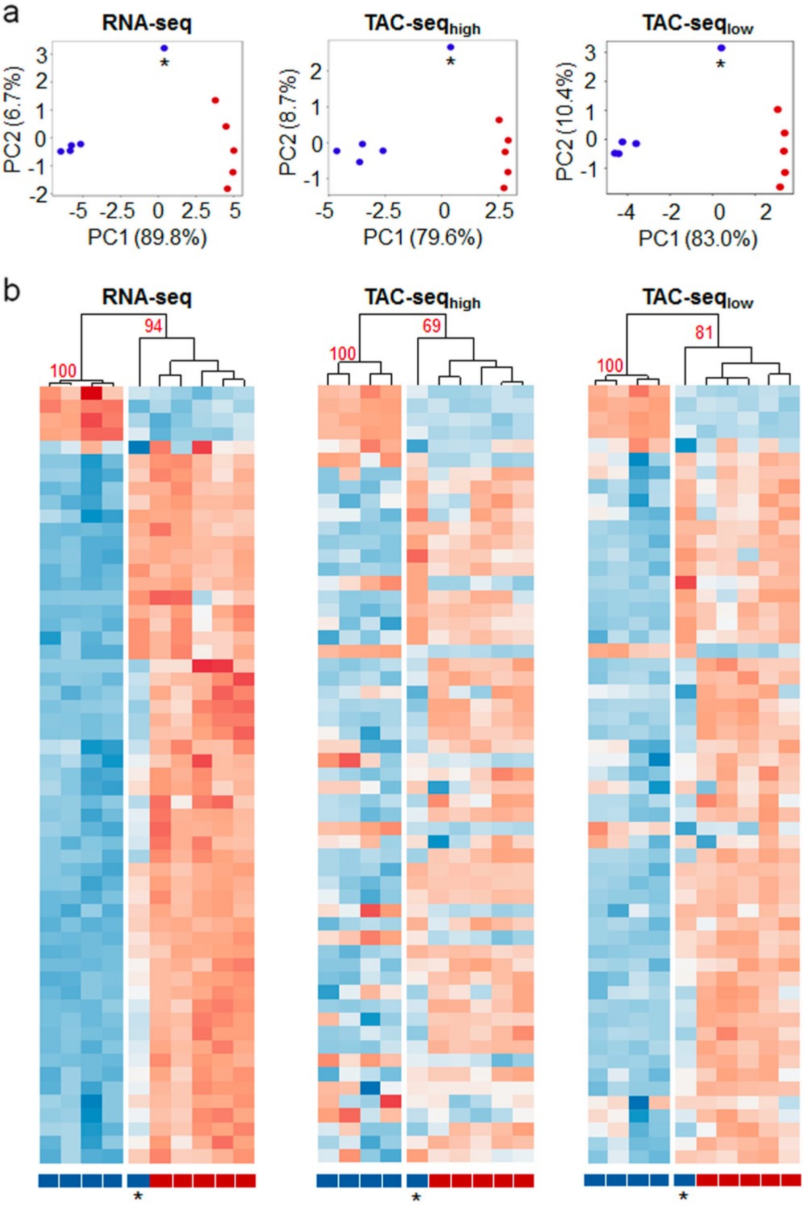

Figure 14. Comparison of the overall predictions for mRNA TAC-seq assay. (A) Principal component analysis of the full transcriptome RNA-seq, high-coverage TAC-seq, and low-coverage TAC-seq of 10 endometrial samples. (B) Heatmaps of the full transcriptome RNA-seq, high-coverage- and low-coverage TAC-seq show the sensitivity to distinguish different endometrial samples according to their receptivity. A pre-receptive sample (marked with an asterisk) shows a similar expression profile and clusters with receptive samples in all three comparisons. The pre-receptive samples are denoted by blue, and the receptive ones by red. The data is presented as log-transformed counts per million (CPM) values, scaled row-wise. The samples are hierarchically clustered column-wise using Pearson correlation, while the genes are ordered row-wise based on the RNA-seq clustering outcomes using Euclidean distance. The clustering probabilities are marked with red numbers. Adapted from (Teder *et al.*, 2018).

Repeated implantation failure with euploid embryos necessitates endometrial receptivity testing to identify maternal factors that may contribute to unsuccessful IVF cycles. One such factor is a shifted or shortened window of implantation, requiring personalized embryo transfers and endometrial testing. Currently, clinical endometrial receptivity tests include the NGS-based ERA® test (Igenomix) (Díaz-Gimeno *et al.*, 2011), RT-qPCR-based ER Map® test (IGLS) (Enciso *et al.*, 2018), RT-qPCR-based WIN-Test (INSERM) (Haouzi *et al.*, 2020) and NGS-based rsERT test (Yikon Genomics Company) (He *et al.*, 2021). Although these tests have been used for over a decade, their high cost has limited their use for many couples. Therefore, this thesis introduces a new approach to developing a cost-effective and precise receptivity test, the beREADY endometrial receptivity test, utilizing a novel DNA sequencing TAC-seq technology. (https://www.medrxiv.org/content/10.1101/2022.06.13.22276318v1.full.pdf).

4.3. Predicted protein-protein interactions between trophectoderm and endometrial cell types (Ref III).

Embryo implantation is a complex process that requires synchronized interplay between the implantation-competent blastocyst and the receptive-phase endometrium, from the first apposition stage until the embryo's attachment to the endometrial epithelial cells and further invasion between the endometrial stromal cells. These actions occur within a short time frame and require specific expression and interaction of surface molecules. The meta signature of receptive endometrium gene expression allows the selection of appropriate marker genes to detect tissue maturation and readiness for blastocyst implantation. When the study was first published, the meta-signature genes were instrumental in developing methods for estimating the endometrial receptivity state. However, it is now known that this approach overlooks the complexity of endometrial tissue structure with multiple cell types and its dynamic gene expression changes during the menstrual cycle (Wang *et al.*, 2019). Therefore, it may not be suitable for precisely defining implantation-ready endometrium. The recently published single-cell endometrial transcriptome studies highlight the significantly different gene expression profiles in the human endometrium cell subtypes (Wang *et al.*, 2020; Garcia-Alonso *et al.*, 2021), emphasizing the need to focus on the specific cell subpopulation transcriptome profiles to model the initial molecular embryo-endometrium dialogue. In addition, to avoid transcriptome changes that may occur during cell cultivation, studying the endometrial gene expression profile *ex vivo* rather than in cultured cells is essential (Krjutškov *et al.*, 2016). Therefore, we aimed to analyze specific transcriptome changes separately between the pre-receptive and receptive endometrium in two central cell populations – epithelial and stromal cells. This approach enables us to combine the data with polar-trophectodermal cells' transcriptome data from blastocyst-stage embryos to predict the protein-protein interactions in the embryo-endometrium crosstalk.

