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HUMAN ENDOMETRIAL RECEPTIVITY AND EMBRYO-ENDOMETRIUM INTERACTIONS

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ABSTRACT

Infertility is an increasing medical and social problem affecting more than 10% of couples worldwide. Many underlying causes of human infertility have been overcome by assisted reproductive techniques; nevertheless, the implantation process remains one of the rate-limiting step as regards the success of the treatment. A prerequisite for successful implantation is the adequate preparation of a receptive endometrium and the establishment and maintenance of a viable embryo. The success of implantation further relies upon a two-way dialogue between the embryo and the endometrium. The molecular bases of these preimplantation and implantation processes in humans are not well known.

The general aim of the current thesis was to add more understanding into the complex mechanism of human embryo implantation; to identify different factors that play role in endometrial and embryo preparation for successful implantation.

In our first approach of identifying **factors important for endometrial maturation to a receptive phase**, we applied single gene analysis and genome expression analysis to fertile women and women with unexplained infertility. In the endometria of fertile women we identify previously known and new genes and pathways expressed in receptive endometrium, and that several of these genes and pathways were dysregulated in the endometria of women with no explainable reason for their fertility complications. These pathways included LIF pathway and JAK-STAT signalling cascade, coagulation cascade, inflammatory responses, lipid metabolism, and others. We also identified genetic variation in genes involved in blood coagulation to influence gene and protein expression levels in the endometrial cells, and their association with unexplained infertility was demonstrated. Further, we found pinopodes, the endometrial morphological markers, to be abundant in fertile endometria, but scarce in infertile endometria at the time of embryo implantation.

Our second study approach was to analyse **factors important for implantation-competent blastocyst development**. For that we analysed human embryos cultured *in vitro*. We found a major wave of transcriptional down-regulation in preimplantation embryos, where one possible down-regulation mechanism could operate via microRNA molecules.

Finally, we aimed to identify **interactions between receptive endometrium and blastocyst-stage embryo**. For that we applied a novel network profiling algorithm HyperModules, which combines topological module identification and functional enrichment analysis. The main curated embryo-endometrium interaction network highlighted the importance of cell adhesion molecules in the implantation process. Also cytokine-cytokine receptor interactions were identified, where osteopontin, LIF and LEP pathways were intertwining. We also identified several novel players in human embryo-endometrium interactions at the time of implantation.

The current thesis gives new insights into the processes involved in successful implantation in humans. Increasing our knowledge in the processes involved in preimplantation and implantation will facilitate the development of strategies to manipulate endometrial function, embryo development, and embryo-endometrium dialogue in order to promote successful implantation or to inhibit infertility.

LIST OF PUBLICATIONS

This thesis is based on the following articles and a manuscript, which will be referred to in the text by Roman numbers.

I. Aghajanova L, **Altmäe S**, Bjuresten K, Hovatta O, Landgren B-M, Stavreus-Evers A. Disturbances in the LIF pathway in the endometrium among women with unexplained infertility. *Fertil Steril. 2009 Jun; 91(6):2602-10.*

II. **Altmäe S**, Salumets A, Bjuresten K, Kallak TK, Wanggren K, Landgren BM, Hovatta O, Stavreus-Evers A. Tissue factor (TF) and tissue factor pathway inhibitors TFPI and TFPI2 in human secretory endometrium – possible link to female infertility. *Reprod Sci. 2011 In press.*

III. **Altmäe S**, Kallak TK, Fridén B, Stavreus-Evers A. Variation in hyaluronan binding protein 2 (HABP2) promoter region is associated with unexplained female infertility. *Reprod Sci. 2011 May;18(5):485-92.*

IV. **Altmäe S**, Martínez-Conejero JA, Salumets A, Simón C, Horcajadas JA, Stavreus-Evers A. Endometrial gene expression analysis at the time of embryo implantation in women with unexplained infertility. *Mol Hum Reprod. 2010 Mar;16(3):178-87*

V. **Altmäe S**, Reimand J, Hovatta O, Zhang P, Kere J, Laisk T, Saare M, Peters M, Vilo J, Stavreus-Evers A and Salumets A. Human embryo-endometrium interactions at the time of implantation. *Submitted.*

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LIST OF ABBREVIATIONS

ADRB	Beta adrenergic receptor
ALCAM	Activated leukocyte cell adhesion molecule
APOD	Apolipoprotein D
BGN	Biglycan
CCR7	Chemokines receptor 7
CD36	CD36 molecule
CD44	CD44 molecule
CDH1	E-cadherin
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
CLDN4	Claudin 4
CMTM5	CKLF-like MARVEL transmembrane domain containing 5
COL1A1	Collagen, type I, alpha 1
COL16A1	Collagen, type XVI, alpha 1
COL4A1	Collagen, type IV, alpha 1
COL4A2	Collagen, type IV, alpha 2
COL4A5	Collagen, type IV, alpha 5
COL4A6	Collagen, type IV, alpha 6
COL7A1	Collagen, type VII, alpha 1
COLEC12	Collectin sub-family member 12
CP	Ceruloplasmin
CTSA	Cathepsin A
CTSB	Cathepsin B
CTSD	Cathepsin D
CTSE	Cathepsin E
CTSH	Cathepsin H
CTSL1	Cathepsin L1
CTSZ	Cathepsin Z
CXCL6	Chemokines ligand 6
CXXC4	CXXC finger protein 4
DAVID	Database for Annotation, Visualisation and Integrated Discovery
DKK1	Dickkopf homolog 1
DNA	Desoxyribonucleic acid
EDN1	Endothelin 1
ESHRE	European Society of Human Reproduction and Embryology

ERKs	Mitogen-activated protein kinases
F3	Coagulation factor III
FAM3B	Family with sequence similarity 3, member B
FAM3D	Family with sequence similarity 3, member D
FBLN1	Fibulin 1
FBLN2	Fibulin 2
FBN1	Fibrillin 1
FGF7	Fibroblast growth factor 7
FSAP	Factor VII-activating protease
FSH	Follicle stimulating hormone
FVII	Factor VII
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAST	Gastrin
GE	Glandular epithelium
GEO	Gene Expression Omnibus
GnRH	Gonadotrophin releasing hormone
GO	Gene ontology
Gp130	Glycoprotein 130
HABP2	Hyaluronan-binding protein 2
HAPLN1	Hyaluronan and proteoglycan link protein 1
HBA	Hemoglobin, alpha
HB-EGF	Heparin-binding EGF-like growth factor
hCG	Human chorionic gonadotrophin
HMMR	Hyaluronan-mediated motility receptor (RHAMM)
HPRD	Human protein reference database
IGF	Insulin-like growth factor
IGF2BP1	Insulin-like growth factor 2 mRNA binding protein 1
IGF2BP3	Insulin-like growth factor 2 mRNA binding protein 3
IGFBP1	Insulin-like growth factor binding protein 1
IHC	Immunohistochemistry
IL	Interleukin
IL6ST	Interleukin 6 signal transducer
IPA	Ingenuity pathway analysis
ITGA1	Integrin, alpha 1
ITGA8	Integrin, alpha 8
ITGA9	Integrin, alpha 9

ITGAE	Integrin, alpha E
ITGAV	Integrin, alpha V
ITGB1	Integrin, beta 1
ITGB8	Integrin, beta 8
IVF	*In vitro* fertilisation
JAK-STAT	Janus kinase/signal transducer and activator of transcription
KDR	Kinase insert domain receptor
KREMEN1	Kringle containing transmembrane protein 1
LAMA1	Laminin, alpha 1
LAMA2	Laminin, alpha 2
LAMA5	Laminin, alpha 5
LAMB3	Laminin, beta 3
LAMC1	Laminin, gamma 1
LAMC2	Laminin, gamma 2
LE	Luminal epithelium
LEP	Leptin
LGALS1	Lectin, galactoside-binding, soluble 1
LH	Luteinising hormone
LIF	Leukaemia inhibitory factor
LIFR	Leukaemia inhibitory factor receptor
MAPK	Mitogen-activated protein kinase
MCAM	Melanoma cell adhesion molecule
miRNA	MicroRNA
MME	Membrane metallo-endopeptidase
MMP	Metalloproteinase
mRNA	Messenger ribonucleic acid
MUC	Mucin
NRP1	Neuropilin 1
OCLN	Occludin
OR	Odds ratio
PAEP	Progestagen-associated endometrial protein
PAP-2	Protease-activated receptor 2
PECAM1	Platelet/endothelial cell adhesion molecule
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PDGFA	Platelet-derived growth factor alpha polypeptide

PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide
PGF	Placental growth factor
POF	Premature ovarian failure
PRKCG	Protein kinase C, gamma
PSG1	Pregnancy specific beta-1-glycoprotein 1
PSG10	Pregnancy specific beta-1-glycoprotein 10
PSG2	Pregnancy specific beta-1-glycoprotein 2
PSG4	Pregnancy specific beta-1-glycoprotein 4
PSG7	Pregnancy specific beta-1-glycoprotein 7
PTGER3	Prostaglandin E receptor 3
PTGES	Prostaglandin E synthase
PTGES2	Prostaglandin E synthase 2
PTGR1	Prostaglandin reductase 1
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
S100A8	S100 calcium binding protein A8
SCGB3A1	Secretoglobin, family 3A, member 1
SDC2	Syndecan 2
SEM	Scanning electron microscopy
SERPINA3	Serpin peptidase inhibitor, clade A, member 3
SNP	Single nucleotide polymorphism
SOCS1	Suppressor of cytokine signalling 1
SPP1	Secreted phosphoprotein 1 (osteopontin)
TF	Tissue factor
TFF3	Trefoil factor 3
TFPI	Tissue factor pathway inhibitor 1
TFPI2	Tissue factor pathway inhibitor 2
TGFB1	Transforming growth factor, beta 1
TJP1	Tight junction protein 1
TNF	Tumor necrosis factor
uNK	Uterine natural killer cell
VCAN	Versican
VEGFA	Vascular endothelial growth factor A
WHO	World health organisation
WISP2	WNT1 inducible signalling pathway protein 2
WNT3A	Wingless-type MMTV integration site family, member 3A

1 INTRODUCTION

More than 10 per cent of couples worldwide are involuntary childless due to infertility. Infertility is not only a medical problem, but has also often social and psychological implications. In Europe, low birth rate is one of the distinctive population features in most countries, and with delayed childbearing the need for infertility treatment is increasing. Although many underlying causes of human infertility have been overcome by assisted reproductive techniques, the implantation process remains rate-limiting step with regards to the success of treatment. A prerequisite for successful implantation is an adequate preparation of receptive endometrium and normally developed viable embryo. The success of implantation further relies upon a two-way communication between the embryo and the uterus. In order to increase success rates, there is, therefore, a continuing need to understand the molecular basis of the preimplantation and implantation processes. Many studies have been performed to improve our understanding of these mechanisms, but the majority of the knowledge regarding human embryo development and implantation is derived from animal models. Studies on human endometrium provide useful information on endometrial preparation for implantation. Nevertheless, there is still a lack of diagnostic and therapeutic tools for implantation dysfunction, and an optimal marker for defining a state of endometrial receptivity is needed.

1.1 FERTILITY AND INFERTILITY

Fertility is the natural capability to give life. Fertility can be measured by the time taken to achieve pregnancy. Time-to-pregnancy is expressed in monthly fecundity rate, which in humans, compared to other mammalian species, is relatively low ~20% (Evers, 2002). It has been estimated that 79% of the population is fertile, 18% subfertile or infertile and 3% superfertile (Evers, 2002). In addition to subfertility, the incidence of embryo wastage and pregnancy loss is relatively high in humans, estimated to be 30% prior to implantation, a further 30% of early pregnancy loss, and over 10% of clinical pregnancies (Blohm, et al., 2008; Macklon, et al., 2002).

In humans, the prevalence of infertility is high, affecting over 10% of couples of fertile age (Boivin, et al., 2007). Infertility is defined as inability of a couple to become pregnant within a year without using any contraceptives (Workshop, 2002). It is estimated that over 72 million women worldwide, aged 20 – 44, are currently infertile, however, only every second couple seeks for infertility medical care (Boivin, et al., 2007). The reason of infertility in a couple could be caused by female (over one-third) or by male factor (over one-third) or by a combination of problems in both partners, or is unexplained (approximately 20%) (1996). Female fertility is regulated by a complex coordination and synchronization of interactions in the hypothalamic-pituitary-ovarian axis. Female fertility can thus be influenced by different diseases or dysfunctions of reproductive tract, neuroendocrine system, and immune system or by any general disease. Major causes of female infertility are disorders in ovulation (most commonly polycystic ovary syndrome, PCOS), tubal factor infertility, endometriosis and

unexplained infertility (Smith, et al., 2003). The most prevalent causes of female infertility according to the diagnostic and treatment guidelines by The ESHRE Capri Workshop (1996; Workshop, 2002) are summarised in Table 1. In a case of male factor infertility, it is generally defined by the finding of an abnormal semen analysis (WHO, 1999).

Table 1. *Aetiology of female infertility.*

Anovulatory infertility	
	Premature ovarian failure (POF) and early menopause
	Polycystic ovary syndrome (PCOS)
Tubo-peritoneal infertility	
	Tubal factor infertility
	Endometriosis
Autoimmunity	
	POF
	Recurrent pregnancy loss
	Autoimmunity associated with infertility
Uterine abnormalities	
	Malformations
	Myomas
Unexplained infertility	

1.2 UNEXPLAINED INFERTILITY

Unexplained infertility is one of the most common diagnoses in a fertility clinic (Adamson and Baker, 2003; Brandes, et al., 2010; Hull, et al., 1985). The estimated incidence of the diagnosis ranges in different studies from 15% (Guzick, et al., 1994) to 30% (Smith, et al., 2003; Templeton and Penney, 1982). The classification as unexplained infertility is applied to an infertile couple whose standard investigations include semen analysis, tests of ovulation and tubal patency have failed to detect any gross abnormalities (Crosignani, et al., 1993). Its aetiology seems heterogeneous, with suggested potential causes ranging from disturbances in endocrinology, immunology, genetics and reproductive physiology (Pellicer, et al., 1998). Unexplained infertility could arise from a defect in fertility that cannot be detected by the routine infertility evaluation, or it could represent the lower extreme of normal distribution of fertility, 70% of these couples achieve pregnancy in 2 years, while 20 – 30% remain infertile even after 9 years (Barnea, et al., 1985; Templeton and Penney, 1982).

Women in couples with infertility of unknown cause have normal ovulatory cycles and hormonal profiles, no organ pathology, and their partners show no evidence of semen quality problems. However, in some cases, these women might be misdiagnosed as unexplained infertile, as thorough evaluation for pelvic pathologies with laparoscopy is not a standard approach in all clinics (Moayeri, et al., 2009). With further evaluation, women misdiagnosed as unexplained infertile may suffer from endometriosis, tubal infertility, premature ovarian aging or immunological infertility (Gleicher and Barad, 2006). It is estimated that older women are more likely to be diagnosed with unexplained infertility, this is due to the negative effect of age on the ovarian reserve (Gleicher and Barad, 2006; Maheshwari, et al., 2008).

Nevertheless, one reason for fertility problems in these women could be abnormalities in endometrial receptivity. Embryos cannot implant in the uterus with defects in the endometrium. Several microarray studies have revealed dysregulation of genes in endometria of women with fertility complications, which may influence endometrial competence (Aghajanova, et al., 2010; Burney, et al., 2007; Horcajadas, et al., 2008; Koler, et al., 2009; Qiao, et al., 2008; Tapia, et al., 2008a). So far, assessment of the endometrium beyond its appearance on ultrasound examination is limited due to lack of clinically useful tests of receptivity. The development of novel tests of endometrial function are likely to reduce the proportion of couples with no clear cause of their infertility, as the contribution of aberrant endometrial maturation becomes clear (Stavreus-Evers, et al., 2011).

