DEPARTMENT OF WOMEN´S AND CHILDREN´S HEALTH
Division of Obstetrics and Gynaecology
Karolinska Institutet, Stockholm, Sweden

ENDOMETRIAL RECEPTIVITY AND HUMAN EMBRYO IMPLANTATION: *IN VIVO* AND *IN VITRO* STUDIES

Sujata Lalit Kumar

Karolinska Institutet

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Cover illustration: Molecular network of endometrial receptivity and human embryo attachment to endometrial construct *in vitro*

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It is not the strongest of the species that survives, nor the most intelligent, but the one most responsive to change.

Charles Darwin

To my family

HE gives the barren woman a home, making her the joyous mother of children. Praise the Lord!

Psalms 113:9

Karolinska Institutet

Department of Women´s and children´s Health

Endometrial Receptivity and Human Embryo Implantation: *In vivo* and *In vitro* studies

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av

Sujata Lalit Kumar
Leg Läk

Huvudhandledare:
Professor Kristina Gemzell Danielsson
Karolinska Institutet
Department of Women´s and Children´s Health
Division of Obstetrics and Gynecology

Bihandledare:
Lalit Kumar PGL
Karolinska Institutet
Department of Women´s and Children´s Health
Division of Obsterics and Gynecology

Fakultetsopponent:
Professor Nick S Macklon
University of Southampton
Complete Fertlity Center
Southampton, United Kingdom

Betygsnämnd:
Professor Stefan Hansson
University of Lund
Clinical Sciences
Department of Obstetrics and Gynecology

Docent Helena Åkerud
Department of Women´s & Children´s Health
Uppsal Universitet,
Uppsala

Docent Kenny Rodriguez-Wallberg
Karolinska Institutet
Department of Clinical Sciences, Intervention and Technology

Stockholm 2013

ABSTRACT

Background: Infertility, one of the common gynaecological disorders, affects 10-15% women in their reproductive years. Despite the advances in assisted reproduction techniques and the best efforts of infertility specialists, implantation rates do not exceed more than 28-30%. Implantation failure is one of the major causes of infertility of couples with unexplained infertility. Understanding the mechanisms of human embryo implantation is important in the attempt to alleviate infertility, improve pregnancy rates and foetal health. This knowledge could also be further explored to develop new modalities for fertility regulation.

Objective: To understand endometrial receptivity and the human embryo implantation process through experimental and translational research using *in vivo* and *in vitro* approaches.

In ***Paper I,*** an *in vitro* three-dimensional embryo-endometrial cell culture model expressing receptivity markers ER-α, ER-β, PR-(A+B), PR-B, VEGF, LIF, IL-1β and COX-2 and $\alpha_V\beta_3$ and MUC1 was developed and tested for its progesterone regulation using anti-progestin mifepristone and gestagen levonorgestrel on the human embryo implantation process. It was found that none of the 15 embryos in the cultures exposed to mifepristone attached to the endometrial construct, whereas 10/17 in control and 6/14 in levonorgestrel groups did attach. This model was further utilised in ***Paper II*** to study the role of Leukemia inhibitory factor (LIF) in human embryo implantation by using a potent LIF inhibitor, PEGLA (PEGylated LIF inhibitor). Inhibition of LIF by PEGLA, inhibited blastocyst attachment to the *in vitro* model, down-regulated its expression of AKT and triggered apoptosis in the inner cell mass, as studied by immunofluorescence and real-time PCR. ***Papers III and IV*** explored large-scale progesterone regulated human endometrial receptivity markers during the implantation window in stromal and epithelial compartments using a laser capture microdissection and microarray analysis. Interestingly, the expression of both the mRNA and the protein for ectonucleoside pyrophosphatase/phosphodiestrase 3 (ENPP3) in the epithelial compartment were not detectable in the progesterone-depleted group, as studied by microarray, real-time PCR and immunohistochemistry. The major canonical pathways altered in epithelial compartment were oxidative phosphorylation and the mitochondrial dysfunction pathway. In the stromal compartment, 101 genes were potentially differentially regulated ($FC > 2$; $p < 0.05$). Real-time PCR analysis showed significant differences in the expression of SFRP4 (6.73; p=0.005), CTSC (2.3; p=0.04), SMARCA 1 (1.66; p=0.02), CPM (16.37; p=0.03), MGN5 (1.82; p=0.03), MT1G (-333; p= 0.003) and MT2A (-4.67; p=0.03) with progesterone. The major canonical pathways differentially regulated with progesterone in stromal cells, as analysed by IPA, were EIF2 signalling and the mitochondrial dysfunction pathway.