4.3.1. The epithelial and stromal cells' gene expression changes from the pre-receptive to the receptive phase

In the cell type-specific approach, RNA-sequencing analysis was performed on freshly isolated pre-receptive (LH+2) and receptive (LH+7/8) stage endometrial epithelial and stromal cells to investigate their transcriptomics. The analysis of transcriptome changes between pre-receptive and receptive endometrium demonstrated similar trends in gene-expression changes in both epithelial and stromal cells. Approximately 80% of the differentially expressed transcripts were up-regulated in cells of the receptive tissue. The up-regulated and down-regulated genes were compared between the epithelial and stromal cells to identify cell-type-specific genes and genes whose expression increased in both studied cell types. In epithelial cells, 373 genes (74.7%) were uniquely up-regulated, and 65 genes (64.4%) were down-regulated. In stromal cells, 455 genes (78.3%) were uniquely up-regulated, and 80 genes (69.0%) were down-regulated. 126 genes were up-regulated, and 36 were down-regulated in both receptive endometrial cell types (Figure 15).

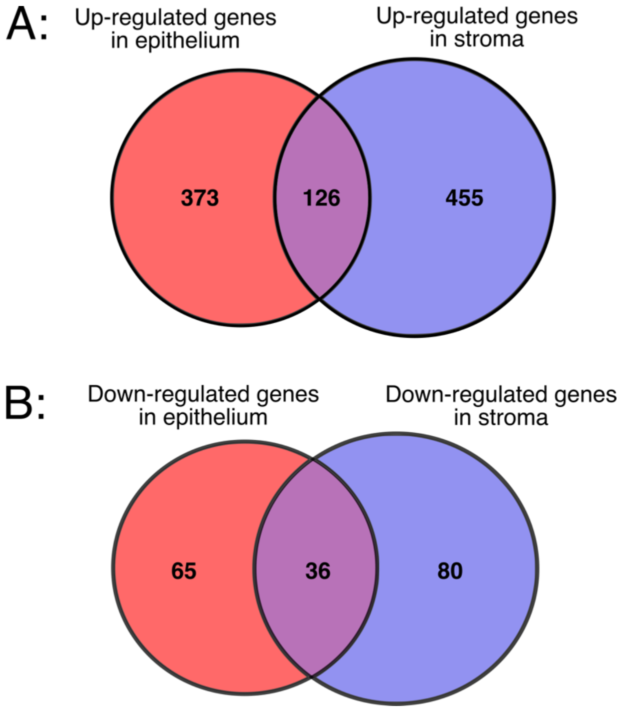

Figure 15. Cell-type specific gene expression in endometrium. **A:** Up-regulated genes in epithelial and stromal cells in receptive *vs.* pre-receptive phase endometrium. **B:** Down-regulated genes in epithelial and stromal cells in receptive *vs.* pre-receptive phase endometrium. Adapted from (Koel *et al.*, 2022).

4.3.2. Predicted protein-protein interactions between trophectoderm and endometrial cell types

Based on previous knowledge that human embryos attach to the receptive endometrial surface with the trophectodermal cells on the polar side of the hatched blastocyst (Johnson, 2012), two distinct datasets were assembled to create the protein-protein interaction (PPI) network for embryo implantation. The first dataset consisted of up-regulated genes from freshly isolated non-cultured receptive endometrial epithelial and stromal cells. The second dataset contained transcripts expressed in the polar trophectoderm of 17 human blastocyst-stage embryos from days six and seven (Petropoulos *et al.*, 2016). The relevance of the polar trophectodermal datasets was confirmed by the recently identified maturation marker of polar trophectoderm, nuclear receptor subfamily 2 group F member 2 (*NR2F2)* (Meistermann *et al.*, 2021).

499 up-regulated epithelial cell transcripts, 581 up-regulated stromal cell transcripts, and 5,578 polar-trophectodermal (pTE) genes were used to predict the PPI network on the embryo side. According to prior research on gene-protein correlation, the proteome encyclopedia from Nusinow et al. (2020) was used to prioritize transcript interactions and involved only these genes whose expression was positively correlating (Spearman R > 0) with the protein expression profile. Based on the UniProt database, the focus was narrowed to genes that encode proteins localized at the cell surface, extracellular matrix (ECM), or secreted by the cells to examine the potential interactions between the receptive endometrium and embryo. These proteins were then used as anchors to assemble protein-protein interaction (PPI) networks. Through these filtration steps, we identified 222 possible interacting proteins (Table 1).

Table 1. Filtration steps to identify molecules involved in the protein-protein network.

Cell type	Up-regulated genes	Positive correlation between the gene and protein expression	Genes encoding cell surface, ECM or secreted proteins	In PPI network
Endometrial epithelium	499	418	62	43
Endometrial stroma	581	458	105	68
Trophectoderm	5,578	4,812	282	161

Among these transcripts, 125 (56.3%) were specific to the pTE cells, while 16 (7.2%) and 38 (17.1%) were unique to the endometrial epithelial and stromal cells, respectively (Figure 16). Interestingly, seven transcripts (3.2%) (*ANXA2*, *CD9*, *CLDN4*, *EFNA1*, *HLA-C*, *B2M*, and *LGALS3*) were expressed in all three cell types, whereas seven transcripts (*HLA-B*, *TIMP1*, *APOL1*, *AGR2*, *SERPING1*, *VCAN*, and *CALCRL*) were expressed in both endometrial cell types, but not in trophectodermal cells.