1.3 HUMAN ENDOMETRIUM

The endometrium is regarded as an endocrine organ, producing several hormones, growth factors and cytokines. The main function of the endometrium is to allow a timely implantation of a viable embryo, and to provide a nurturing local environment that supports establishment of a successful pregnancy. Endometrial functions include the ability to trigger its own destruction in the absence of pregnancy, and protection against invading pathogens (Johnson and Everitt, 2000).

The endometrium consists of a basal and a functional layer (Figure 1). The basal layer, adjacent to the myometrium, remains after menstruation and undergoes only limited changes during the menstrual cycle (Aplin, et al., 2008). This is the layer from which the endometrium regenerates after menstrual shedding. The other layer, the functional layer, is highly sensitive and responsive to oestrogen, progesterone and androgens and is subsequently discharged during menstruation (Tabibzadeh, 1998). This layer has a cycle of proliferation, secretion and degeneration. The purpose of the functional layer is to prepare the endometrium for embryo implantation.

The endometrium can be divided into two hormone responding tissue types: a single layer of columnar epithelium, and stromal connective tissue, that contains fibroblasts, endothelial cells and leukocytes (Aplin, et al., 2008). The types of epithelium in the endometrium are luminal epithelium (covers the endometrial surface) and glandular epithelium (lining of glands). Luminal epithelium provides the sites of implantation, as it is the first maternal surface for the trophoblast cells of the implanting embryo to encounter (Meseguer, et al., 2001). In addition, luminal epithelium acts as a blood-uterine lumen barrier, which prevents substances to enter the uterus from the blood (McRae and Kennedy, 1983). Glandular epithelial cells secrete several autocrine and paracrine factors required for endometrial maturation and embryo implantation. Fibroplasts are the dominant cell type of the stroma, and produce extracellular matrix, metalloproteinases (MMPs), and other proteins (Aplin, et al., 2008). Stromal endothelial cells locate in the wall of arteries and veins, and are essential for the formation of new vessels from the existing ones, i.e. angiogenesis. Leukocytes are part of the immune system, mediating inflammatory response. They vary in number and type throughout the menstrual cycle (Critchley, et al., 1999; Salamonsen and Lathbury,

2000) and include T and B cells, mast cells, natural killer cells (uNK), macrophages, and neutrophils (Jabbour, et al., 2006).

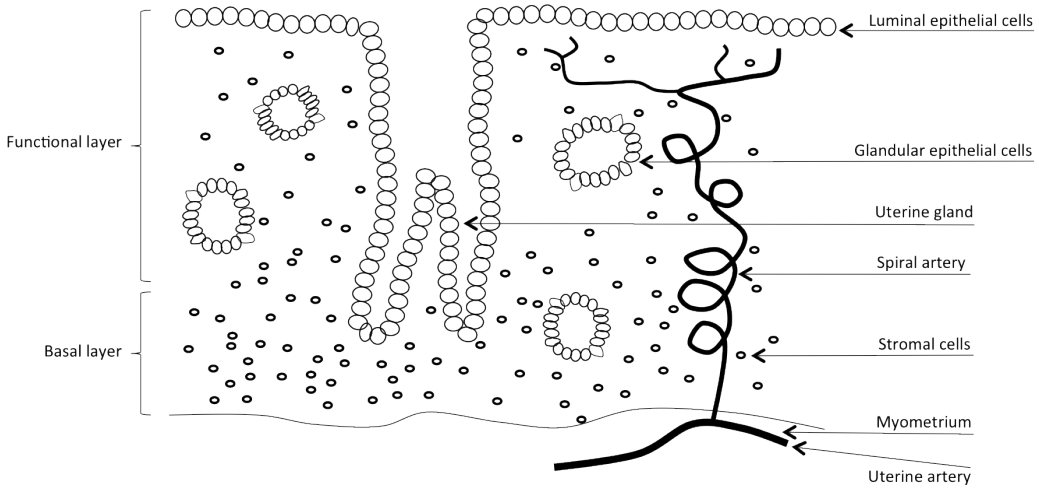

Figure 1. *Simplified schematic figure of human endometrium.*

1.4 HORMONAL REGULATION OF ENDOMETRIAL CYCLE

The menstrual cycle consists of a follicular phase (menstruation and proliferative phase) and a luteal (or secretory) phase, separated by ovulation (Figure 2). A normal cycle has approximately the same length in each time, from 25 to 35 days with an average of 28 days. The series of classic morphological changes in the endometrium occur in response to cyclical ovarian activity (Noyes, et al., 1975).

Ovarian function is under the control of luteinising hormone (LH) and follicle stimulating hormone (FSH), which bind to their receptors in the ovary and regulate its function by promoting sex steroid production and folliculogenesis (Hillier, et al., 1994). The hypothalamus secretes pulses of GnRH, which regulates the pituitary gland to produce gonadotrophins in a similar pulsatile pattern. When gonadotrophins act on the follicles, the production of oestrogens increases and reaches its maximal level in the preovulatory follicle. Estradiol is the main oestrogen synthesised and has dual action in gonadotrophin secretion – at low circulating levels it exerts negative feedback control over FSH and LH production by inhibiting GnRH secretion, meanwhile at high circulating levels positive feedback becomes a dominant force and LH and FSH surge is induced, followed by the ovulation. Ovarian follicular phase coincides with the endometrial proliferative phase (Figure 2). Growing levels of estradiol exert mitogenic effects on the endometrium, leading to cell division and growth of the endometrium and angiogenesis (Aplin, et al., 2008).

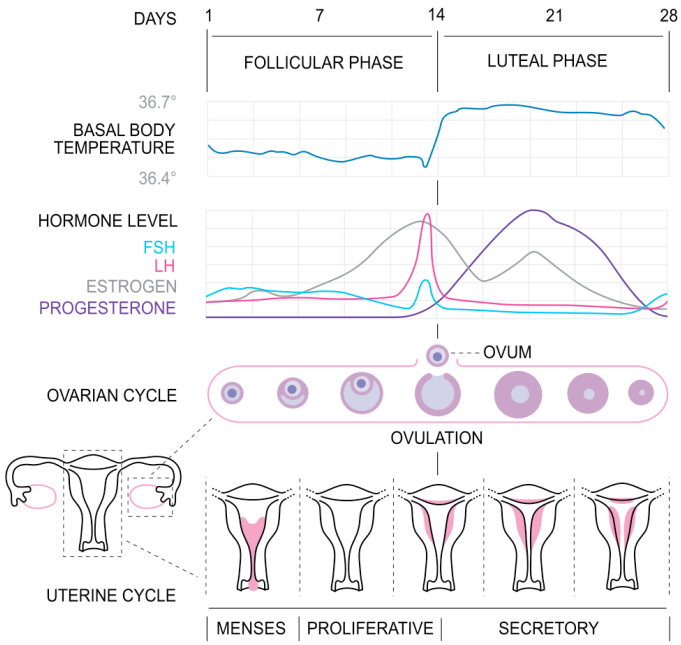

Figure 2. *The menstrual cycle. Cycle days, basal body temperature, hormone levels, ovarian cycle and uterine cycle are indicated (www.wikipedia.org)*.*

After ovulation, the oocyte moves along the Fallopian tube for potential fertilization and the dominant post-ovulatory follicle transforms into corpus luteum. LH promotes luteinisation of mature follicles and maintains progesterone production from the corpus luteum. High levels of progesterone, in the presence of oestrogen, form a negative feedback action that suppresses gonadotrophin secretion. This coincides with the start of the endometrial secretory phase (Figure 2). Progesterone is the main hormone that regulates the endometrial maturation for blastocyst implantation. The progesterone-dominated latter half of the menstrual cycle is constituted by an early, mid-, and late secretory phase. Implantation takes place during the mid-secretory phase. The pattern of sex steroid receptor expression in the secretory endometrium reflects the fact that the early secretory phase is regulated by both oestrogen and progesterone; the mid-secretory phase is regulated by progesterone alone; and the late secretory phase is associated with progesterone withdrawal and, consequently, menstruation (Snijders, et al., 1992). The histological features characteristic for the receptive endometrium are increased glandular volume, secretion, edema, coiling of spiral arteries and decidualisation of the stroma (Jabbour, et al., 2006). In the absence of pregnancy, the corpus luteum degenerates, resulting in decrease of circulating steroids, that lead to enhanced secretion of FSH and the initiation of a new cycle (Speroff and Fritz, 2005).

1.5 ENDOMETRIAL RECEPTIVITY

Endometrial receptivity refers to the time when luminal epithelium is favourable for blastocyst implantation. This limited time period, called also as implantation window, is restricted to approximately days 19 to 24 of a menstrual cycle of 28 days (Harper, 1992; Lopata, 1996). This corresponds to the time when the embryo hatches, day 6 after the LH surge, and it is subsequently ready for implantation within the following 24 hours (Aplin, et al., 2008).

The development of a receptive endometrium depends on adequate secretory transformation of the oestrogen-primed endometrium in response to progesterone. Significant developmental changes occur in luminal epithelium, glandular epithelium as well as in endometrial stroma. Many molecules have been identified in the involvement of this process, such as integrins and their ligands (e.g. osteopontin), mucins, growth factors (HB-EGF), cytokines (LIF, leptin, IL-1, IL-11), homeobox transcription factors (HOXA gene products), lipids and other molecules (Aghajanova, et al., 2008b; Giudice, 1999; Stavreus-Evers, et al., 2011; Wang and Dey, 2006).
The availability of new methods for investigating human endometrium has provided deeper knowledge in the molecular regulation of endometrial receptivity. Gene expression microarrays allow studies on the expression levels of thousands of genes simultaneously. In recent years, the global gene expression analysis has been successfully applied in several endometrium transcriptome studies and distinct regulation of hundred of genes in different menstrual cycle phases has been demonstrated (Borthwick, et al., 2003; Carson, et al., 2002; Feroze-Zaidi, et al., 2007; Haouzi, et al., 2009a; Haouzi, et al., 2009b; Horcajadas, et al., 2004; Kao, et al., 2002; Mirkin, et al., 2005; Riesewijk, et al., 2003; Talbi, et al., 2006). Although each study has revealed many candidate genes for endometrial receptivity, the number of common genes distinguished is relatively limited (Horcajadas, et al., 2007).

Opening of the implantation window is characterised by remarkable ultrastructural changes in endometrial epithelial cell morphology (Nikas, 1999). In several studies the time-point of implantation is coincided with the presence of endometrial pinopodes (Martel, et al., 1991). Pinopodes are cytoplasmic protrusions of the endometrial surface, arising from the apical surface of the epithelial cells and extending into the uterine cavity. Although some groups have questioned the correlation between pinopodes and endometrial receptivity (Quinn and Casper, 2009), the timed correlation of pinopode expression, period of blastocyst hatching, and preference of human blastocyst to attach to pinopodes suggest pinopodes as structural markers of receptive endometrium (Achache and Revel, 2006; Aghajanova, et al., 2008a; Bentin-Ley, et al., 1999). Furthermore, pinopode formation and maintenance is shown to be hormone dependent, where progesterone is crucial for their appearance, and oestrogen interferes with the formation or induces regression (Martel, et al., 1991; Stavreus-Evers, et al., 2001). Further, the co-expression of pinopodes and other markers of endometrial receptivity has been demonstrated, such as with integrin $\alpha_v\beta_3$ (Nardo, et al., 2003b), osteopontin (Lessey, 2003), glycodelin (Stavreus-Evers, et al., 2006), progesterone receptors (Stavreus-Evers, et al., 2001).

The relative lack of clinical investigation of the endometrium is partly from the lack of objective tools for examining endometrial receptivity in a clinical setting. As a result, most current therapeutic interventions aimed to modulate endometrial receptivity are empirical, with little evidence base to support their use (Stavreus-Evers, et al., 2011). Nevertheless, recent developments in understanding the molecular regulation of endometrial receptivity are offering novel insights into the role of the endometrium in determining whether or not implantation could be successful. Indeed, studies comparing endometrium at the pre-receptive and receptive phases in fertile women and women with poor reproductive success demonstrate that implantation failure is, at least in part, due to a failure of the endometrium to differentiate into a receptive state (Sharkey and Smith, 2003).

1.5.1 LIF signalling pathway

Numerous paracrine factors, mainly cytokines and growth factors are involved in the regulation of the endometrium towards the receptive phase. Leukaemia inhibitory factor (LIF) is the most intensively studied factor regarding uterine receptivity. LIF is a pleiotropic cytokine, which crucial role in successful implantation was established in mice (Stewart, et al., 1992). Animals lacking *LIF* gene produced normal blastocysts failed to implant in the LIF-deficient uterus, but were capable of implanting in a wild-type uterus (Stewart, et al., 1992). Administration of LIF in LIF-deficient animals restored implantation in these mice (Chen, et al., 2000; Stewart, et al., 1992). There is evidence that LIF is involved in implantation in several other species, such as rhesus monkey and sheep (Vogiagis, et al., 1997; Yue, et al., 2000). However, the importance of LIF in human implantation is still unclear.

LIF involvement in human endometrial receptivity has been studied by several groups (reviewed by Aghajanova *et al.* (Aghajanova, 2010)). LIF is expressed in the human endometrium in a menstrual cycle-dependent manner, maximal LIF secretion coincides with the window of implantation (Arici, et al., 1995; Chen, et al., 1995). Although *LIF* expression is an indicator of receptive endometrium, its role in the assessment of implantation potential in humans is controversial (Aghajanova, 2010), and use of recombinant human LIF has failed to improve the implantation and pregnancy outcomes after assisted reproductive techniques in women with recurrent implantation failure (Brinsden, et al., 2009).

LIF acts through binding to its receptor LIFR, which forms a heterodimer with a specific subunit common to all members of that family, the gp130 signal transducing subunit. This leads to activation of the JAK-STAT (Janus kinase-signal transcuder and activator of transcription), MAPK (mitogen activated protein kinase) or P13/AKT (phosphatidylinositol-3-kinase/Akt) pathways (Figure 3) (Carino, et al., 2008; Cheng, et al., 2001; Heinrich, et al., 2003). LIF signalling in the uterus occurs mainly through the JAK-STAT pathway (Cheng, et al., 2001). Activation of JAKs causes phosphorylation of a family of transcription factors STATs (signal transducers and activators of transcription) (Schindler and Darnell, 1995). Negative regulation of the LIF pathway is exerted via the suppressors of cytokine signalling (SOCS), which

negatively regulates JAK-STAT signalling cascade by interacting with JAKs (Paiva, et al., 2009).

LIFR and *gp130* (*IL6ST*) knock-out mice, contrary to LIF-deficient mice, are fertile and do not have implantation problems, indicating that LIF can act through an alternative pathway (Dani, et al., 1998). Nevertheless, LIFR-deficient mice display different abnormalities, which culminate in early perinatal lethality (Ware, et al., 1995), and *gp130* knock-out mice have impaired heart development and die before birth (Yoshida, et al., 1996). Human preimplantation embryos express LIF and both of its receptors (Wanggren, et al., 2007). Gp130 is present in human luminal and glandular epithelium throughout the menstrual cycle (Cullinan, et al., 1996). This suggests that LIF signalling pathway is necessary for a cross-talk with the implanting embryo, mediating paracrine signals to embryonic tissue and autocrine/paracrine signals in the endometrial tissue (Aghajanova, 2010). Indeed, it has been shown that LIF plays a role in both adhesive and invasive phases of human embryo implantation due to its anchoring effect on trophoblast and regulation of trophoblast differentiation (Dimitriadis, et al., 2010; Tapia, et al., 2008b).