Conclusion: In this study, an *in vitro*-three dimensional embryo-endometrial cell culture model to investigate the human embryo implantation process was established and this shed more light onto the role of LIF in the embryo implantation process. This model could be used to examine the embryo-endometrial interaction that leads to successful implantation, as well as to develop new contraceptive agents. A novel endometrial epithelial progesterone-regulated receptivity marker, ENPP3, was identified and its potential application in ART needs to be explored further. The knowledge gained from the expression of progesterone-regulated genes in the endometrial, glandular and stromal compartments could assist in understanding the molecular mechanisms involved in endometrial receptivity and would be beneficial for improving fertility rates in women, as well as paving way to the development of new endometrium-based fertility regulating agents.

Key words: *3D-endometrial cell construct, human blastocyst implantation, receptivity markers, LIF, PEGLA, stromal cells, epithelium, progesterone regulation, ENPP3, endometrial receptivity.*

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals I-IV:

I. Lalitkumar PG, **Lalitkumar S**, Meng CX, Stavreus- Evers A, Hambiliki F, Bentin-Ley U, Gemzell-Danielsson K. **Mifepristone, but not levonorgestrel, inhibits human blastocyst attachment to an *in vitro* endometrial three-dimensional cell culture model.** *Human Reproduction, 2007, Nov 22:3031-7*

II. **Lalitkumar S**, Boggavarapu NR, Menezes Judith, Evdokia Dimitriadis, Jian-Guo Zhang, Nicos A Nicola, Gemzell-Danielsson Kristina, Lalitkumar P.G.L. **LIF inhibition blocks human blastocyst adhesion and implantation by down regulating AKT and triggering apoptosis.** *Fertil Steril. 2013 Oct; 100(4): 1160-1169.*

III. **Lalitkumar S**, Boggavarapu NR, Joshua Vijay, Byström Birgitta, Gemzell-Danielsson Kristina, Lalitkumar P.G.L. **Global gene expression profile of human endometrial epithelium: ENPP3 a novel progesterone regulated receptivity marker during the implantation window.** *In manuscript*

IV. **Lalitkumar S**, Boggavarapu NR, Joshua Vijay, Byström Birgitta, Gemzell-Danielsson Kristina, Lalitkumar P.G.L. **Progesterone mediated gene expression in human endometrial stromal compartment during the implantation window.** *Submitted for publication*

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LIST OF ABBREVIATIONS

ART	Assisted reproductive technique
CD	Cluster domain
EDTA	Ethylene diamine tetra acetate
ENPP3	Ectonucleotide pyrophosphatase 3
ER	Estrogen receptor
ET	Endometrial thickness
FC	Fold change
FSH	Follicle stimulating hormone
hCG	Human chorionic gonadotropin
HOXA 10	Homeobox A10
HPO	Hypothalamus pituitary ovarian axis
ICM	Inner cell mas
IHC	Immunohistochemistry
IPA	Ingenuity Pathway Analysis
IVF	*In vitro* fertilization
IW	Implantation window
LA	LIF antagonist
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
LIFR	LIF receptor
MMPs	Matrix metalloproteinase
P	Progesterone
PDGF	Platelet derived growth factor
PEG	Polyethylene glycol
PEGLA	PEGylated LIF antagonist
PR	Progesterone receptor
RT-PCR	Real-time PCR
TIMP	Tissue inhibitory of metalloproteinase
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

The intersection of assisted reproduction and genomic knowledge challenges us with innovative opportunities and intriguing questions. But the question is: how can human beings find a way to serve the worthy human needs without eroding human freedom and dignity? And if a way is found, how shall we do it? The ethical and physiological constrains involved in *in vivo* human embryo implantation research makes it difficult or nearly impossible to pursue. The research in this thesis introduces experimental and translational concepts in human female infertility, focusing on *in vivo* and *in vitro* studies. The *in vitro* studies were conducted in an *in vitro* human embryo-endometrial cell culture/implantation model.