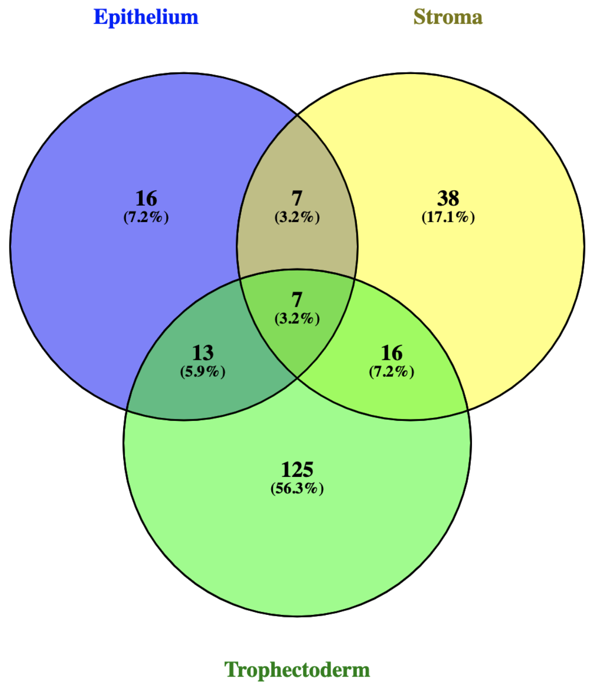

Figure 16. Overlap of the PPI network's epithelial, stromal, and trophectodermal proteins.

To identify the most significant PPIs for implantation processes, the predicted interactions were clustered using the Cytoscape software with AutoAnnotate (http://apps.cytoscape.org/apps/autoannotate) using default settings, as previously described (Kucera *et al.*, 2016). The resulting clusters were labeled alphabetically (A, B, C, D, E) based on the number of interacting proteins, and a total of 15 sub-networks were identified. The top five clusters, which consisted of more than 18 proteins, were subjected to enrichment analyses using g:Profiler (Reimand *et al.*, 2016).

4.3.2.1. The predicted protein-protein interactions regulate cell attachment and adhesion.

During the attachment phase of embryo implantation, the positioned blastocyst induces stable, shear stress-resistant adhesion, which depends on several cellular adhesion molecules. Two sub-clusters were identified in the PPI network enriched with proteins related to cell adhesion (adj. p-value 6.5×10^{-20}) (Cluster A) and cell-cell adhesion (adj. p-value 1.4×10^{-7}) (Cluster D) that could be relevant for embryo attachment to the endometrial cells (Figure 17). In cluster A (Figure 17A), the protein with the highest degree of interaction was integrin β1 (ITGB1). This transmembrane protein is expressed on both epithelial and trophectodermal cells and could be essential for both interacting partners. Indeed, ITGB1 role in the endometrial receptivity (Chen *et al.*, 2016) and blastocyst implantation process (Brakebusch *et al.*, 1997) has been shown and discussed previously. In the formed PPI network, seven secreted proteins (VEGFA, MDK, SDC1, IGFBP2, LGALS3, MET, VTN) interact with either trophoblast or epithelial integrin β1 and could regulate its functions. Previously it has been demonstrated that galectin-3, through ITGB1 regulation, mediates trophoblast cells invasion (Bojić-Trbojević *et al.*, 2019), insulin-like growth factor binding protein-2 (IGFBP2) binds with integrin β1 and regulate cell migration and invasion (Han *et al.*, 2014).

Twelve transmembrane proteins (ANPEP, BSG, CD63, CD81, ITGA5, JAM3, PROM1, SLC3A2, ALCAM, CD44, CD9, ENG) that could be involved in embryo attachment through integrin β1 were also highlighted. The integrin β1 interaction with basigin (BSG, expressed in stromal and trophectodermal cells) has been described to regulate embryo implantation (Lee *et al.*, 2013). Also, the role of epithelial CD44 in the early stages of embryo attachment has been demonstrated in in vitro study (Berneau *et al.*, 2019). In addition, the transmembrane protein CD44 interaction with trophectodermal syndecan-1 (SDC1) has previously been proposed to be involved in the trophoblast invasion regulation (Ibrahim *et al.*, 2017).

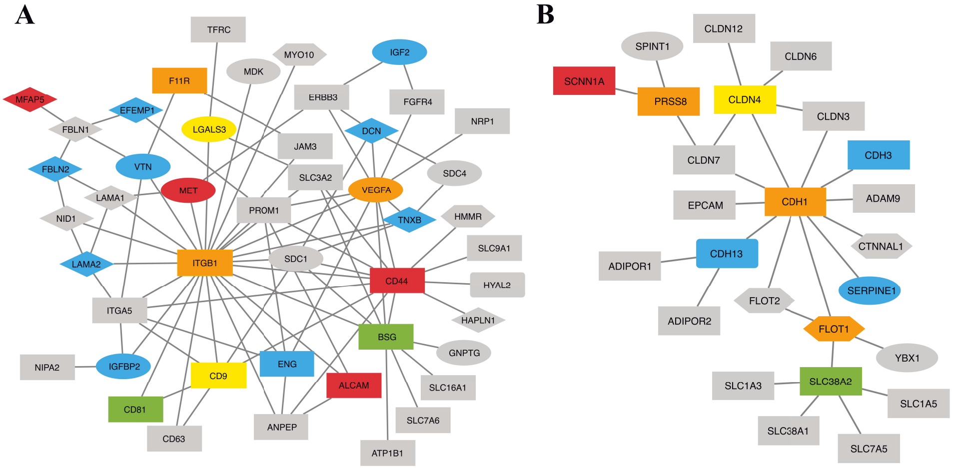

Figure 17. Molecular interactions of polar trophectodermal cells with endometrial epithelial and stromal cells. A: Cluster A, B: Cluster D

Blue: stromal proteins, red: epithelial proteins, grey: trophectodermal proteins, orange: epithelial and trophectodermal proteins, green: stromal and trophectodermal proteins, yellow: epithelial, stromal, and trophectodermal proteins. Ellipse: secreted proteins, diamond: extracellular matrix proteins, octagon: membrane-bound proteins, rectangle: transmembrane proteins. Modified from (Koel *et al.*, 2022).