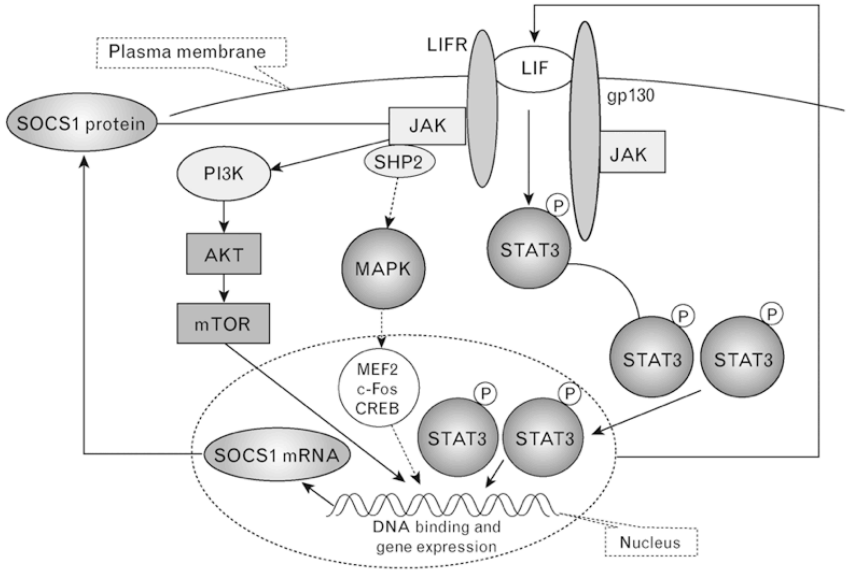

Figure 3. *Leukaemia inhibitory factor (LIF) signalling pathway. LIF receptors LIFR (leukaemia inhibitory factor receptor) and gp130 (glycoprotein 130), JAK-STAT (janus kinase/signal transducer and activator of transcription) and MAPK (mitogen-activated protein kinase) cascades, and LIF signalling pathway inhibitor SOCS1 (suppressor of cytokine signalling 1) are indicated (Aghajanova, 2010).* *Published with permission from Wolters Kluwer Health.*

1.5.2 Vascular regulation

Angiogenesis is an essential component of endometrial regeneration and maturation. It is a process involving growth of new blood vessels from pre-existing vessels. Angiogenesis occurs periodically at three distinct stages in human endometrium: during menstruation for repair of the vascular bed; at the proliferative phase during rapid endometrial growth; and during secretory stage when spiral arterioles exhibit growth and coiling (Gargett, et al., 1999). Additionally, vascular development is important during implantation and early placentation (Torry, et al., 2007). At the time of implantation, adequate uterine vascularity is needed to provide a richly vascularised endometrium, and following the implantation, development and expansion of placental villous vasculature is needed to facilitate transport of nutrients and oxygen to the embryo (Torry, et al., 2007). It has been demonstrated in mice that inhibition of angiogenesis either before, or shortly after implantation interrupts placentation and results in resorption of all embryos (Klauber, et al., 1997), thus highlighting the critical role of angiogenesis in normal implantation and placentation. Further, derangements in endometrial haemostasis are believed to represent a potential source of implantation failure and subsequent pregnancy complications in humans (Azem, et al., 2004; Coulam, et al., 2006; Lockwood, et al., 1994). During the mid-secretory phase, when endometrial stromal cells decidualise around blood vessels, these cells are temporally and spatially positioned to create a local haemostatic milieu. This can counteract the threat of haemorrhage during the implantation process, as blastocyst-derived trophoblasts penetrate the spiral arteries to establish the primordial utero-placental circulation (Lockwood, et al., 1999).

A wide range of soluble factors, some with well established angiogenic functions (e.g. VEGF, PIGF, FGF, TGFβ) (Torry, et al., 2007) as well as other factors, can contribute to vascular development and maintenance at the maternal-foetal interface.
Hyaluronan-binding protein 2 (HABP2), also known as factor VII-activating protease (FSAP), is an extracellular serine protease, which has been shown to inhibit angiogenesis (Jeon, et al., 2006). HABP2 has also been shown to promote the coagulation cascade by acting as an activator of factor VII, independently of tissue factor, and in the fibrinolytic pathway activating pro-urokinase (Roemisch, et al., 2002; Romisch, et al., 1999) (Figure 4). Moreover, HABP2 seems to be involved in additional vascular regulation, since it has been shown to regulate migration of vascular smooth muscle cells (Kannemeier, et al., 2004), and vascular integrity (Mambetsariev, et al., 2010). In the endometrium, *HABP2* expression was significantly lower in subfertile women (Horcajadas, et al., 2005; Riesewijk, et al., 2003) and in women with inert intrauterine device (Horcajadas, et al., 2006).

Tissue factor (TF), also known as coagulation factor III (F3), is the key initiator of the blood coagulation cascade. TF initiates haemostasis through binding to factor VII, while tissue factor pathway inhibitor (TFPI) inhibits the cascade (Figure 4). TF has been proposed to have a central role in the connection of haemostatic, angiogenic, and pro-inflammatory pathways (Krikun, et al., 2009), as TF acts as a signalling molecule that binds FVIIa and cleaves protease-activated receptor 2 (PAR-2), which plays a role in angiogenesis, inflammation, and tumor progression (Chen, et al., 2006; Chen and

Hogg, 2006; Wolberg, et al., 1999). Knock-down of *TF* gene resulted in embryo lethality in mice (Toomey, et al., 1996), while in the presence of low levels of TF, the *TF*-null embryos were rescued (Parry, et al., 1998). Endometrial *TF* is explicitly up-regulated at the time of implantation, and any alteration in the expression can lead to various pathologies of the endometrium including infertility (Kato, et al., 2005; Krikun, et al., 2009).

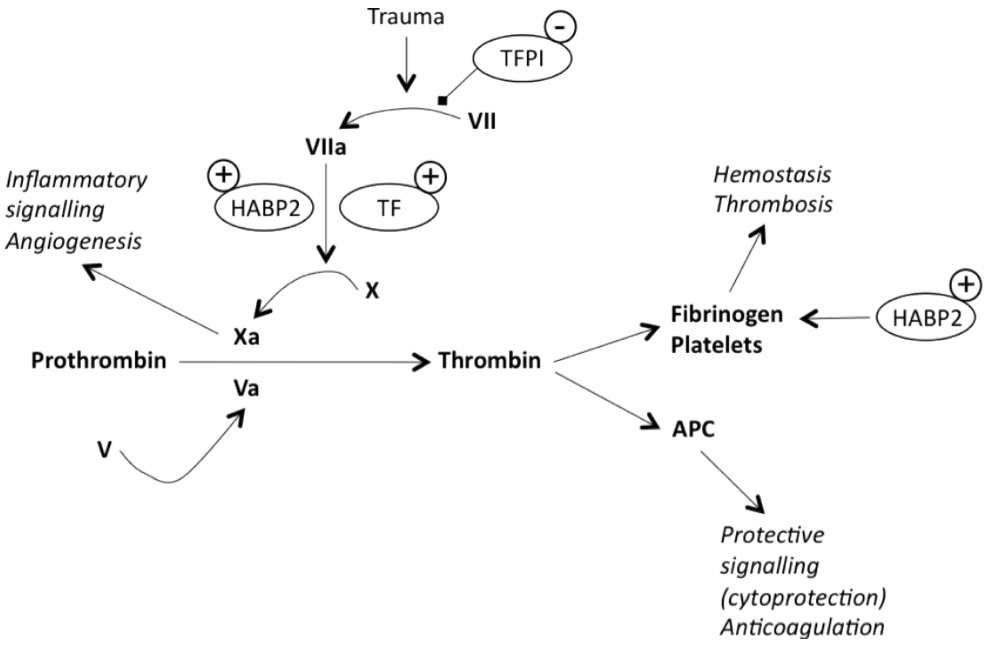

Figure 4. *Simplified schematic illustration of coagulation cascade and related signalling, where factors tissue factor (TF), tissue factor inhibitor (TFPI), hyaluronan-binding protein 2 (HABP2, also known as factor VII-activating protease) are indicated. Central proteins factors VII, X and V and their activated forms (a) are also shown. APC – activated protein C. '+' indicates activators and '–' refers to inhibitor.*

1.6 PREIMPLANTATION EMBRYO DEVELOPMENT

After fertilisation, the human preimplantation embryo develops during the transport through the Fallopian tube into the uterus, a journey taking four days. The Fallopian tube provides space and biological environment for fertilisation of the oocyte, which is then actively transported by cilia and muscle contractions towards the endometrium (Croxatto, 2002).

The embryo undergoes several changes including cleavage, embryonic genome activation, compaction, and cavitation during its development to form a blastocyst (Figure 5). At the 4-cell stage, the transcription starts to change from maternal to embryonic genome activation, and the maternal mRNAs are gradually degraded (Duranthon, et al., 2008). Maternal to embryonic gene activation shows two principal

transient waves of *de novo* transcription, where the first wave peaks between the 2- and 4-cell stages and the second wave peaks at the 8-cell stage and precedes morula-to-blastocyst formation, as shown in mice (Bell, et al., 2008; Hamatani, et al., 2004).

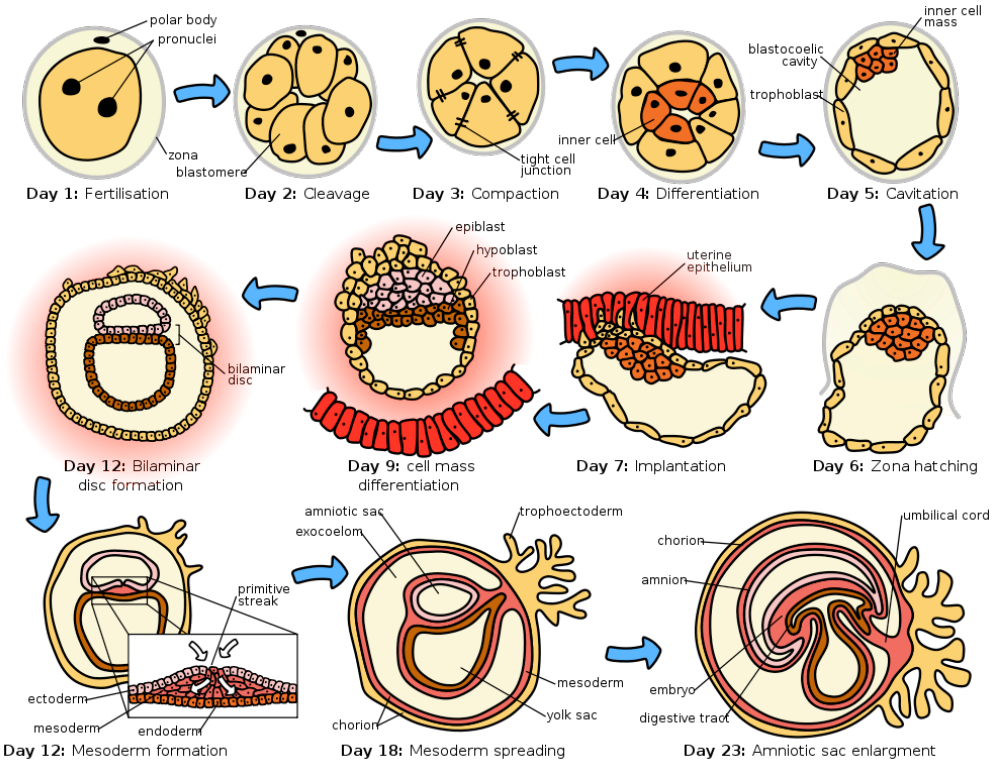

***Figure 5**. Human embryo development (www.wikipedia.org)*.*

Within 24 hours after fertilisation, the zygote undergoes a regulated series of mitotic cell divisions. The zygote will enter the blastomere stage, then becomes a morula (16 cell-stage), which will undergo compaction where the trophoblast cells (outer cell-layer) form a compact epithelial structure, connected by tight junctions and microvilli. Synchronically, the blastocyst cavity is created and a blastocyst is formed, which will hatch from the zona pellucida. The zona pellucida is a glycoprotein membrane surrounding the oocyte, which prevents the embryo from falling apart and prevents two genetically distinct conceptuses from sticking together. The blastocyst cells are totipotent, meaning that these cells have the ability to differentiate into any cell type in the developing embryo. Trophoblast cells play a crucial role in the implantation process and establishment of pregnancy, as they are the first cells to reach the maternal surface, invade and establish the embryo–maternal dialogue (Meseguer, et al., 2001). Trophoblasts produce a number of cytokines, growth factors and many other factors, including hCG, IGF-1, MMPs, TNFα, CDH1, and interleukins, which facilitate communication with the maternal tract and thereby promote implantation and ongoing embryo development through paracrine, juxtarine and/or autocrine actions (Chen, et al., 2005; Merviel, et al., 2001).

When the endometrium reaches the receptive stage, it is able to respond to signals from the preimplantation embryo for implantation. The so-called crosstalk requires several different molecules secreted both in an autocrine and paracrine manner from both the trophoblast cells and the endometrium. The involved molecules include integrins, matrix-degrading enzymes and their inhibitors, a variety of growth factors and cytokines, and their receptors and modulator proteins (Giudice, 1999; Nardo, et al., 2003a). Disturbances in this two-way dialogue are believed to represent a major reason why over 60% of all pregnancies are terminated at the end of the peri-implantation period (Herrler, et al., 2003; Macklon, et al., 2002; Wilcox, et al., 1999).

1.7 IMPLANTATION

Implantation spans from the moment of hatching of the blastocyst to the formation of a primitive placental circulation system, and is initiated by the contact between the blastocyst and the endometrium. Implantation is a dynamic process, which involves embryo apposition, adhesion to the endometrial epithelium and invasion into the stroma (Loke, et al., 1995). The 4 days old embryo arrives to uterine cavity at the stage of morula or early blastocyst and implantation is believed to take place 6-7 days after fertilisation (Figure 5) (Croxatto, 2002). In the apposition phase the embryonic trophectoderm comes closely apposed to the uterine luminal epithelium and a loose connection is established. During the adhesion step, the contact between blastocyst and endometrium is sufficiently increased to resist dissociation by flushing. There is an active communication between the blastocyst and the endometrium at this stage, conveyed by receptor-ligand-interactions (Aplin and Kimber, 2004). When adhesion is established, trophoblast cells differentiate into syncytiotrophoblasts on the outside and cytotrophoblast on the inside. Invasion starts with lytic activity of syncytiotrophoblast cells that weaken endometrial structures, thus enabling the penetration of the blastocyst. The function of the decidua is to control trophoblast invasion into the spiral arteries, to provide nutrition for the embryo, and to protect the embryo from maternal immunological response. During the subsequent invasion phase of implantation, blastocyst penetrates endometrial luminal epithelium and enters the endometrial stroma. The goal of embryo invasion is to reach the decidua and to ensure the contact with endometrial blood supply (Enders, et al., 1986). By day 9, the embryo is completely implanted in the endometrium. The role of the endometrium is now the opposite of that in the initial adhesion phase; it is to actively limit the invasive course of action by the embryo. The balance between trophoblast invasion and maternal restraint on invasion is a requirement for a procedure that must be beneficial for the embryo without being detrimental to the mother.