1.1 INFERTILITY

Infertility is one of the major increasing gynaecological problems worldwide (Pandian, et al., 2005). Besides being a medical problem, it has considerable psychological morbidity on the individual couple with strong social implications (Edelmann and Connolly, 1986). When comprehensive investigations of the infertile couple are normal, about 15-30 % of couples still will have no apparent reason for their infertility and are thus classified as `unexplained infertility´(Templeton and Penney, 1982). Assisted reproductive technology, has given a new ray of hope for such couples. Despite the best efforts of assisted reproductive technology, the pregnancy rate is not more than 30%. Oftentimes, repeated IVF attempts add to the agony and frustration that couples face, not to mention a huge economical burden, both on the couple and society. Successful embryo implantation is a synchrony between the pre-implantation blastocyst and a receptive endometrium. Early events in human pregnancy - apposition, adhesion and attachment of the pre-implantation embryo to the maternal receptive endometrium still remain an enigma.

Despite the transfer of the best quality embryo, women still fail to achieve pregnancy. The issue behind this failure is a lack of methods to precisely

estimate the molecular maturity of the receptive endometrium and the reproductive potential of the embryo. The standard investigations presently available to evaluate the endometrium, which include examining endometrial thickness by ultrasound and histology, are insufficient to identify the molecular defects in the endometrium. The concept of natural selection of human embryos with influences on the endometrial decidualization may be central to the process of conception (Teklenburg 2010). Such a state where the endometrium is receptive for an embryo is called `receptive endometrium´. In this relationship between the embryo and the endometrium, three fundamental questions need to be addressed:

1. What is the molecular signature of a receptive endometrium and its influence on the development/viability of pre-implantation embryo?
2. Are there signals from the pre-implantation blastocyst to the endometrium that initiate the implantation process?
3. Is it a reciprocal mechanism between the endometrium and pre-implantation stage embryo that leads to successful implantation?

1.2 INFERTILITY-HISTORICAL PERSPECTIVES

1.2.1 Fertility in ancient writings

Women have been the symbol of fertility since antiquity. Fertility is a concept deeply rooted in all societies and cultures, evolved from the Mother Nature: wells, springs and rivers reflecting an awareness of rain and water as having power to regenerate the parched and *barren* earth. Procreation and fertility, a fundamental evolutionary process since the dawn of human race is essential to sustain life on planet Earth.

Fertility symbols were clearly identified in the pre-historic times of ancient civilization in all parts of the world. The fundamental role of gods in the treatment of infertility and childbirth was evident in all major ancient civilizations: Indus, Mesopotamian, Chinese and Mayan, with depictions in cave paintings, monuments and sculptures. Infertility was considered to be divine punishment until the medieval renaissance, when it was recognised as any other ailment demanding investigation and treatment. The early records of infertility date back to the pre-historic time of ancient civilization.

1.2.1.1 Biblical Concept

The first chapter of the first book in the Bible quotes `Be fruitful, and multiply: fill the earth and subdue it´ (Genesis 1:28) as a command from God to Adam and Eve. Both, in the Old and New Testament, there is a strong relevance to the divine grace for continued human progeny. The desperate and long agony of childlessness is pronounced both in men and women, and this is narrated in the story of Jacob and Rachel (Genesis 30:2). Further on, fertility blessing was more evident, when Abraham´s wife Sara, who was beyond her reproductive years of fertility, could bring forth a child. This emphasized that fertility was essential for survival, besides reflecting the symbol of wealth. The very fact that women were forbidden to have intercourse during the time of menstruation and 7 days thereafter (5+7=12 day of the menstrual cycle), only to commence after a ritual bath, reveals that the ancient biblical civilization (>3000 years) already knew about the most fertile days of the menstrual cycle.

1.2.1.2 Vedic concept

Evidences of the first writings regarding childlessness were found 5000 years ago in Vedas. The ancient Sanskrit texts of Hindu Wisdom acknowledged that men could also suffer from the anguish of childlessness.