Cluster D (Figure 17B) is also related to cell-cell adhesion. The hub protein transmembrane E-cadherin (CDH1) was predicted to bind other transmembrane proteins, including claudins (CLDN3, CLDN4, CLDN7), and cadherins (CDH3, CDH13), extracellular membrane proteins flotillins (FLOT1, FLOT2) and alpha-catulin (CTNNAL1), as well as trophectodermal-specific plasminogen activator inhibitor-1 (SERPINE1). Although the importance of E-cadherin in cell-cell adhesion during the implantation process has been discussed, 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.

4.3.2.2. The predicted protein-protein interactions mediate extracellular matrix organization and cell migration

During the implantation process, the embryo invasion phase involves the extracellular matrix (ECM) remodeling to facilitate the trophectoderm cells anchoring to the implantation site. As cellular gene expression constantly changes, the initial interactions between the endometrium and blastocyst regulate subsequent embryo invasion. In the context of the PPI network, secreted ECM proteins were expected to be involved in preparation for blastocyst migration. In the PPI network, subclusters enriched with proteins undergoing post-translational protein modifications (Cluster B) (adj. p-value 9.1×10^{-26}), regulating secretion processes (Cluster C) (adj. p-value $2.4x10^{-7}$), or involved in an ECM organization (Cluster E) (adj. p-value 1.8×10^{-6}) were detected. Some of these proteins are shown to mediate cellular signaling between distinct cell types, which could be relevant for intermediate communication between the blastocyst and maturing endometrium.

In cluster B (Figure 18A), seven proteins were detected to be secreted uniquely from the epithelial cells (CLU, CP, IGFBP5, and SPP1) or both epithelial and stromal cells (TIMP1, APOL1, and SERPING1). One of the most extensively studied proteins among these molecules is osteopontin (SPP1), secreted by the endometrial epithelial cells. Osteopontin has been repeatedly identified in transcriptome studies as an endometrial receptivity-specific molecule (Altmäe *et al.*, 2017; Wang and Yu, 2018). Furthermore, its role in cell-to-cell adhesion during the early stages of implantation has been shown in *in vitro* models (Kang *et al.*, 2014; Wu *et al.*, 2015; Berneau *et al.*, 2019) and in other species (Johnson *et al.*, 2001). The PPI network predicted osteopontin's integration with several trophoblast extracellular and transmembrane proteins, indicating its role in blastocyst attachment and migration. For instance, interaction with trophectodermal ECM protein galectin-1 has been associated with blastocyst attachment, trophoblast migration (Barrientos *et al.*, 2014), and interaction with trophectodermal transmembrane integrin α5β1 with cell adhesion and migration (Barry *et al.*, 2000).

In Cluster B, versican (VCAN), an extracellular matrix protein expressed by both endometrial epithelial and stromal cells, plays a central role. Previous studies have proposed its involvement in the implantation (Altmäe *et al.*, 2012). VCAN is predicted to interact with several trophectodermal proteins, including APOE, APOA1, APOA2, LAMB1, LAMC1, LGALS1, and MXRA8, all of which are involved in cell adhesion and migration processes.

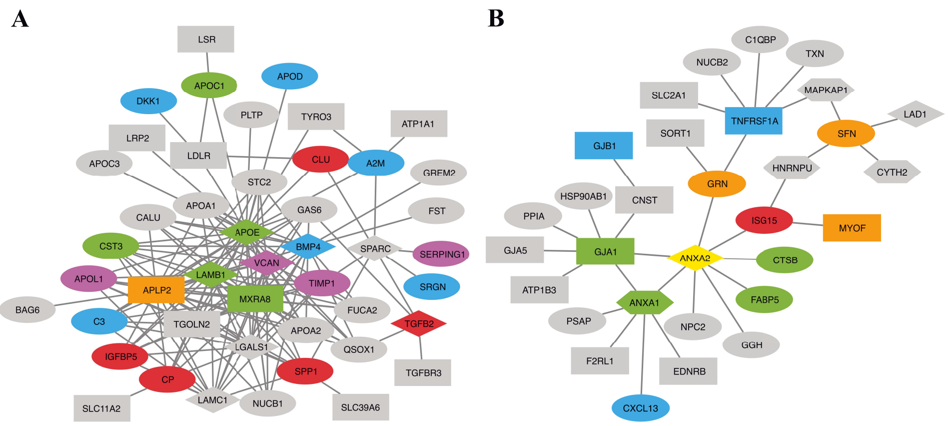

Figure 18. Molecular interactions of polar trophectodermal cells with endometrial epithelial and stromal cells. A: Cluster B, B: Cluster C

Blue: stromal proteins, red: epithelial proteins, grey: trophectodermal proteins, violet: epithelial and stromal proteins, orange: epithelial and trophectodermal proteins, green: stromal and trophectodermal proteins, yellow: epithelial, stromal and trophectodermal proteins. Ellipse: secreted proteins, diamond: extracellular matrix proteins, octagon: membrane-bound proteins, rectangle: transmembrane proteins. Modified from (Koel *et al.*, 2022).

Cluster C (Figure 18B) was enriched with proteins involved in secretion processes, including 18 secreted proteins. Two proteins, stratifin (SFN) and granulin (GRN) were expressed in the epithelial-trophectodermal cells, and one interferon-stimulated gene, *ISG15*, was uniquely expressed in the epithelial cells. Previous studies have shown that endometrial ISG15 secretion in response to pregnancy is critical for pregnancy maintenance in humans and other mammals, including bovine and ovine (Henkes *et al.*, 2015). In the PPI network, ISG15 was predicted to interact with extracellular matrix protein annexin A2 (ANXA2), which is known to play a role in trophoblast outgrowth and embryo adhesion to the human endometrium (Garrido-Gómez *et al.*, 2012). This finding supports the importance of these proteins in the preparation of the blastocyst migration and suggests their potential involvement in the communication between the blastocyst and maturing endometrium during the implantation process.