Both, adequate preparation of receptive endometrium and the development of a viable embryo are essential for successful implantation. The limited window of implantation ensures coordinated embryonic and endometrial development, thus minimising the risk of late implantation of compromised embryos. Besides its receptive role in the implantation, the endometrium appears to have a ‘selective’ role (Teklenburg, et al., 2010a). Recently it was demonstrated that decidualising endometrial stromal cells assess the quality of embryos that have breached the luminal epithelium, serving as

biosensors of embryo quality (Teklenburg, et al., 2010b). This introduces a novel functional window 'the window of natural embryo selection', which enables maternal recognition and elimination of compromised pregnancies, regardless of the embryonic karyotype (Teklenburg, et al., 2010a).

The cellular events that define various stages of implantation are known, but the molecules and molecular genetic pathways that are crucial to this process (and how they interact) are not well understood. It is ethically and practically impossible to study human implantation process *in vivo*, and we lack an ideal model with which to study embryo implantation in humans. Animal models do provide important information to the process regulating implantation, but as the process varies across species (Carson, et al., 2000), the results cannot always be extrapolated to humans. *In vitro* co-culture systems allow to study the signalling between embryo and endometrium, however, the current systems are relatively crude representations of the dynamic *in vivo* situation (Teklenburg and Macklon, 2009).

Previous studies on the implantation process in humans have focused exclusively on analysis of the endometrium or the embryo. As a novel step in identifying the genetic players in the early dialogue between implanting embryo and endometrium, a recent study compared global gene expression pattern between blastocyst cells and endometrial cells in women undergoing *in vitro* fertilisation (IVF) treatment (Haouzi, et al., 2011). They detected several cytokines (*PDGFA*, *PGF*, *IGF2BP1*, *IGF2BP3*) to be up-regulated in blastocyst trophectoderm cells, while some of the corresponding receptors (*PDGFRA*, *KDR*) were highly expressed in the endometrium. Also several adhesion molecules (*MCAM*, *ALCAM*, *CEACAM1*, *PECAM1*), extracellular matrix proteins (*LAMA1*, *LAMC1*, *LAMA2*, *FBN1* and others), integrins (*ITGAE*, *ITGB8*, *ITGAV*, *ITGA9* and *ITGB1*), lectins (*COLEC12*, *LGALS1* and others), and proteoglycans (*HAPLN1*, *HMMR*, *CD44* and *SDC2)* were identified in the embryo-endometrium cross-talk (Haouzi, et al., 2011). Stable adhesion through integrins expressed in blastocyst and in the endometrium, their extracellular matrix ligands, and different adhesion molecules are required for implantation (Kimber and Spanswick, 2000; Lessey and Castelbaum, 2002; Reddy and Mangale, 2003). The importance of integrins and cytokines in implantation has been acknowledged by several previous studies (Aghajanova, 2010; Aplin, 2006; Aplin and Kimber, 2004; van Mourik, et al., 2009). The initial attachment of the blastocyst to the uterine wall also involves low-affinity carbohydrate ligand-binding molecules, such as selectins and galectins (Bazer, et al., 2009).

2 AIMS OF THE STUDIES

The general aim of the current thesis was to add more understanding into the complex mechanism of human embryo implantation; to identify different factors that play role in endometrial and embryo preparation for successful implantation.

Accordingly, the studies had the following aims:

1. To examine the expression of leukaemia inhibitory factor (LIF) pathway in the pathogenesis of unexplained infertility, by analysing LIF, its receptors LIFR and gp130, and its inhibitor SOCS1 in endometria from fertile women and infertile women with unexplained infertility.

2. To study tissue factor (TF) and its inhibitors TFPI and TFPI2 in secretory endometrium from fertile women and from women with unexplained infertility in relation to endometrial receptivity. Additionally, the aim was to analyse common variation in the regulatory area of *TF* and *TFPI* genes in association to gene expression levels and unexplained female infertility.

3. To compare the genetic variation in hyaluronan-binding protein gene (*HABP2*) together with the protein expression in the endometrium at the time of implantation between women with unexplained infertility and fertile controls. Additionally, to investigate the possible role of HABP2 in human endometrial function.

4. To investigate the endometrial gene expression profile in women with unexplained infertility in comparison with fertile controls at the time of embryo implantation in order to find potential predictive markers of uterine receptivity and to identify the molecular mechanisms of infertility.

5. To analyse molecule-molecule interactions between the embryo and the endometrium at the time of implantation. Also, to identify genes and pathways that are activated within the preimplanting embryo and in the receptive endometrium.

3 MATERIALS AND METHODS

3.1 PARTICIPANTS

The study group of healthy fertile women consisted of volunteers from Stockholm or Uppsala, Sweden (Studies I, II, III, V) and from Valencia, Spain (Study IV). All these women had regular checkups at the gynaecologist, and they had no fertility-related disease. Some women for DNA analysis were post-menopausal, but the majority of the women, including all women for endometrial biopsy analysis, were of fertile age.

The study group of infertile women consisted of participants undergoing assisted reproduction in Stockholm or Uppsala (Studies I, II, III, IV). All the biopsies were obtained during a natural, non-stimulated cycle. Unexplained infertility was diagnosed by means of a standard set of tests that included hormonal analyses and at least 2 analyses of semen from the partner. All women had normal hormone levels, normal ovarian function and normal mid-secretory endometrial thickness assessed by transvaginal ultrasound scan. All infertile women showed normal tubal passage as demonstrated by hysterosonosalpingography and no recognisable endometriosis according to symptoms and clinical examination in transvaginal ultrasonography or diagnostic laparoscopy.

Both fertile and infertile women in the studies donated endometrial material and/or blood sample for research (Table 2). A diagnosed group of infertile women undergoing IVF treatment at Örebro or Uppsala donated pre-implantation embryos (day 3 and day 5 after fertilisation). The donated embryos had been frozen for future infertility treatment, and when there was a wish for no further treatment, they were donated for research.

***Table 2**. Number of women (n) who donated material for studies.*

		Article I	Article II	Article III	Article IV	Article V
Endometrium	P (F)	2				4
	ES (F)	6	10			
	MS (F)	14	20	8	5	4
	MS (Inf)	14	20	19	4	
	LS (F)	4	11			
Blood	F		108	151		
	Inf		65	105		
Embryos	Day 3					68
	Day 5					60

F – fertile women; Inf – women with unexplained infertility; P – proliferative phase; ES – early secretory, MS – mid-secretory, and LS – late secretory phases.

3.2 BIOPSY SAMPLES

All women scheduled for endometrial biopsy sampling underwent transvaginal ultrasonographic examination prior to biopsy. Serum progesterone concentration was measured to ascertain ovulatory cycles and the day of the LH surge was determined. Endometrial biopsies were obtained from the anterior wall of the uterine cavity. None of the women had used hormonal contraceptives for at least 3 months prior to the study, or used any intrauterine device for at least 6 months prior to the study. The obtained biopsies were further prepared for analysis by scanning electron microscopy (SEM), immunohistochemistry, real-time polymerase chain reaction (PCR), and microarray analysis. Day 3 and day 5 embryos were stored as frozen until RNA extraction for microarray analysis.

3.2.1 SEM

SEM is a unique method to study the surface of unsectioned samples. SEM was used to study the endometrial surface ultrastructure, to detect the presence of pinopodes. Endometrial biopsies were fixed in a solution containing glutaraldehyde which does not denature proteins and is therefore good for preservation of ultrastructure. Samples were then washed, dehydrated and dried. The specimens were then mounted on a holder, coated with a thin layer of platinum to make the specimen conductive by creating a layer, from which the scanning electron impulse is generated.

3.2.2 Immunohistochemistry

Immunohistochemistry (IHC) was used to determine the presence and cellular distribution of different proteins studied, such as LIF, LIFR, gp130, SOCS1, TF, TFPI, TFPI2 and HABP2. Relative amounts of protein can be detected by this semi-quantitative measurement, although the absolute amount cannot be determined. In general, IHC staining technique enables to visualise antigens in frozen and paraffin-embedded tissues via the sequential application of a specific antibody to the antigen (primary antibody), a biotinylated antibody to the primary antibody (secondary antibody), an enzyme conjugate (tertiary component) and a chromogenic substrate. The enzymatic activation of the chromogen results in a visible reaction product at the antigen site. We run each sample twice or three times to confirm the reproducibility of the experiment. Two observers, unaware of the identity of the slides, evaluated the staining intensities of the samples. A scoring system was used to determine the intensity of staining.

Western blot was used to verify the specificity of TF, TFPI and TFPI2 antibodies used for IHC analysis in Study II. Term human placenta was used for tissue sample, as the expression of these proteins in placenta has been shown before. Placenta tissue pieces were homogenised. The gel electrophoresis was applied for separating placental proteins by size. Thereafter the separated proteins in the gel were transferred to a membrane, and then incubated with the primary and secondary (fluorescent conjugated)

antibodies. Antibodies that specifically bind to the protein of interest were visualised using fluorescent signals.

3.2.3 Total RNA isolation

Endometrial tissue was immediately snap-frozen and stored in liquid nitrogen until further analysis. Also the cultured embryos were stored as frozen until RNA extraction. Total RNA was extracted from samples for gene expression analysis of a single genes or the whole gene expression pattern.
The cells were homogenised and the total RNA was extracted using commercial kit or the 'TRIzol method'. The quality of the purified RNA was measured by a spectrophotometer (Article I) or Agilent bioanalyser (Articles II, IV, V).

3.2.3.1 Real-time PCR

Synthesis of the complementary DNA from total RNA was performed using reverse transcriptase. SYBR Green was used to detect the amplification of single genes in interest, such as *LIF*, *LIFR*, *gp130* and *SOCS1* (Article I); *TF*, *TFPI* and *TFPI2* (Article II); and *MMP26*, *HABP2*, *HBA*, *S100A8*, and *IGFBPI* (Article IV).
SYBR Green is a fluorogenic dye that exhibits little fluorescence when in solution, but emits a strong fluorescent signal upon binding to double-stranded DNA. SYBR Green provides the simplest and most economical format for detection and quantification of PCR products in real-time reactions. The advantages of SYBR Green are that it is inexpensive, easy to use, and sensitive. The disadvantage is that SYBR Green binds to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products. Nevertheless, for single PCR product reactions with well-designed primers, SYBR Green can work extremely well, with spurious non-specific background only showing up in very late cycles. We used glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) as internal control in real-time PCR; quantitative gene expression data are normalised to the expression levels of the housekeeping gene. To quantify the results obtained by real-time PCR, standard curve method was used. In this method, a standard curve is first constructed from RNA of known concentration. This curve is then used as a reference standard for extrapolating quantitative information for mRNA targets of unknown concentrations. We used different PCR platforms, ABI 7500 Fast Real-Time PCR System (Studies I, II) and LightCycler platform (Study IV).

3.2.3.2 Microarray analysis

Microarray analysis was performed to study gene expression profiles in human receptive endometrium *vs*. endometrium of infertile women (Article IV), human receptive *vs.* non-receptive endometrium (Article V), and day 5 *vs.* day 3 embryos (Article V).
Microarray analysis was performed using the Whole Human Genome Oligo Microarray (Agilent Technologies) (Article IV) and Affymetrix HG-U133 Plus 2.0 (Affymetrix) platforms (Article V). Total RNA was processed (reverse transcribed, amplified, labelled and hybridised) according to the instructions by Agilent Technical Manual or Affymetrix two-cycle GeneChip Eukaryotic small sample target labelling assay. Initial array data analyses were performed by using the R-statistical software system. The

primary microarray data are available in the public database Gene Expression Omnibus (GEO) repository (GSE16532) (Article IV), and in ArrayExpress repository (E-MEXP-3111) (Article V).

***Table 3**. Summarising table of used methods.*

Method	Article
Immunohistochemistry (IHC)	I, II, III
Scanning electron microscopy (SEM)	I, II, IV
Real-time PCR	I, II, IV
Restriction fragment length polymorphism analysis (RFLP)	II
TaqMan SNP genotyping assay	III
Western blot	II
Microarray analysis	IV, V
Migration assay	III
Statistical analysis	I, II, IV, III, V
Mathematical modelling	V

3.3 BLOOD SAMPLES AND GENOTYPING

Blood samples were collected for polymorphism analysis in *TF*, *TFPI* (Article II) and *HABP2* genes (Article III). Genomic DNA was extracted from blood using QIAamp DNA Blood Maxi kit. We used different genotyping methods, restriction fragment length polymorphism (RFLP) and TaqMan.

3.3.1 RFLP

We analysed single nucleotide polymorphisms (SNPs) *TF* -603 A/G (rs1361600), *TFPI* -399 C/T (rs10153820) and *TFPI* -287 T/C (rs10931292) using RFLP method. RFLP refers to a variance between samples of homologous DNA molecules as a result of differing locations of restriction sites. In the RFLP analysis, DNA strand is digested with restriction enzyme and the obtained restriction fragments are separated according to their lengths by gel electrophoresis. We used Agilent DNA 1000 Kit system for restriction fragment analysis, where interconnected set of microchannels are used for separation of fragments based on their size as they are driven electrophoretically through the chip. This system provides high resolution of smaller fragments, is easy and fast to use, however, it is more expensive than simple gel-electrophoresis.

3.3.2 TaqMan assay

We analysed polymorphisms rs7080536, rs1157916 and rs2240879 in *HABP2* gene using TaqMan assays. TaqMan chemistry allows detection of PCR products via the generation of fluorescent signal. TaqMan probes depend on the 5'- nuclease activity of the DNA polymerase used for PCR to hydrolyze an oligonucleotide that is hybridized to the target sequence. TaqMan probes are oligonucleotides that have a fluorescent

reporter dye attached to the 5' end and a quencher moiety coupled to the 3' end. These probes are designed to hybridize to an internal region of a PCR product. In the unhybridised state, the proximity of the fluor and the quench molecules prevents the detection of fluorescent signal from the probe. Well-designed TaqMan probes require very little optimization, however, TaqMan probes can be expensive to synthesize, with a separate probe needed for each mRNA target being analyzed.

3.4 MIGRATION ASSAY

We used migration assay in Boyden Chamber format for assessing the ability of human trophoblast and endometrial endothelial cells to migrate towards a chemoattractant, HABP2, in order to investigate the role of HABP2 in the implantation process. Boyden Chamber is a popular traditional tool in studying cell migration *in vitro*. This assay is ideal to study migration via a chemoattractant gradient. However, the Boyden Chamber assay format can only be used for quantitative and endpoint analysis. Figure 6 illustrates the Boyden Chamber method. Cultured cells are placed in an upper chamber in serum-free media, where the bottom is a porous membrane (pore size is dependent on the cell type being studied). The membrane serves as a barrier to discriminate migratory cells from non-migratory cells. Serum or another chemoattractant is placed in the well below.

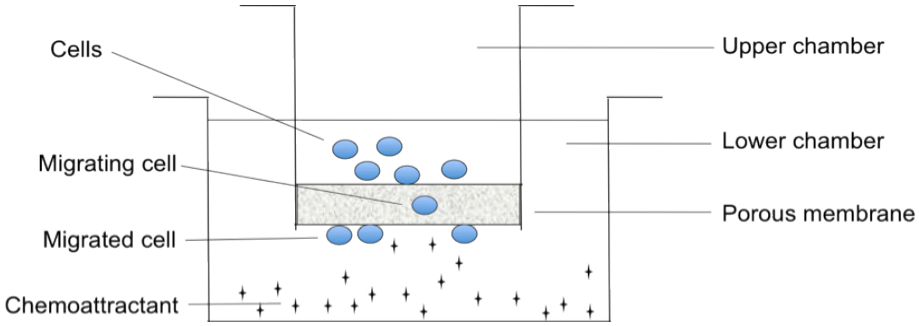

Figure 6. *Schematic illustration of Boyden Chamber migration assay.*

3.5 STATISTICAL ANALYSIS

For statistical analyses different software was used – Statistical Package for Social Sciences statistical software (SPSS) (Articles I, II, III, IV) and the R2.3.1 A Language and Environment (Articles IV, V). Additionally, for further analysis of microarray data we used MeV software and for functional analyses of dysregulated genes Database for Annotation, Visualisation and Integrated Discovery (DAVID) and Ingenuity Pathway Analysis (IPA) (Article IV). Software g:Profiler, Multi Experiment Matrix web tool and GraphWeb tool were used for complex **mathematical modelling** of embryo-endometrium interactions (Article V).

In general, data are presented as mean ± SD, unless otherwise indicated. Nominal variables were analysed by χ^2 tests. Allele frequencies were calculated to investigate the deviation from Hardy-Weinberg equilibrium and also to calculate the odds ratios (ORs). All continuous variables were assessed for normal distribution and in a case of deviation, square root transformation was conducted. We assessed the differences in continuous data between study groups by using parametric tests. In the case of deviation from normal distribution and in the case of limited sample size, the non-parametric tests were used. For all analyses, a p value < 0.05 was considered statistically significant.

Table 4. *Summary of the five studies presented in the current thesis.*

Study	**Subjects (n)**	**Studied variables**
I	Healthy fertile women (26) Women with unexplained infertility (14)	Pinopode formation LIF, LIFR, gp130, SOCS1 mRNA and protein expression in endometrium
II	Healthy fertile women (140) Women with unexplained infertility (65)	Pinopode formation TF, TFPI, TFPI2 mRNA and protein expression in endometrium *TF* -603 A/G *TFPI* -399 C/T *TFPI* -287 T/C
III	Healthy fertile women (158) Women with unexplained infertility (116) Primary endometrial endothelial cells Human trophoblast cells	HABP2 protein expression in endometrium *HABP2* Gly543Glu (Marburg I) *HABP2* -70 A/G *HABP2* G/A in promoter region Migration assay of endothelial cells and trophoblast cells with HABP2
IV	Healthy fertile women (5) Women with unexplained infertility (4)	Endometrial genome expression Pinopode formation Real-time PCR of *MMP26*, *HABP2*, *HBA*, *IGFBP1*, *S100A8* genes
V	Healthy fertile women (8) Human embryos (128)	Gene expression profile of non-receptive *vs.* receptive endometria Gene expression profile of day 3 *vs.* day 5 embryos Embryo-endometrium interaction networks

4 RESULTS

4.1 FACTORS INFLUENCING ENDOMETRIAL RECEPTIVITY

4.1.1 LIF pathway (Studies I and V)

First we set up to assess the expression of LIF pathway – LIF, LIFR, gp130 and its inhibitor SOCS1, in the endometrium of fertile women throughout the menstrual cycle. The mRNA expression levels of *LIF*, *LIFR*, *gp130* and *SOCS1* did not differ significantly in different cycle phases. On protein expression level, gp130 was significantly intensely expressed in luminal and glandular epithelium during mid-secretory phase, meanwhile SOCS1 demonstrated apical staining in epithelial cells, however, no significant difference was detected throughout the cycle. Furthermore, SOCS1 correlated negatively with LIFR expression in luminal epithelial cells (Figure 7).

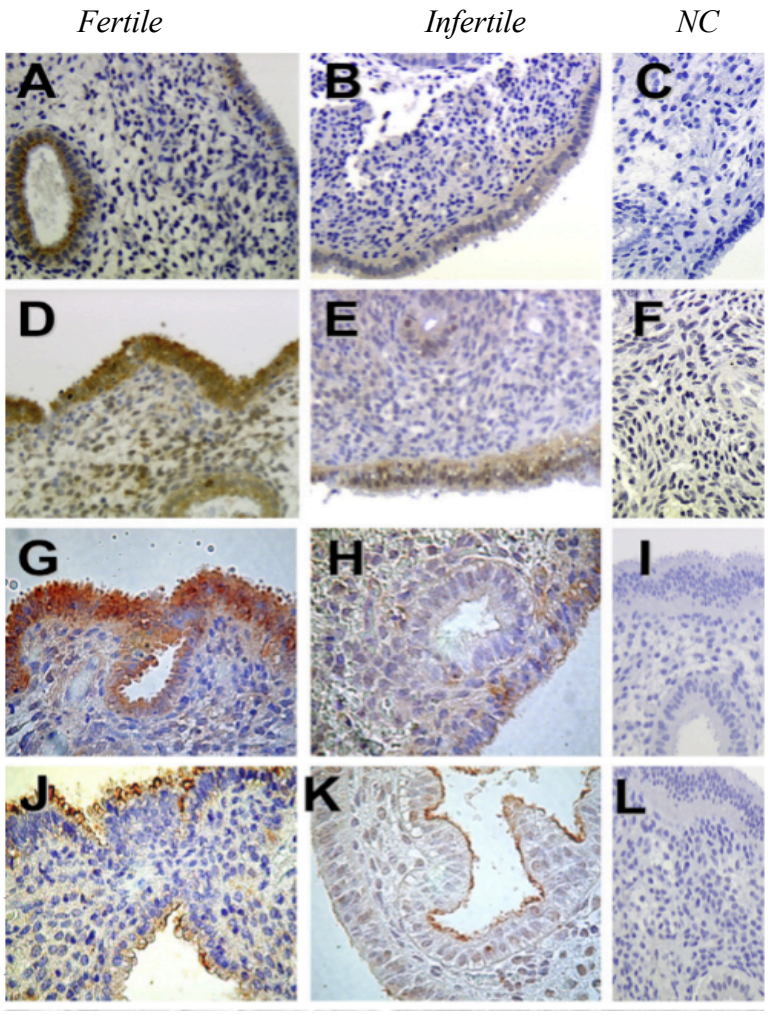

Figure 7. *Immunostaining of LIF (A, B, C), LIFR (D, E, F), gp130 (G, H, I) and SOCS1 (J, K, L) in fertile and infertile endometrium at the time of embryo implantation. NC – negative control.*

Immunostaining of LIF and LIFR in fertile endometrium has been assessed in our previous study, where the expression correlated positively with the pinopode appearance, being highest in the mid-secretory phase (Aghajanova, et al., 2003). Also in our next study (Article V), where we compared the global gene expression levels in non-receptive (proliferative phase) and receptive phase endometria of fertile women, we detected significant up-regulation of *LIF* and *gp130* at the time of embryo implantation.

The comparison of LIF pathway endometrial expression in fertile and infertile women revealed that unexplained infertility in some women might be explained by disturbances in the LIF pathway. In fertile mid-secretory endometrium high levels of LIFR and gp130 protein expression correlated with low SOCS1 expression, meanwhile the opposite expression pattern was detected in most women with unexplained infertility (Table 5, Figure 7).

Table 5. *Mid-secretory phase endometrial samples from fertile and infertile women showing strong immunostaining of LIF, LIFR, gp130 and SOCS1. LE – luminal epithelium, GE – glandular epithelium.*

Strong staining	Fertile % (n)	Infertile % (n)
LIF LE	85% (12)	64% (9)
LIF GE	100% (14)	78% (11)
LIFR LE	100% (12)	**28%** (4)
LIFR GE	100% (12)	**35%** (5)
Gp130 apical staining LE	100% (11)	**14%** (2)
Gp130 apical staining GE	81% (9)	**0%** (0)
Gp130 cytoplasmic staining LE	91% (10)	**35%** (5)
Gp130 cytoplasmic staining GE	45% (5)	28% (4)
SOCS1 apical staining LE	100% (14)	78% (11)
SOCS1 apical staining GE	85% (12)	85% (12)
SOCS1 cytoplasmic staining LE	0% (0)	**28%** (4)
SOCS1 cytoplasmic staining GE	0% (0)	**93%** (13)

4.1.2 Tissue factor pathway (Study II)

We assessed the expression of tissue factor and its inhibitors throughout the secretory phase endometrium of fertile women. We detected TF and TFPI protein to have similar expression pattern in luminal and glandular epithelium, being highest at the time of embryo implantation (Figure 8). In most samples no TFPI staining in the stroma was detected. TFPI2 protein was highly expressed throughout the secretory phase, and in stroma showed descending pattern. On mRNA level, the expression pattern was different; *TFPI* mRNA transcripts were gradually rising through the secretory phase. Differences in mRNA and protein expressions could arise from different sample preparation method, as for real-time PCR the whole tissue sample is homogenised, and the upper part of the functional layer forms only a part of it. Additionally, the differences could arise from further gene expression regulation (e.g. epigenetic

modification) and/or post-translational modification. We also cannot rule out the interindividual variance as fewer samples were used for real-time PCR than for IHC analysis.

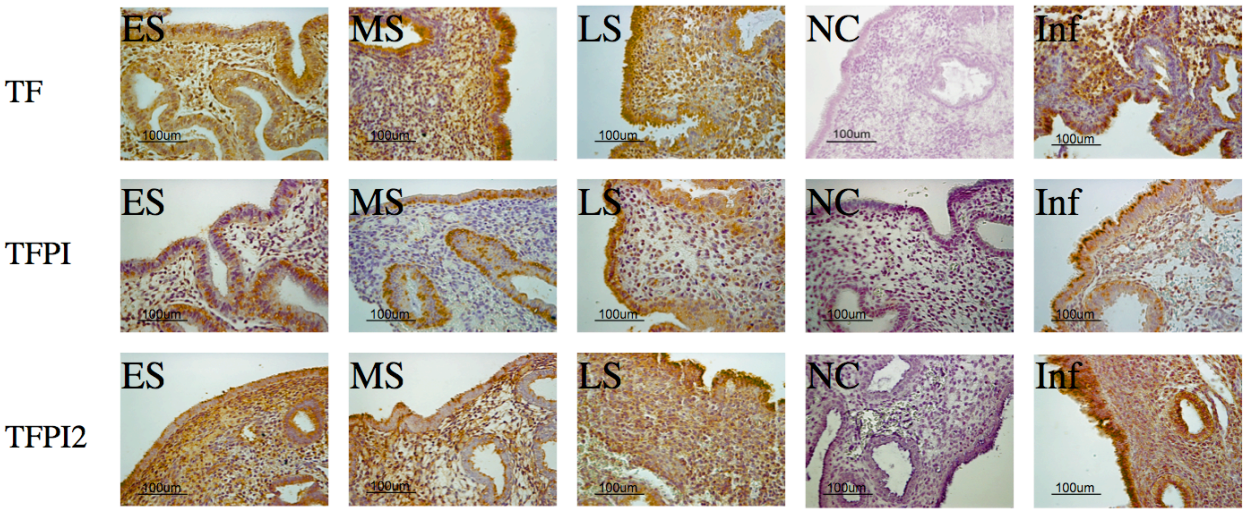

Figure 8. *Immunostaining of TF, TFPI and TFPI2 in early (ES), mid- (MS) and late secretory (LS) endometrium in fertile women, and in women with unexplained infertility (Inf) in the mid-secretory phase.*

We then compared TF and its inhibitors' expression patterns in fertile women and infertile women at the time of implantation, and detected significantly higher *TF* mRNA and TFPI protein expression in the luminal epithelium in endometria of infertile women (Figure 8). In line with the high TFPI expression, *TFPI* -287 T/C polymorphism associated with unexplained infertility – infertile women presented more frequently T allele, and T allele has been shown in previous studies to favour protein expression (Amini Nekoo and Iles, 2008; Skretting, et al., 2010). In *TF* gene, -603 A/G SNP associated with endometrial protein expression level, being highest in women with GG genotype (Figure 9). However, no association with unexplained female infertility was detected.

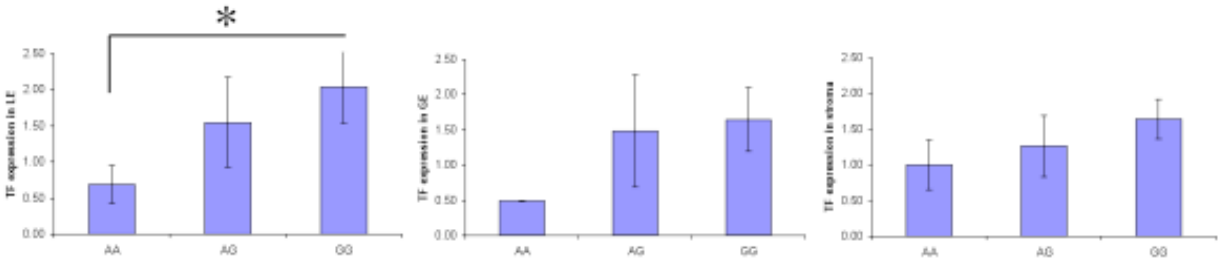

Figure 9. *TF -603 A/G genotypes and TF immunostaining in the endometrium of women with unexplained infertility during mid-secretory phase. LE – luminal epithelium, GE – glandular epithelium. *Statistically significant difference in TF immunostaining intensity between genotypes.*

4.1.3 HABP2 (Studies III, IV, and V)

In the study of global gene expression analysis in fertile endometrium at the time of implantation, we detected the presence of *HABP2* gene (Article V). When compared the endometrial gene expression profiles at the implantation window between fertile and infertile women, significantly lower levels of *HABP2* were detected in women with unexplained infertility (Article IV), which was also confirmed by real-time PCR (Figure 10).

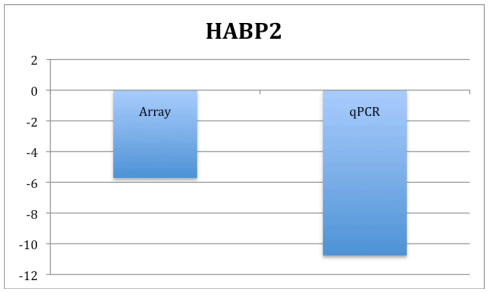

Figure 10. *HABP2 gene expression in the endometrium of infertile women vs. fertile women detected by microarray analysis and real-time PCR.*

Next we set up to analyse the HABP2 protein expression and localisation in the mid-secretory endometrium (Study III). HABP2 was predominantly expressed in stromal cells and in vessels in fertile endometrium. In infertile women, HABP2 expression was again significantly reduced (Figure 11). As genetic variation in *HABP2* gene has been shown to influence the expression (Sedding, et al., 2006), we conducted polymorphism analysis in these fertile and infertile women. We analysed rs1157916 in the promoter area, rs2240879 in 5'UTR and rs7080536 (Gly534Glu) in exon 13 of *HABP2* gene. Minor rs1157916 A and the major rs2240879 A alleles, together with AA genotypes were significantly less frequent in women with unexplained infertility, who demonstrated lower *HABP2* mRNA and protein expression levels.