Proteins involved in the organization of the extracellular matrix were enriched in sub-network E (Figure 19), suggesting their potential involvement in the migration of trophoblast cells between endometrial stromal cells. The predicted interaction between estrogen-regulated anterior gradient 2 (AGR2), secreted by epithelium/stroma cells, and trophectodermal transmembranous Ly6/PLAUR domain-containing protein 3 (LYPD3) and dystroglycan 1 (DAG1) could enhance blastocyst invasion through the regulation of receptor adhesion and interaction with the ECM (Salmans *et al.*, 2013; Arumugam *et al.*, 2015). The interaction between trophoblast cell-secreted procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 (PLOD3), and extracellular matrix protein COL12A1 in the stroma could also regulate cells' invasion and migration (Li *et al.*, 2020). Additionally, other secreted stromal proteins, such as SERPINE1 (in Cluster D), related to trophectoderm spheroid adhesion (Evans *et al.*, 2020) and insulin-like growth factor binding protein-2 (IGFBP2) (in Cluster C), regulating cell migration and invasion (Han *et al.*, 2014), could regulate cell migration.

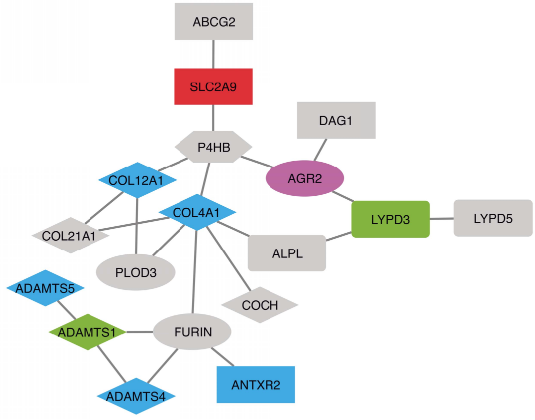

Figure 19. Molecular interactions of polar trophectodermal cells with endometrial epithelial and stromal cells. Cluster E

Blue: stromal proteins, red: epithelial proteins, grey: trophectodermal proteins, violet: epithelial and stromal proteins, orange: epithelial and trophectodermal proteins, green: stromal and trophectodermal proteins, yellow: epithelial, stromal and trophectodermal proteins. Ellipse: secreted proteins, diamond: extracellular matrix proteins, octagon: membrane-bound proteins, rectangle: transmembrane proteins. Modified from (Koel *et al.*, 2022).

The embryo-endometrium interactomics study provides valuable insights into the molecular mechanisms underlying endometrial receptivity by identifying and characterizing key molecules involved in the early stages of implantation, including embryo apposition, attachment, and initiation of invasion events. However, this study has limitations, such as using a static snapshot analysis for a dynamic process and restricting prediction analysis to known database interactions. Additionally, some critical interactions were not analyzed due to ethical considerations, such as the effects of seminal plasma on endometrial gene expression, immune cell effects, and secreted extracellular vesicles during natural conception *in vivo*. Nonetheless, using non-cultured and sorted cells has helped bypass some of the limitations of the gene expression profiling (Krjutškov *et al.*, 2016; Suhorutshenko *et al.*, 2018), providing more interpretable results. As a result, identifying putative molecular networks between the polar trophectodermal cells of the implantation-competent blastocyst and the receptive endometrium's epithelial and stromal cells is a valuable resource for future reproductive research, enabling the exploration of unknown mechanisms of implantation.

4.4. Optimizing BMP4-mediated differentiation of human embryonic stem cells into trophoblast-like cells (Ref IV)

During embryo implantation, the differentiation of trophectodermal cells into villous cyto- and syncytiotrophoblasts is critical for the maturing blastocyst's anchoring. Bone morphogenetic protein 4 (BMP4) is a key molecule in trophoblast cell differentiation from human embryonic stem cells. Interestingly, BMP4 is significantly upregulated in the receptive phase endometrium stromal cells (**Ref III**). However, it has been shown that this cellular differentiation also requires co-regulation with inhibiting fibroblast growth factor 2 (FGF2) and transforming growth factor beta (TGF-β)/activin/nodal signaling pathways (Amita *et al.*, 2013). The debate on the role of TGF-β and FGF2 pathway inhibition in the BMP4-triggered hESC differentiation (Roberts *et al.*, 2014) aims to investigate how the inhibitors of these pathways change the gene expression compared to primary BMP4 treatment during the first 12 days of differentiation (Figure 9).

4.4.1. The hESC line H9 differentiation under four different conditions of BMP4 induction

To determine the direction of hESC differentiation, we investigated the expression of known marker genes for hESC, primitive streak, mesoderm, endoderm, ectoderm, and trophoblast-like cells from a previous study by Lee (Lee *et al.*, 2016) using RNA-sequencing data and confirmed by RT-qPCR. Due to the low depth of RNA sequencing, some low-expressed marker genes were below the detection limit and not detected by RNA sequencing. In all conditions, the expression of hESC-specific genes such as *POU5F1*, *POLR3G*, *NANOG*, *DPPA4*, and *MYC* was down-regulated, indicating the loss of pluripotency. While RNA-sequencing did not detect other analyzed embryonic cell fate markers, slight up-regulation of the primitive streak and ectoderm markers (*T* and *NCAM*, respectively) were detected by RT-qPCR. However, *NCAM* up-regulation has also been associated with the differentiation of endovascular trophoblast cells (Blankenship and King, 1996), making it unsuitable for detecting a specific cell fate.

The pan-trophoblast marker cytokeratin 7 (KRT7) was studied to explore the hESC differentiation to trophoblast-like cells. The immunofluorescence (IF) analysis revealed that on day 5, cytokeratin 7 was detected in most cells with 'iFGF2 + BMP4' and BMP4 alone but only in a few cells with 'iFGF2 + iTGFb + BMP4' and 'iTGFb + BMP4' conditions. Two main trophoblast subtypes, extravillous (EVT)- and syncytiotrophoblast (ST) marker genes, were explored. EVT marker genes *HLA-G*, *CDH5*, *ITGA1*, and *ASCL2* were not detected, but syncytiotrophoblast marker genes *CGB5*, *CGA*, *MMP2*, and *MMP9* were expressed in all the other conditions except 'iTGFb + BMP4'. However, the late syncytiotrophoblast fusion marker *GCM1* (glial cells missing transcription factor 1) was expressed at a very low level, starting from day 8 of differentiation, indicating the incomplete differentiation of cytotrophoblast cells toward syncytiotrophoblasts. This was confirmed by detecting EVT cells, cytotrophoblast cells, or stem trophoblast cells (Cole, 2007) secreted molecule hyperglycosylated human chorionic gonadotropin (hCG-H) in the cell growth media from day 8 onwards only in conditions where FGF2 inhibitor was added (Figure 20).

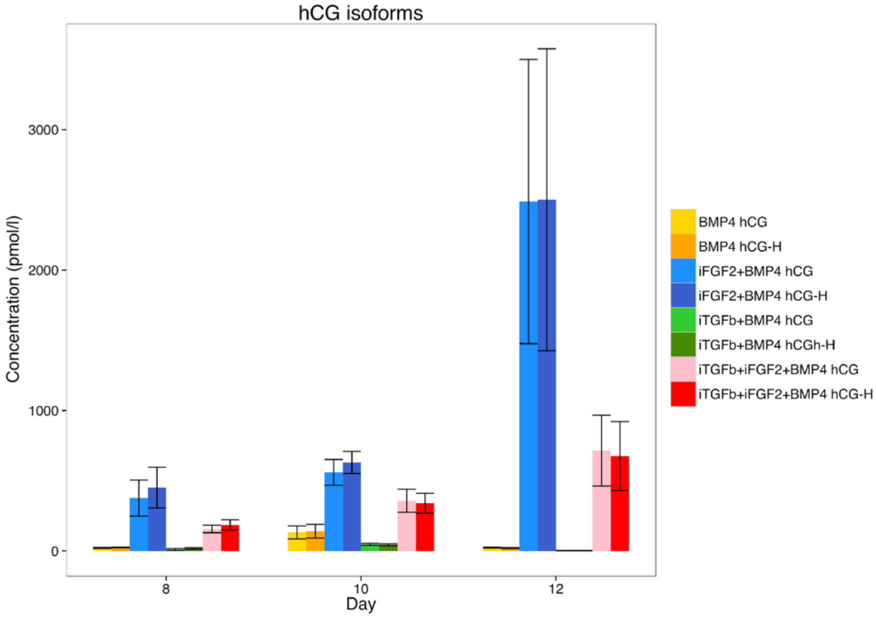

Figure 20. **ELISA measurement of total hCG and hyperglycosylated hCG (hCG-H) concentration in the cells' growth media**. Different treatment groups are shown with different colors. The error bars show the minimum and maximum values of the two replicates. Adapted from (Koel *et al.*, 2017).

4.4.2. TGF-β/activin/nodal inhibition has a general suppressive effect for BMP4-mediated hESC differentiation

Gene expression profiling revealed that the effects of TGF-β/activin/nodal inhibitors were not noticeable during the initial 6–8 days of differentiation, as no significantly differentially expressed genes were found when compared to BMP4 induction alone. A hierarchical clustering analysis was performed to study the impact of the inhibitors in more detail (Figure 21). The results showed four clusters: (i) day 0 hESC samples; (ii) days 1 and 2 in all conditions; (iii) days 4 and 6 in all conditions, and days 8, 10, and 12 with the TGF-β/activin/nodal inhibition condition; and (iv) day 8 and beyond in all conditions except the TGF-β/activin/nodal inhibition condition. These findings suggest that TGF-β/activin/nodal inhibition has a general suppressive effect after a week of treatment, as the transcriptome profile for days 8–12 with TGF-β/activin/nodal inhibition and BMP4 activation is like that of days 4–6 in the rest of the conditions. Furthermore, the differential expression analysis showed that the number of differentially expressed genes increased progressively for all conditions, except for 'BMP4' and 'iTGFb + BMP4,' where the growth rate decreased on day 8.

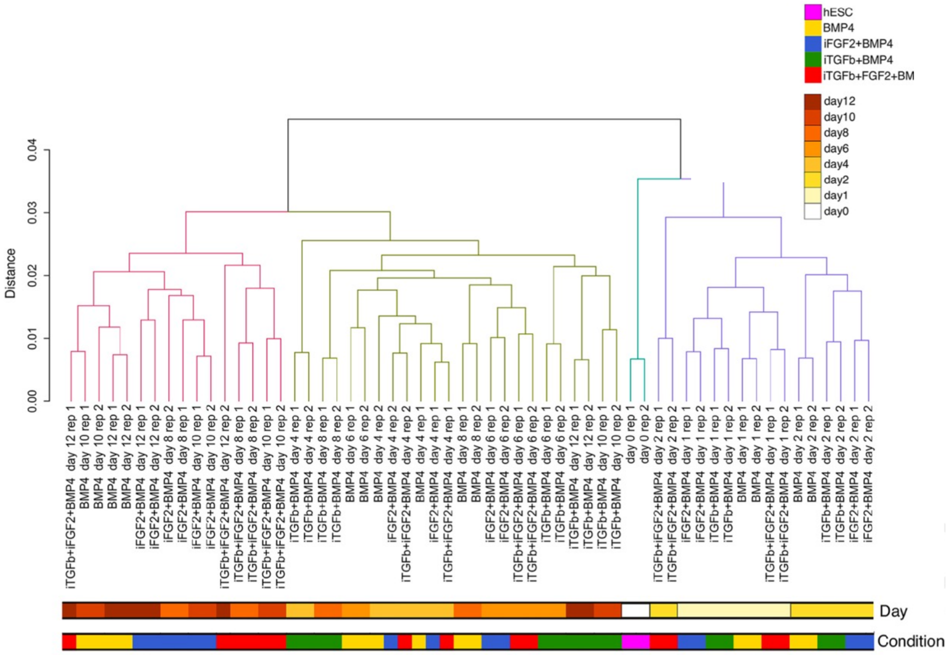

Figure 21: **Unsupervised hierarchical clustering of gene expression profiles for hESC line H9 grown under four different conditions of BMP4 induction with or without inhibitors**. Four formed clusters are marked with different colors: Blue: Day 0, hESC samples; violet: Days 1 and 2, all conditions; green: Days 4 and 6, all conditions, as well as days 8, 10, and 12 with the 'iTGFb + BMP4' condition; red: Day 8 and beyond, all conditions, except the 'iTGFb + BMP4' condition. The clustering of the samples is based on Pearson correlation and average linkage. Adapted from (Koel *et al.*, 2017).