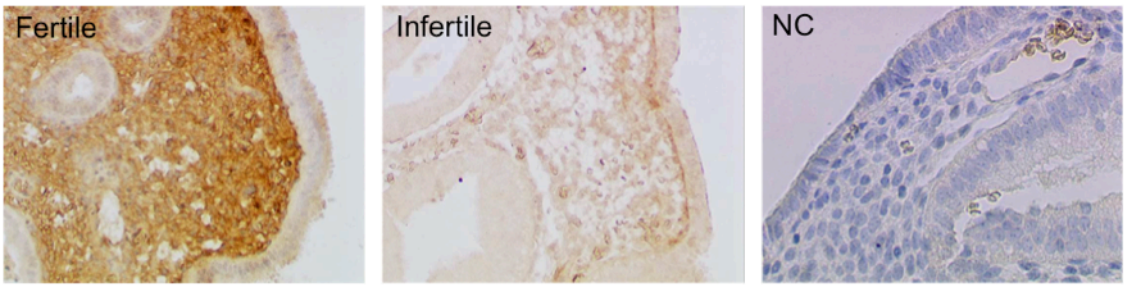

Figure 11. *Immunohistochemical staining of HABP2 in the endometria of a fertile woman and an infertile woman (Inf) at the time of implantation. Negative control (NC) is indicated.*

In order to study HABP2 possible role in the endometrium at the time of implantation, we set up a migration assay to analyse trophoblasts and endometrial endothelial cells migration toward HABP2 protein (the chemoattractant). The mean numbers of endometrial endothelial cells migrating toward HABP2 were significantly increased when compared with the positive control of 10% foetal calf serum. No difference in migrating trophoblast cell numbers was detected.

4.1.4 Pinopodes (Studies I, II and IV)

In fertile women, no pinopodes were observed in early secretory endometrial samples, while in mid-secretory phase pinopodes were detected in the majority of samples (≥88%) and on some samples from late secretory phase (≤20%) (Figure 12).

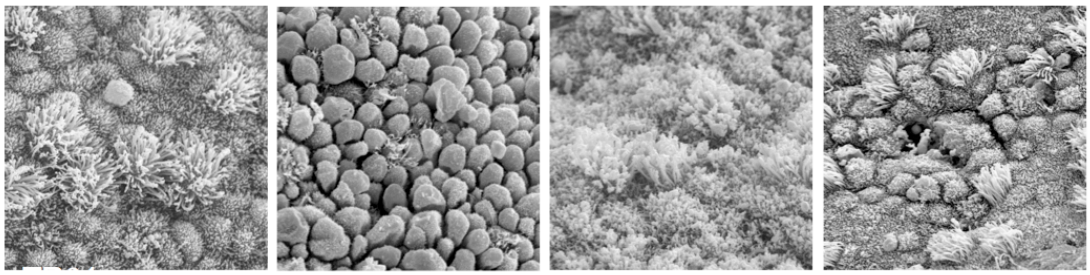

Figure 12. *SEM micrographs of endometrium obtained form fertile women during secretory phase (ES – early, MS – mid- and LS – late secretory) and women with unexplained infertility at mid-secretory phase (Inf).*

The appearance of pinopodes during the time of implantation in the endometrial samples was higher when compared to the early and late phase samples. In the endometria of fertile women the presence of pinopodes correlated positively with LIFR expression in luminal epithelium, and TF expression in glandular epithelial cells and in stroma. In women with unexplained infertility, the presence of pinopodes was reduced, and the number of pinopodes was significantly lower (Table 6).

Table 6. *Presence of pinopodes on the endometrial surface in fertile and infertile women at the time of embryo implantation (mid-secretory phase).*

Study	Group of women	Total no. of samples	Samples with pinopodes (%)	Few pinopodes
I	Fertile	17	15 (88)	
	Infertile	14	8 (57)	+
II	Fertile	16	15 (93.8)	
	Infertile	15	12 (80)	+
IV	Infertile	4	3 (75)	+

4.1.5 Global gene expression pattern (Studies III and V)

We compared the genome expression pattern of receptive endometrium *vs.* non-receptive endometrium in fertile women in order to identify genes and gene networks activated at the time of embryo implantation. We detected up-regulation of 920 genes and down-regulation of 1257 genes. The down-regulated gene list presented pregnancy-specific functions, such as gland development, the progesterone-mediated oocyte maturation pathway, and a maternal process involved in pregnancy. The up-regulated gene list was characterised by response to external stimulus, positive regulation of the immune system, extracellular matrix-receptor interaction, acute inflammatory response, innate immune response and macrophage activation during immune response. Functional categories such as cell adhesion, integrin cell surface interactions, and regulation of cell proliferation indicate endometrial preparation for embryo implantation.

We then studied protein-protein interaction networks within the receptive endometrium. The analysis applied was based on the assumption that significantly induced genes may establish permanent and transient protein-protein interactions to create protein complexes and initiate signal transduction. For that, up-regulated genes in receptive endometrium were mapped to the Human Protein Reference Database (HPRD). The mapping resulted in endometrial network of 264 genes and 324 interactions, which was further clustered into 144 modules. Next we based on the assumption that interacting proteins with many shared interactions are biologically more relevant. Such assessment of functional importance identified 10 of the most significant biological process, cell components, molecular functions and pathways for endometrial networks (Figure 13). The identified functions and pathways included various immune and inflammatory responses, the JAK-STAT signalling pathway, cell-cell adherens junctions, focal adhesion, complement and coagulation cascades, and others.

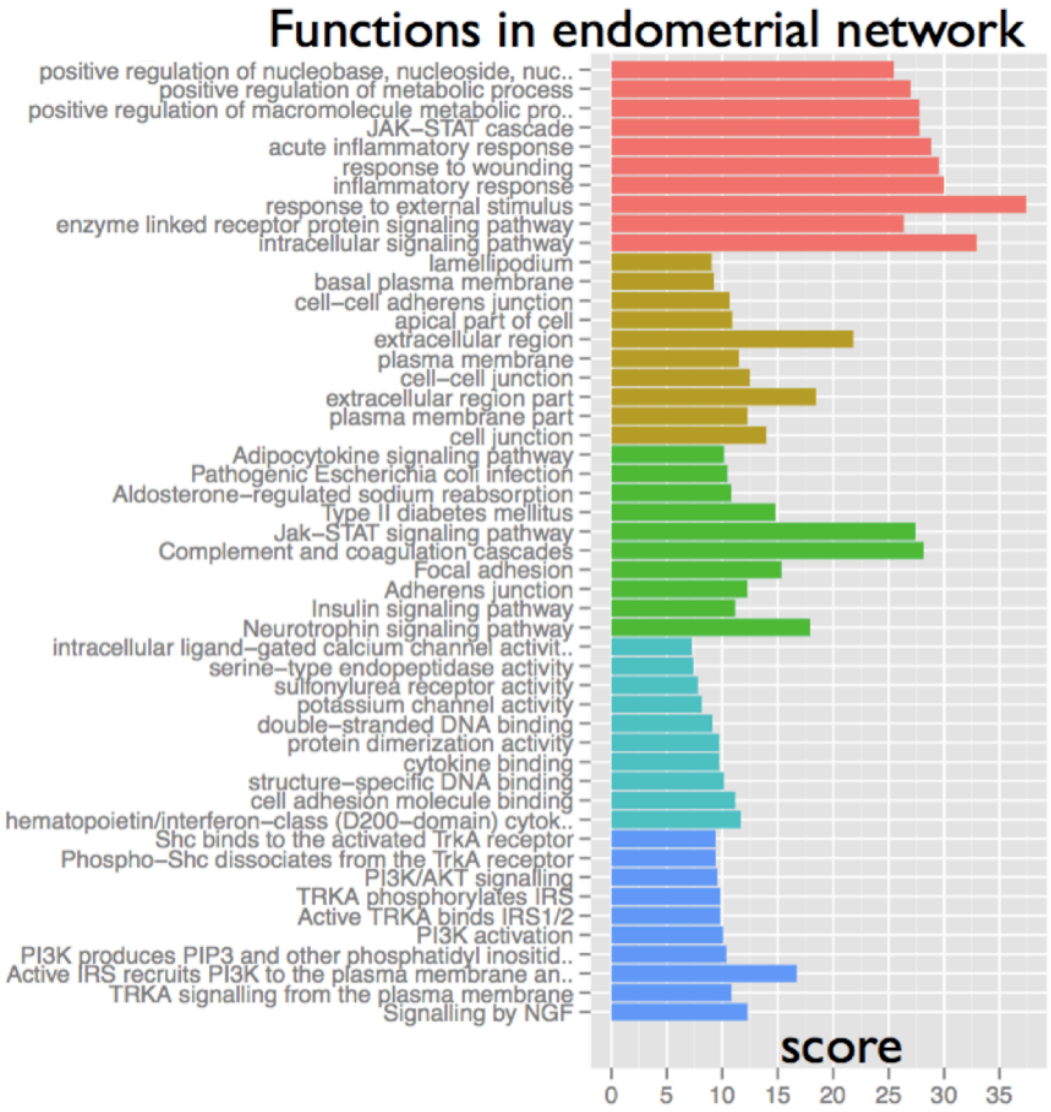

Figure 13. *Functional enrichment analysis of endometrial interaction networks. Red denotes Gene Ontology (GO) biological process, brown GO cellular component, green KEGG pathway, marine GO molecular function, and blue indicates Reactome pathway.*

We also compared endometrial genome expression between fertile women and women with unexplained infertility at the time of embryo implantation, in order to find potential predictive markers of uterine receptivity and to identify molecular mechanisms of infertility. Our cluster analysis demonstrated clear distinction between fertile and infertile women based on the differentially regulated genes (Figure 14). We identified a total of 145 significantly up-regulated and 115 down-regulated genes in the endometria of infertile women *vs.* controls. Genes involved in immune responses, signal transduction, binding, transport, lipid metabolism and extracellular matrix components, among other functions, showed elevated expression levels among infertile women. 24% of the up-regulated genes were with unknown function, and 38% of down-regulated genes were without known function. Down-regulated genes included

genes encoding transmembrane receptors, transcription factors, proteins involved in lipid metabolism and transmembrane transport, and other genes. We detected several potential target molecules that were dysregulated in infertile women, such as mucins (*MUC4*, *MUC5B*), the mucin-associated peptide *TFF3*, insulin-like growth factor binding protein-I (*IGFBPI*), metalloproteinases (*MMP8*, *MMP10*, *MMP26*), cytokines and chemokines (*SCGB3A1*, *FAM3D*, *FAM3B*, *CCR7*, *CXCL6*, *IL21*, *CMTM5*), integrins (*ITGA8*, the integrin-binding protein *COL16A1*), immunomodulators, lipids, genes involved in Wnt signalling (*WISP2*, *WNT3A*, *CXXC4*, *PRKCG*), and others.

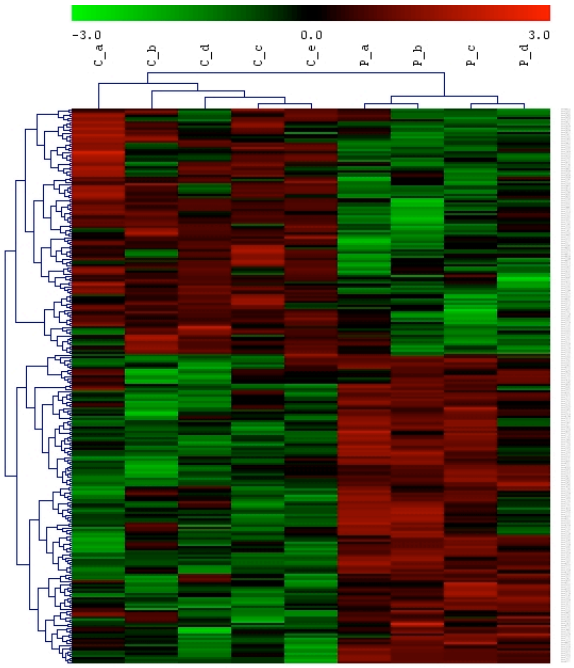

Figure 14. *Cluster analysis of dysregulated gene expression in the endometrium during the time of implantation in fertile women (C) and in women with unexplained infertility (P).*

Functional analysis of differentially regulated genes using DAVID database revealed that 21.1% of the aberrantly expressed genes in infertile *vs.* fertile women were involved in localization and 18.8% of the genes were involved in transport. In addition, a significant proportion of the genes were involved in ion transport, defence responses, digestion, and metabolic processes of multicellular organisms. A significant number of dysregulated genes were located in extracellular regions, regions integral to the plasma membrane, and in the extracellular matrix. As regards molecular functions, many genes were involved in transporter activity, and especially transmembrane transporter activity. The biological pathway analysis using IPA revealed that aberrant endometrial gene expression in infertile women could influence pathways involved in leukocyte extravasation signalling, lipid metabolism, and detoxification. Analysis of molecular relationships between differentially expressed genes in infertile women showed a complex network where the majority of signals were mediated through mitogen-activated protein kinase (ERKs, P38MAPK, Jnk) complexes, where different genes such as those for beta adrenergic receptors (ADRBs), metalloproteinases, IGFBPI and others seem to play important roles (Figure 15).

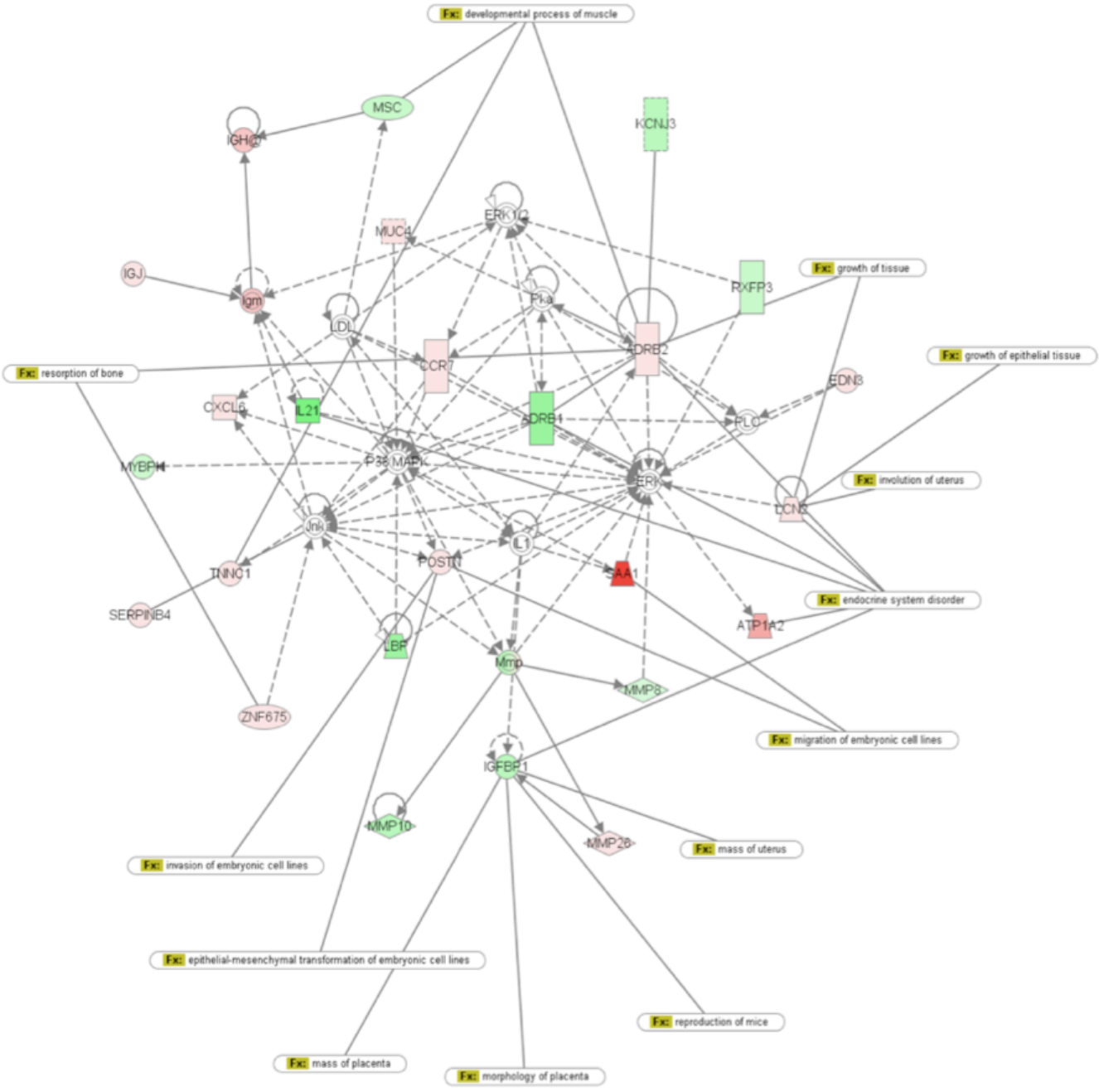

Figure 15. *A network of the molecular relationships between differentially expressed genes in infertile women. The intensity of the node colour indicated the degree of up- (red) or down- (green) regulation. A white node indicates a gene that is not part of our dataset, but is interacting in the network.*

4.2 FACTORS IMPORTANT FOR IMPLANTATION-COMPETENT BLASTOCYST DEVELOPMENT (STUDY V)

We analysed the genome expression in blastocyst (day 5 embryos) and compared the pattern with day 3 embryos, in order to identify genes and gene networks activated in implantation-competent embryos. 2812 genes were up-regulated and 2824 genes down-regulated in blastocysts. The list of down-regulated genes comprised a large fraction of transcription factors, also GO categories such as sexual reproduction, brain development, and pattern specification process were identified. The up-regulated gene list was characterised by genes involved in metabolic processes, development, and localisation. On single gene level, E-cadherin, transforming growth factor beta-1 (*TGFB1*), *gp130*, different cathepsins (*CTSB*, *CTSH*, *CTSD*, *CTSZ*, *CTSL1*, *CTSE*, *CTSA*), prostaglandins (*PTGES2*, *PTGES*, *PTGR1*, *PTGER3*), and pregnancy-associated glycoproteins (*PSG1*, *PSG2*, *PSG4*, *PSG7*, *PSG10*) were present.