4.4.3. FGF2 inhibition enhances BMP4-mediated trophoblast-like cell differentiation

To investigate the impact of FGF2 inhibition on trophoblast formation, the 'iFGF2 + BMP4' condition was compared to control samples treated only with BMP4. Differential gene expression was observed at the last two time points on days 10 and 12. Among the 45 up-regulated genes identified, 42 were present in the placental transcriptome listed in The Human Protein Atlas. However, the three undetected genes, such as calbindin 1 (*CALB1*), are likely associated with developing human trophoblasts rather than a fully formed placenta, as previously suggested (Cole, 2007). The up-regulated genes were found to be involved in pathways related to cellular focal adhesion, extracellular matrix (ECM)-receptor interactions, regulation of the actin cytoskeleton, tight junctions, and leukocyte transendothelial migration pathway, which have been previously reported to be important in the biology of trophoblasts during embryo implantation. Interestingly, these pathways were related to the downregulated genes in the 'iTGFb + BMP4' condition compared to BMP4 alone. The combination of both FGF2 and iTGFb inhibitors in the 'iFGF2 + iTGFb + BMP4' condition resulted in the regulation of pathways identified under both inhibitory conditions separately, indicating the effect of the two added inhibitors is likely, not additive. Furthermore, combining the two inhibitors led to fewer up-regulated genes specific to the placenta than FGF2 inhibition alone, suggesting that sole FGF2 inhibition with BMP4 activation is sufficient to improve syncytiotrophoblast formation. The study also revealed that when BMP4 activation was combined with FGF2 inhibition, several genes linked to cell cycle, DNA replication, and pyrimidine/purine metabolism were downregulated. This implies that FGF2 inhibition plays a role in regulating cell cycle arrest and cellular senescence, which are known to occur during syncytiotrophoblast formation (Chuprin *et al.*, 2013).

This study and approximately 40 other publications presented so far contribute to the BMP-mediated trophoblast cell differentiation model. Current consensus suggests that trophoblast-like cells, mesoderm, or endoderm can be generated from hESCs by adjusting the relative concentration of FGF2, ACTIVIN-A, and BMP4 in the culture medium. To promote trophoblast differentiation in this study, external FGF2 and Activin A were not added, and endogenously produced FGF2 or Activin-A was diminished by using inhibitors such as A83-01 and PD173074. The BAP (BMP4 + A83-01 + PD173074) medium used in this study drove hESCs towards trophoblast-like cells, albeit relatively slowly compared to other publications. hCG-H secretion was detected at lower levels and later stages than comparable studies, indicating a slower overall differentiation rate. The study also found that TGF-β inhibition was somewhat suppressive for trophoblast lineage formation rather than potentiating it. Notably, recent proposals suggest that some company BMP4 supplementation may be responsible for these effects, emphasizing the importance of evaluating the amount, timing, quality, and potency of BMP4 to optimize the trophoblast differentiation model (Roberts *et al.*, 2018). Hence, the data presented in this comprehensive study provides valuable information for modeling the trophoblast-like cell differentiation and, therefore, studying the early development of human trophoblasts in normal and complicated pregnancies.

CONCLUSIONS

This doctoral thesis aimed to address challenges emerging from the increasing demand for ART. To this end, it investigated the molecular preparation for embryo implantation, specifically the communication between the endometrium and trophoblast cells. Transcriptomic studies of the receptive endometrium were used to determine the endometrial receptivity meta-signature that can be used to evaluate the endometrial receptivity status. The study emphasized the roles of immune response-related signaling and exosome-based communication. A novel TAC-seq method was developed for the evaluation of the endometrial receptivity of endometrial tissue with high sensitivity, parallel processing capability, and affordability. Further research aimed to the identification of possible protein-protein interaction partners, some of which may play a significant part in interaction between the endometrial and trophoblast cells as well as highlighted the differential contribution of different endometrial cell types to the endometrial receptivity status and blastocyst implantation. The study also identified the optimal conditions for differentiating human embryonic stem cells into trophoblast-like cells.

In summary, the main outcomes of the thesis are as follows:

- Identification of an endometrial receptivity meta-signature list of 57 genes, with potential as valuable biomarkers for evaluating the endometrial receptivity.
- The development of a novel high throughput TAC-seq method for quantitative measuring biomarker levels with high sensitivity.
- The identification of specific molecules in different endometrial cell types that would enable the elucidation of the molecular mechanisms underlying endometrial receptivity and predict the relevant molecular pathways that are involved in initial implantation events.
- Identification of the optimal protocol for differentiation of human embryonic stem cells into trophoblast-like cells that involves the activation of BMP4 and inhibition of FGF2 signaling.

While the research presented here was conducted with the best knowledge available, there were also several limitations. Those included the focus on only two endometrial cell types, a static analysis of a dynamic implantation process, and the fact that *in vivo* cell-to-cell signaling and direct interactions were not investigated. However, the new knowledge acquired provides a valuable resource for future reproductive research, improves the understanding of the complex embryo implantation process in humans, and potentially leads to new prognostic and diagnostic biomarkers and therapeutic approaches for infertility.

SUMMARY IN ESTONIAN

Trofoblastide ja endomeetriumi rakkude molekulaarsed interaktsioonid embrüo implantatsioonil

Uue elu algus ja rasedus võivad esmapilgul tunduda üks loomulikumaid etappe naise elus, samas on see osutunud paljude paaride jaoks tõsiseks proovikiviks. Kuigi kehavälise viljastamise ehk IVF protseduuri abiga sünnib igal aastal üha enam lapsi ja meetodid arenemisvõimeliste embrüote tuvastamiseks on muutunud järjest paremaks, siis endiselt on kehavälise viljastamise edukus alla 35 aastastel naistel vaid ligikaudu 50% ja väheneb ema vanuse kasvades kiiresti. Selleks et mõista, miks vaatamata hea kvaliteediga IVF embrüote siirdamisele emakasse ei õnnestu paljudel naistel rasestuda, on vaja tunda molekulaarseid mehhanisme, mis reguleerivad embrüo kinnitumist emaka limaskestale ehk endomeetriumile implantatsiooni ehk pesastumise protsessis. Õigesti toimunud implantatsioon on nii raseduse tekkimiseks kui püsimiseks ülioluline. Raseduse edukaks lõpuni kandmiseks on samuti vajalik õigesti arenev platsenta mis võimaldab lootel normaalselt kasvada ja areneda. Toimiva platsenta moodustumiseks on määrava tähtsusega trofoblasti rakkude diferentseerumine erinevate ülesannetega rakkudeks ning paljude raseduse komplikatsioonide põhjustest aru saamiseks on vajalik seda protsessi sügavuti mõista. Paraku on mõistetavatel eetilistel põhjustel inimese embrüo pesastumist ja varaste trofoblasti rakkude kujunemist keeruline uurida naise organismis, mistõttu kasutatakse embrüo kinnitumise iseloomustamiseks ja trofoblasti diferentseerumise kirjeldamiseks erinevaid mudelsüsteeme.

Antud doktoritöö keskendubki molekulaarsele suhtlusele endomeetriumi ja embrüo trofoblasti rakkude vahel, et paremini mõista implantatsiooni molekulaarseid mehhanisme. Doktoritöösse kaasati autori poolt läbi viidud uurimustööd, mis kirjeldasid ja hindasid naise endomeetriumi koe küpsemist ja embrüo kinnitumiseks valmistumist, ning selle protsessi seost geenide avaldumise mustri muutustega endomeetriumi põhilistes rakutüüpides – strooma rakkudes ja epiteeli rakkudes. Kuigi mitmed varasemad teadusuuringud on kirjeldanud geenide avaldumise muutusi retseptiivses ehk embrüo kinnitumiseks valmisolevas endomeetriumis, on uuringute valimid olnud enamasti väikesed ja kasutatud metoodika varieeruv. Seetõttu võeti antud doktoritöös käsitletud artiklites kasutusele varem loodud bioinformaatiline meetod, mis võimaldas erinevate uuringute põhjal saadud geeninimekirju kombineerides leida unikaalne nimekiri 57 geenist, mille avaldumise muutus iseloomustab endomeetriumi retseptiivset faasi. Esmakordselt hinnati ka nende geenide avaldumise muutusi eraldi strooma ja epiteeli rakkudes. Paljud geenid, mille avaldumises esinesid muutused erinevate rakutüüpide vahel, kodeerivad valke mis mängivad olulist rolli immuunsuse regulatsioonis ja vesikulaarsel suhtlusel endomeetriumi ja implanteeruva embrüo vahel.

Kasutades retseptiivset endomeetriumit iseloomustavat geeninimekirja, töötati välja uus kulutõhus geenide aktiivsuse analüüsi meetod, mille abil määratakse endomeetriumi retseptiivsuseks oluliste geenide avaldumist, eesmärgiga leida viljatusravi saava naise jaoks embrüo siirdamiseks parim aeg. Loodud TAC-seq metoodika võimaldab oluliselt vähendada laboris tekkivat viga retseptiivsuse geenide avaldumise mõõtmisel ning seeläbi ajastada embrüo siirdamist täpsemini.

Kuigi endomeetriumi retseptiivsuse eest vastutavate geenide uurimine võimaldas hinnata üldist endomeetriumi koe valmisolekut embrüo kinnitumiseks, on hiljutised teadustööd näidanud, et selline lähenemisviis ei ole piisavalt täpne et kirjeldada implantatsioonil toimuvat embrüo ja emaka vahelelist valk-valk interaktsioonide abil toimuvat molekulaarset suhtlust. Sellest tulenevalt keskenduti rakutüübi-põhistele geenide avaldumise andmetele emaka limaskesta epiteeli ja strooma rakkudest, et tuvastada valke mille ennustuslik seondumine trofoblasti rakkude pinnavalkudele vahendab embrüo implantatsiooni. Teadustöö tulemusena kirjeldati ligikaudu 550 valk-valk seost, mis moodustavad molekulaarse võrgustiku embrüo ja emaka limaskesta rakkude vahel ning on vajalikud uue elu alguseks.

Mõistmaks platsentat moodustavate trofoblasti rakkude diferentseerumist uuriti käesolevas doktoritöös ka meetodeid inimese embrüonaalsete tüvirakkude diferentseerimiseks trofoblasti-laadseteks rakkudeks. See osa tööst aitas hinnata TGBβ ja FGF2 inhibiitorite rolli trofoblasti-laadsete rakkude diferentseerumisel BMP4 aktivatsiooni tingimustes ning seeläbi optimeerida seda tüüpi uurimistöös kasutatavat rakukultuuri keskkonda.

Käesolev doktoritöö uuris ühte huvitavaimat rakkudevahelist seost ja samal ajal ühte komplitseeritumat süsteemi – naise ja areneva embrüo vahelist kontakti. Seetõttu tuli uuringutes paratamatult kasutada erinevaid mudeleid ja lihtsustusi, et kirjeldada seda äärmiselt dünaamilist ja mitmetahulist protsessi fikseeritud ajahetkel ja kindlate rakutüüpide vahel. Sellest hoolimata on saadud teadmised oluliseks panuseks tulevastesse reproduktiivuuringutesse ning loovad uusi võimalusi embrüo edukaks siirdamiseks. Lisaks on doktoritöö tulemused heaks aluseks uute prognostiliste ja diagnostiliste biomarkerite välja arendamiseks, mida saab tulevikus kasutada viljatuse diagnostikas ning ravi optimeerimises.

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Suhorutshenko, M., Kukushkina, V., Velthut-Meikas, A., Altmäe, S., Peters, M., Mägi, R., Krjutškov, K., **Koel, M.**, Codoñer, F.M., Martinez-Blanch, J.F., Vilella, F., Simón, C., Salumets, A., Laisk, T., 2018. Endometrial receptivity revisited: endometrial transcriptome adjusted for tissue cellular heterogeneity. Hum. Reprod. Oxf. Engl. 33, 2074–2086. https://doi.org/10.1093/humrep/dey301

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