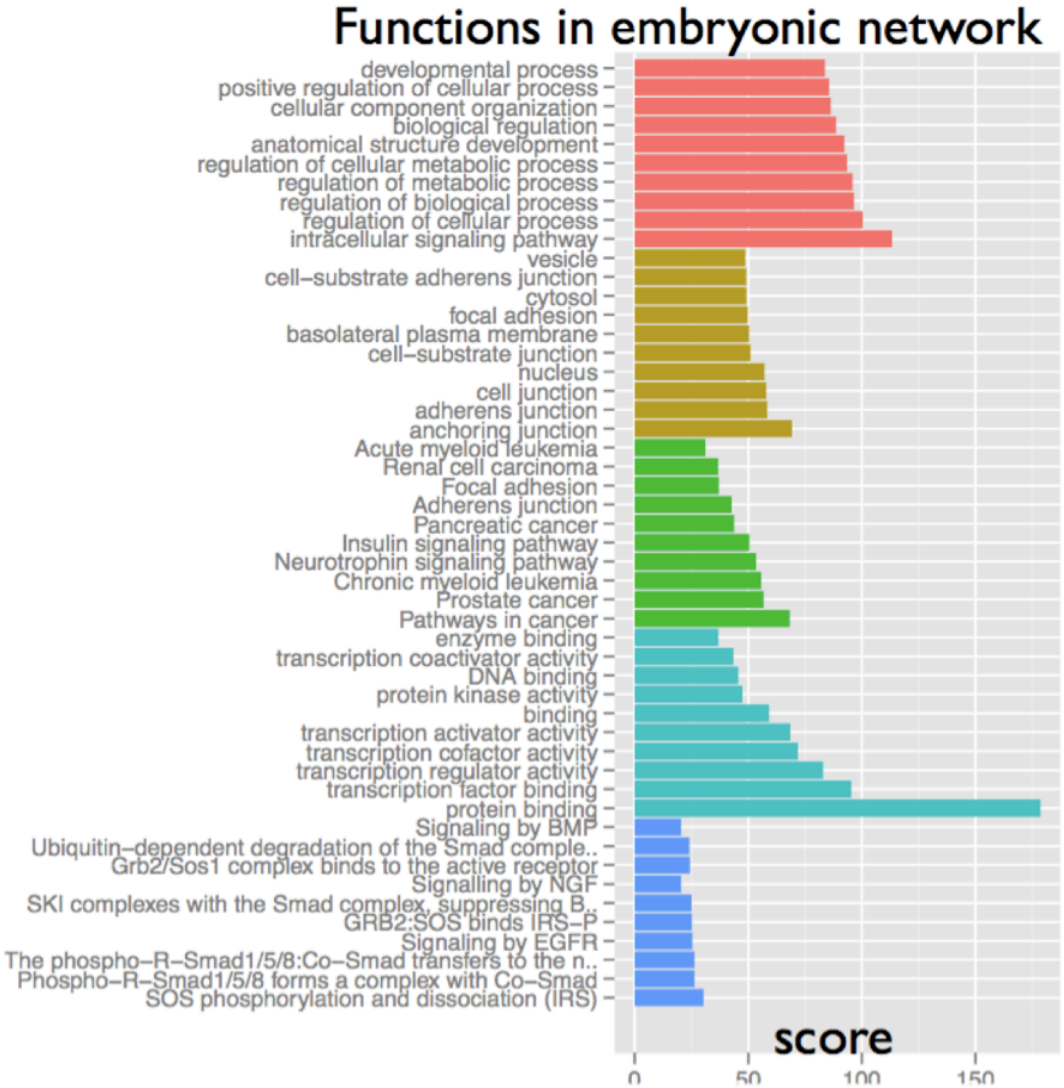

Figure 16. *Functional enrichment analysis of embryo interaction networks. Red denotes Gene Ontology (GO) biological process, brown GO cellular component, green KEGG pathway, marine GO molecular function, and blue indicates Reactome pathway.*

Next we studied protein-protein interaction networks within the blastocyst. The analysis was carried out the same way as described earlier in the section 4.1.5, the focus being on the list of up-regulated genes in blastocyst. The analysis resulted in embryonic network of 1096 genes and 1956 interactions, which were clustered into 325 modules. The functional importance of the 10 most significant biological processes, cellular components, molecular functions and pathways for embryo networks are presented in Figure 16. The identified functions and pathways included transcription regulation, developmental processes, regulation of cellular metabolic processes, intracellular signalling pathways, protein binding, and pathways in cancer, focal adhesion and adherens junction.

Further, we implemented a computational procedure for revealing possibly active microRNAs (miRNAs) and their likely targets, based on genomic locations of miRNA genes and their predicted target sites, using public databases. We assumed that miRNA genes located in the immediate vicinity of their host genes (e.g. a miRNA gene located on the opposite strand of a host gene) may be subject to transcription in concert with the host gene. First, we compiled lists of miRNA genes that were potentially expressed in embryonic tissues because their host genes were differentially up-regulated in the samples. We considered a miRNA to be active if a significantly over-represented number of its predicted target genes were down-regulated in embryos. We identified numerous examples of active miRNAs in embryos, including hsa-mir-9, hsa-mir-19a, hsa-mir-20a, hsa-mir-548b, and others (Figure 17). We found a considerable proportion of target genes inhibited by miRNA as transcriptional regulators according to GO analysis.

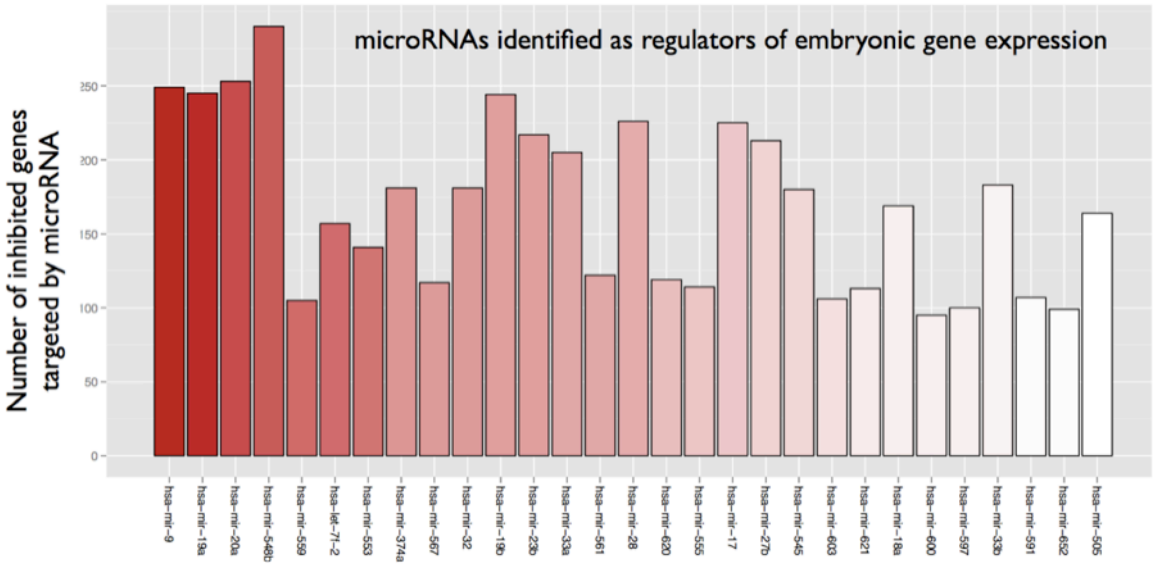

Figure 17. *miRNAs and the number of putative target genes identified among embryonic genes. Bar colour intensity represents statistical significance of target gene enrichment ($p < 0.05$).*

4.3 INTERACTIONS BETWEEN RECEPTIVE ENDOMETRIUM AND BLASTOCYST-STAGE EMBRYO (STUDY V)

In the last study we set out to describe the inter-tissue interface that is initiated during implantation. We constructed an embryo–endometrium interaction network that encompassed genes induced in both endometrial and embryonic tissues. We extracted known protein–protein interactions from the HPRD that spanned the two tissues, such that each interaction comprised one gene induced in the embryo and the other induced in the endometrium. The interactions in the embryo–endometrium interaction network were further filtered using GO cell component annotations. We focused on proteins known to be localised near the outer cell boundaries such as membranes and the extracellular matrix, and excluded proteins localised within the cell cytoplasm and organelles, and nucleus. Proteins with no cellular component annotations were included in the analysis. In total we identified 105 modules, where functional enrichment analysis revealed functions and pathways, such as cell adhesion, focal adhesion, cell–cell junctions, tight junctions, integrin cell surface interactions, extracellular matrix structural constituents, and others. We then created a high-confidence variant of the embryo–endometrium interface by careful literature curation, which resulted in the high-confidence network comprising of 96 genes, 87 interactions and 22 connected network components (Figure 18). The largest curated network was built up of 35 interacting molecules between the two tissues belonging to the protein families of collagens (COL1A1, COL4A1, COL4A2, COL4A5, COL4A6, COL7A1), integrins (ITGA1, ITGB8), laminins (LAMA1, LAMA2, LAMA5, LAMB3, LAMC1, LAMC2) and fibulins (FBLN1, FBLN2), together with other molecules involved in cell adhesion (CD36, CD44, HABP2, TGFB1, VCAN, VEGFA). The second largest interaction network, of 14 genes, represented proteins involved in cytokine–cytokine receptor interaction, where osteopontin (SPP1), apolipoprotein D (APOD), leptin (LEP) and LIF pathways intertwine. The third largest interaction network united 4 molecules that are involved in tight junctions, including TJP1, occludin (OCLN) and claudin 4 (CLDN4). The next network in size demonstrated a novel interaction network in the human implantation process, comprising the hormone gastrin (GAST), the metalloprotein ceruloplasmin (CP), membrane metallo-endopeptidase (MME), and endothelin 1 (EDN1). Several additional novel interactors in the embryo–endometrium interface were detected in our study, including the molecules Dickkopf 1 (DKK1), kringle containing transmembrane protein 1 (KREMEN1) and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1).

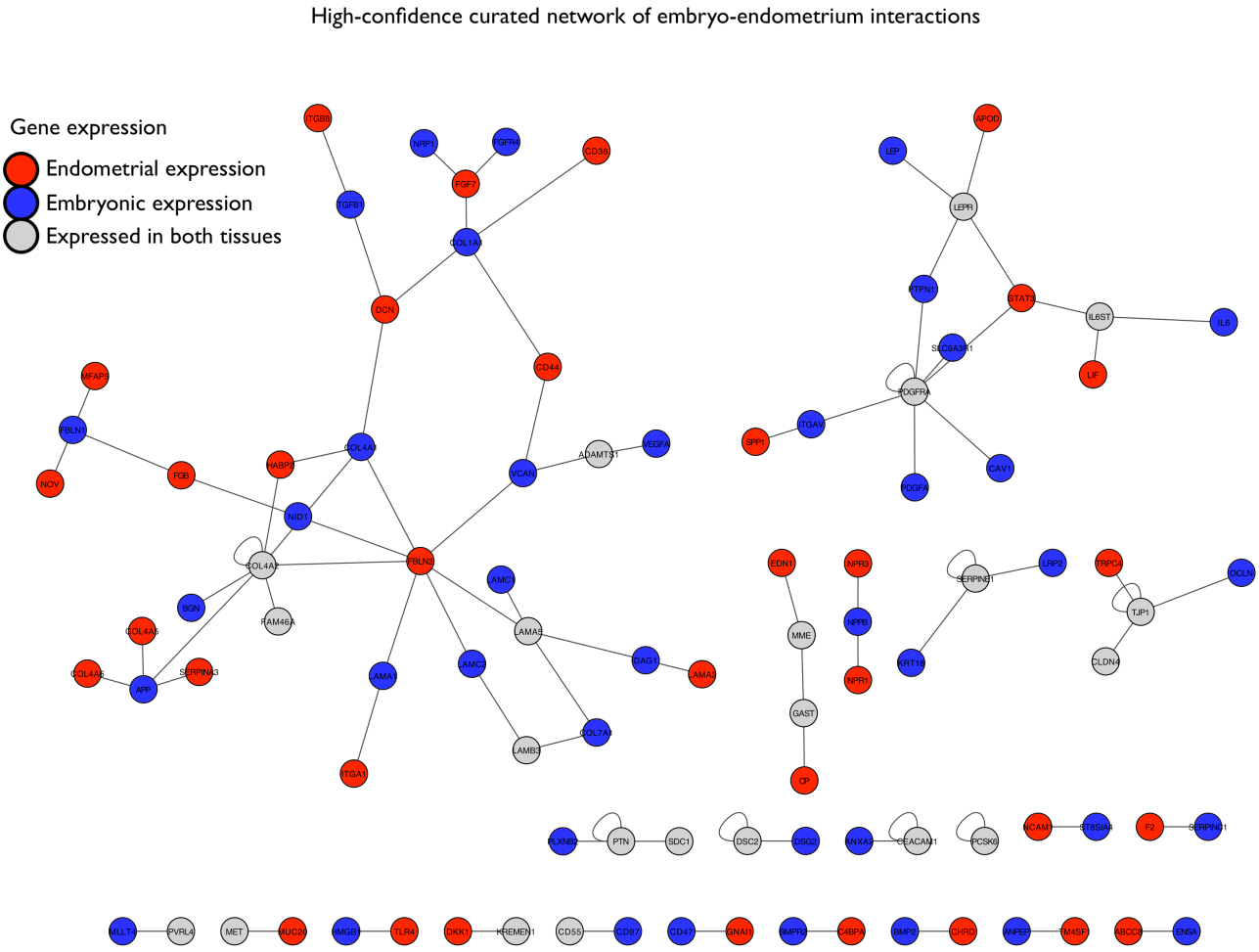

Figure 18. *Embryo-endometrium interaction network from protein-protein interaction data and literature curation. Node colour represents tissue-specific differential gene expression; blue – expressed in embryo, red – expressed in endometrium, grey – expressed in both tissue.*

5 DISCUSSION

5.1 FACTORS INFLUENCING ENDOMETRIAL RECEPTIVITY

Receptive endometrium is crucial for blastocyst attachment and implantation, process in which both structural and functional changes take place. Many molecules have been identified as being important for the endometrial preparation to a receptive state, such as integrins and their ligands, mucins, growth factors, cytokines, lipids and other molecules (Aghajanova, et al., 2008a; Aghajanova, et al., 2008b). In the current thesis, applying genome-wide expression analysis, we detect the expression of many genes in the normal receptive endometrium that have previously been identified in connection with uterine receptivity, including **LIF**, **HABP2**, IL15, PAEP, SPP1 and others. In addition, we identify several relevant gene networks in the adequate preparation of receptive endometrium, such as those connected with the **JAK-STAT signalling pathway**, complement and **coagulation cascades**, focal adhesion, adherens junctions and inflammatory responses with major localisation in extracellular regions. The next finding in the current thesis is that in women with otherwise no reason for their infertility, several of these molecules and pathways involved in endometrial receptivity were dysregulated, such as mucins, metalloproteinases, cytokines, chemokines, integrins, and pathways involved in inflammatory responses, lipid metabolism, and detoxification.

Next, our approach of single gene analyses is in line with the results from genome expression studies. We analysed **LIF pathway**, the activator of the JAK-STAT signalling cascade, expression in the endometrium of fertile and infertile women. The main finding of this study is that LIF signalling pathway expression was impaired in women with unexplained infertility (Figure 20), indicating that the reason for their fertility complications could be explained by disturbances in the LIF pathway at the time of embryo implantation, that less efficient LIF action could lead to aberrant endometrial receptivity.

Our study of single gene approach was focusing on the factors involved in the blood **coagulation cascade**, which besides to maintaining the haemostatic balance, is related to other signalling pathways such as angiogenesis, inflammatory signalling, and protective signalling. We show distinct expression pattern of **tissue factor pathway** in the fertile secretory endometrium, and the spatial and temporal TF and TFPI expression during the mid-secretory phase of the menstrual cycle reflect their involvement at the time of embryo implantation. We also demonstrate that alteration in this well-regulated process of coagulation, specifically elevated **TFPI** protein expression may contribute to female infertility through unbalancing haemostatic balance at the site of embryo implantation toward reduced coagulation. Indeed, bleeding during the first trimester has been associated with risk of miscarriage (Hasan, et al., 2009).

HABP2 was the next factor in the coagulation pathway that we analysed in the endometria of fertile and infertile women. Interestingly, HABP2 gene expression was

detected in our both array studies, being up-regulated in the fertile endometrium at the time of implantation and being down-regulated in women with no clear reason for their fertility complications. In fact, HABP2 is one of the few genes that has been detected in several independent array studies, and it is proposed as one of the few endometrial receptivity markers (Horcajadas, et al., 2007). The function of HABP2 in endometrial receptivity is not established, therefore we analysed the possible role of this protein in the endometrium at the time of implantation. The high HABP2 protein expression in stroma and vessels in fertile women, and not in epithelial cells, together with increased endometrial endothelial cell migration toward HABP2 protein refer to the role of HABP2 in the blood coagulation pathway and angiogenesis. In infertile women HABP2 endometrial expression pattern was significantly lower. This lower expression of HABP2 in infertile women is in line with the tissue factor pathway results, that lower level of this coagulation-pathway-initiator may lead to reduced coagulation in infertile women. Haemostatic balance may prove to be critical during the formation of new blood vessels i.e. angiogenesis that is needed for endometrial maturation and at the time of implantation, when the blastocyst interacts with the endometrium and trophoblast cells breach endometrial blood vessels, thereby establishing the primordial uteroplacental circulation.

It is known that genetic variation within the gene and its regulatory area can influence gene expression level. An additional finding in the current thesis demonstrates that **polymorphisms** in the regulatory area of *TF*, *TFPI* and *HABP2* genes may influence mRNA and/or protein expression levels in the endometrium (Figure 19).

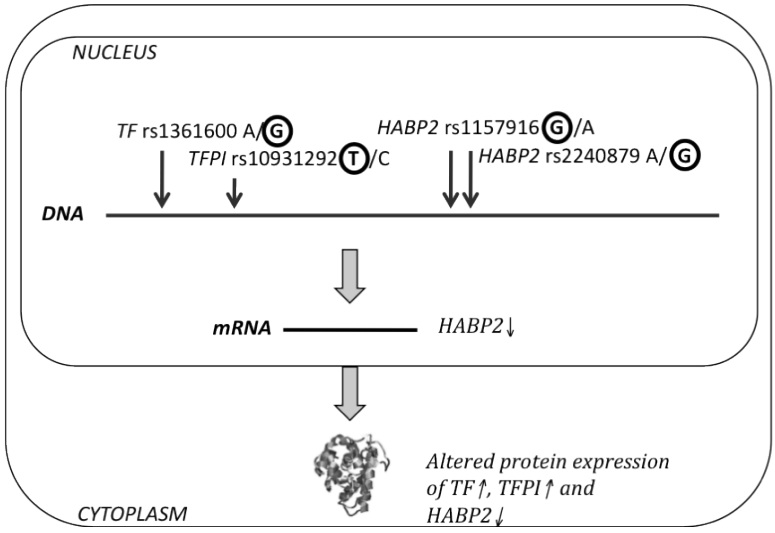

Figure 19. *Illustration of genetic variation in TF, TFPI, HABP2 genes and their influence on gene and/or protein expression levels in the endometrium.*

Women with *TF* rs1361600 G allele show higher TF immunostaining intensities in different cell types, and women carrying *TFPI* rs10931292 T allele were more frequently infertile and had higher TFPI immunostaining in the luminal epithelium. Our findings are in agreement with the previous studies, where the *TF* rs1361600 G allele

has been associated with increased plasma TF level (Arnaud, et al., 2000; Reny, et al., 2004), and the *TFPI* rs10931292 T allele has been associated with higher TFPI expression and is proposed as a genetic protective factor for deep vein thrombosis (Amini Nekoo and Iles, 2008). Our findings of *HABP2* gene polymorphisms are novel. Polymorphisms rs1157916 G/A and rs2240879 A/G in *HABP2* gene could serve as genetic risk factors for female infertility, the rs1157916 G allele and the rs2240879 G allele were more frequent among infertile women, meanwhile these women had lower *HABP2* mRNA and protein levels than controls.

Endometrial maturation to the receptive phase is also characterised by structural changes. One of these characteristics are endometrial **pinopodes**, that arise from the apical surface of the epithelial cells and cover the endometrial surface at the time of embryo implantation (Nikas, 1999). Pinopodes are believed to serve as endometrial receptivity morphological markers, although opposing results exist. In the current thesis, we detect the co-expression of endometrial receptivity factors and pinopodes. The presence of pinopodes significantly correlate with LIFR expression in the luminal epithelium, and with TF expression in the glandular epithelium and in stroma in fertile endometrium. Additionally we found that pinopodes are more abundant in endometria from fertile women than in endometria from women with unexplained infertility, referring to the aberrant endometrial maturation in these women.

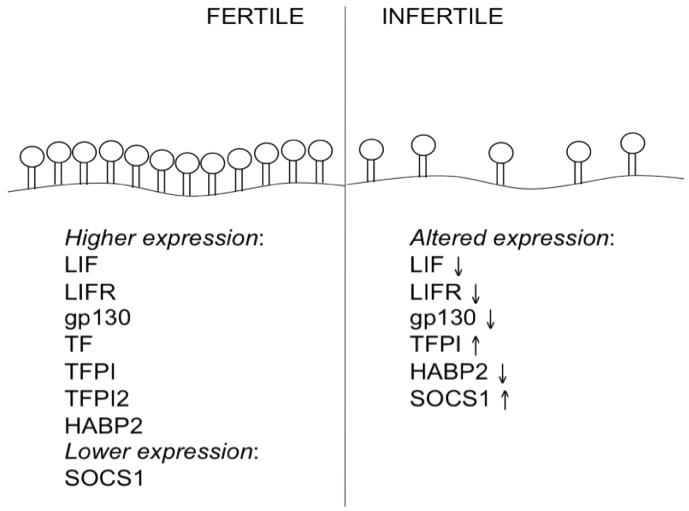

Figure 20. *Illustration of studies of single gene analysis of endometrial receptivity factors in fertile women and women with unexplained infertility.*

5.2 FACTORS IMPORTANT FOR IMPLANTATION-COMPETENT BLASTOCYST DEVELOPMENT

Formation of the implantation-competent blastocyst is as important as a receptive endometrium for successful embryo implantation. Next we analysed the whole gene expression pattern of embryos for identifying genes and gene networks activated within the blastocyst-stage embryos. Preimplantation development of embryos includes critical events such as the transition from maternal to embryonic genome activation, compaction, cavitation and blastocyst formation (Bell, et al., 2008). However, given the

scarcity of human embryos for research, the molecular mechanisms within the developing embryo are not well understood. One important finding in the current thesis is that a big proportion of the expressed genes in the blastocyst-stage embryos are involved in transcription regulation, and especially transcriptional down-regulation. Our finding is the human is well in line with the existence of transcriptional programmed waves identified in mice embryos, where maternal to embryonic gene activation shows two principal transient waves of *de novo* transcription, where the first wave peaks between the 2- and 4-cell stages and the second wave peaks at the 8-cell stage and precedes morula-to-blastocyst formation (Hamatani, et al., 2004).

Another important finding in the current thesis is the identification of one possible regulation mechanism for the extensive transcriptional re-programming in the developing embryos, namely identification of miRNAs. miRNAs are a class of small non-coding RNAs that regulate gene expression at the post-transcriptional level via directing either target mRNA degradation or translational repression (Bartel, 2004; Bartel, 2009), and they are involved in many biological processes, including development, tissue morphogenesis, cell growth, differentiation, apoptosis, metabolism, and others (Zhao and Srivastava, 2007). With our study, we are the first to carry out an extensive miRNA analysis in human embryos based on an *in silico* prediction model, and we identified several miRNAs in human blastocyst-stage embryos. Additionally, our miRNA prediction approach also demonstrates that a considerable proportion of miRNA target genes of transcriptional regulators are down-regulated in preimplantation embryos.

5.3 INTERACTIONS BETWEEN RECEPTIVE ENDOMETRIUM AND BLASTOCYST-STAGE EMBRYO

Successful implantation depends on the embryo's development into an implantation-competent blastocyst and the synchronised transformation of the uterus into a receptive stage. Endometrium secretes molecules that influence embryo development, while the presence of an embryo in the uterus triggers specific molecular and cellular responses within the uterus (Carson, et al., 2000). The success of the implantation further relies upon the two-way dialogue between the blastocyst and the endometrium. In the last study of the current thesis we focus on identifying the molecule-molecule interactions between the blastocyst and the endometrium at the time of implantation in humans. For that we applied, in collaboration with biostatisticians, a novel mathematical modelling approach.

It is ethically impossible to study human implantation *in vivo*, and most information concerning the molecular mechanisms involved in implantation is derived from animal models, which cannot fully be extrapolated to humans. One promising alternative is to apply computational analysis and integration methods to the knowledge obtained separately from human endometrial tissue analysis and from human embryos cultured *in vitro*. Our novel systems-biology analysis of human endometrial tissue and cultured embryos reveals several known and new genes and gene networks in the implantation process in humans.

The main interaction network in our study highlights the importance of cell adhesion molecules, including integrins, collagens and laminins in the implantation process. Indeed, in the initial stage of implantation the blastocyst interacts with the endometrium using adhesion molecules, followed by stable adhesion (Aplin, 1997). Also in focus of the first interacting molecules, we found cytokine–cytokine receptor interactions to be important, where osteopontin and LIF and LEP pathways intertwine. We also propose several new players in human embryo–endometrium interaction, including APOD, BGN, EDN1, FBLN2, FGF7, GAST, KREMEN1, NRP1, SERPINA3, VCAN and others.

Global gene expression analyses clearly demonstrate the complexity of a tissue regulation on molecular level, and that not solely one factor is crucial for implantation success in humans. As for instance seen in the case of LIF gene. LIF is one of the most important and studied genes related to implantation, and its crucial role in mice implantation has been demonstrated (Stewart, et al., 1992). In the current thesis we see clearly LIF pathway involvement in human embryo implantation. However, although *LIF* expression is an indicator of receptive endometrium, its role in the assessment of implantation potential in humans is controversial (Aghajanova, 2010), and the use of recombinant human LIF has failed to improve the outcome of IVF treatment in women with recurrent implantation failure (Brinsden, et al., 2009). Although the role of LIF in the human implantation process has been proved to be important, it seems not to be crucial, but rather a part of a highly coordinated orchestra. The complex implantation process is, like other systems of nature, in interrelationships of opposing forces, where affecting one factor in this balance, the opposing counterpart has to adjust in parallel for avoiding imbalances. This theory of equilibrium (Weghofer and Gleicher, 2009) could well explain the countless number of unsolved questions in human implantation process – there is not only a single answer since every answer is relative, depending on where the balance of any given system lies.

6 CONCLUSIONS

Adequate preparation of receptive endometrium and the establishment and maintenance of a viable embryo are prerequisites for successful embryo implantation. Research in the field of implantation in humans is actively ongoing, nevertheless the molecular bases of these preimplantation and implantation processes are not well known.

The main findings of the current thesis are:

- Identification of new and also previously known genes and pathways involved in endometrial preparation for the receptive phase, including JAK-STAT signalling pathway, coagulation cascade, focal adhesion, inflammatory responses, and others.

- Several genes and pathways that are important for endometrial receptivity are dysregulated in women with otherwise no clear reason for their fertility problems, including LIF pathway, coagulation pathway (TF pathway, HABP2), inflammatory responses, lipid metabolism, and detoxification.

- Abundant presence of pinopodes in fertile mid-secretory endometrium and their co-expression with LIFR and TF proteins in endometrial cells; meanwhile women with unexplained infertility demonstrate very few pinopodes at the time of implantation.

- Polymorphisms in the regulatory area of genes involved in blood coagulation pathway (*TF*, *TFPI* and *HABP2* genes) influence protein expression levels in endometrial cells, and the variants of *TFPI* and *HABP2* could serve as potential genetic risk factors for unexplained female infertility.

- Implantation-competent embryos demonstrate significant down-regulation of genes involved in transcription regulation, where one possible mechanism for this transcriptional re-programming could operate via microRNA molecules.

- Identification of new and previously suggested molecules and molecular pathways involved in embryo-endometrium dialogue at the time of implantation, including adhesion molecules, LIF pathway, gastrin, endothelin, versican and many others.

In conclusion, the current thesis gives new insights into the process of embryo implantation in humans. With different analysis methods applied, we detect several factors that are believed to have role in the preimplantation and implantation processes, and we identify several new molecules and molecular pathways in these molecular processes. Additionally, we demonstrate that dysregulation of these pathways could lead to fertility complications in women with unexplained infertility. Increasing our understanding of the genes and pathways involved in the key mechanisms of implantation will facilitate the development of strategies to manipulate endometrial function, embryo development and embryo-endometrium dialogue in order to promote successful implantation and alleviate infertility; or on the contrary, to inhibit fertility.

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