1.2.1.3 Egyptian papyrus

The first systematic records were found in Egyptian papyrus documents, the gynaecological Papyrus (1825 BC), where women´s ailments were cured either by their faith in the fertility gods (Nephtys) or magic responses (Dawson, 1967). The Egyptians excelled in the development of diagnosis of early pregnancy by 40%, where the pregnant woman was asked to urinate on a cloth bag containing wheat and barley, only to wait for the germination. If both the seeds germinated, she would likely bear a child. This observation was based on the evidence that human chorionic gonadotrophin (hCG) promoted adventitious root in cuttings of *Begonia semperflorens* and *Vitis vinifera* (Leshem and Lunenfeld, 1968). Additionally, the Egyptians addressed male infertility. Soranus of Ephesus (177-98), a great Greek scholar and practising physician in Rome, described the female anatomy,

physiology and related pathological conditions in his book *Gynaecology* (Soranus and Temkin, 1956).

1.2.1.4 Greek concept of infertility

Little was known until the era of Hippocrates (460-377 BCE), when he brought a rationalistic perspective to medicine, rather than magic thinking. The gynaecological and obstetrical pathologies were addressed with relevance to many infertility treatments in his books: *Diseases of Women, Nature of Women, The generating seed and the Nature of child (King, 1998).* Thus medieval physicians followed these treatment concepts up to the 19th century.

1.2.1.5 Arabic concept

More knowledge with high level scientific reasoning and hypotheses for factual deductions were supplemented from records of the Arab School, AD 700-1200. Avicenna (980-1037) introduced the concept of masculine and feminine causes of infertility in his writings: The book of *Healing* and *The Canon of medicine (Qanun)*.

1.2.1.6 Medieval Renaissance and the early 20th century

Medieval medicine was largely practised based on the knowledge rooted from the Greeks and the writings of Hippocrates. With the dawn of scientific renaissance, the understanding of infertility changed with the elegant illustrations of the female pelvis by Leonardo da Vinci (*Corporis Fabrica,* 1543). Some of the greatest discoveries of Leeuwenhoek, *the microsope* and *the sperm*, (correcting an earlier misconception that sperm came from bones), enlightened what had been the mystery of fertilization. However, the ovum was not discovered until 1826 (Von Baer, 1956), and then later in 1875, it spurred the novel conclusion: fertilization occurs when spermatozoa penetrate the egg (Austin, 1961, Hertwig, 1906) . Thus 19th-20th century medicine embarked on a remarkable path of progress in the diagnosis and treatment of infertility. Diagnostic methods began to emerge at the beginning of 20th century with the advent of the Aschiem Zondek test, Huhner´s postcoital test, the tubal insufflation test, etc. However, fertility endocrinology was still on its way to establish its worth with limited therapeutic application until the

middle of the century. The discovery of human chorionic gonadotropin (hCG) by Carl Gemzell was a big leap in the modern management of infertility, as it lead to the development of routinely used pregnancy test and ovarian stimulation, which is the cardinal step in assisted reproductive technology (ART)(Gemzell, 1962, Gemzell, et al., 1966). Later, in 1978, Professor RG Edwards and his team successfully recorded the birth of the first IVF baby in England. Since then, the understanding of the human embryo implantation process has rapidly progressed and remarkable discoveries have been made in the history of infertility treatment.

1.3 THE UTERUS

One cannot refuse the fact that uterus is a highly dynamic endocrine organ, dependent on the effects of ovarian steroid hormones for executing cyclical synchronous events dedicated to prepare fertile land for the seed to be sowed (Fritz and Speroff, 2010). The uterus, mostly referred as the `Womb`, was first described by Soranus of Ephesus in the second century AD (Soranus and Temkin, 1956).

1.3.1 Embryonic development of the uterus

As early as the 10^{th} week of embryonic life, the uterus, Fallopian tubes and the upper one-third of the vagina develop from the fusion of Mullerian ducts (Acien, 1992). The endometrium differentiation is complete by the 20^{th} week, which is derived from the mucosal lining of the fused Mullerian ducts. By the 22^{nd} week, the canalization is complete with the formation of the uterine cavity, cervical canal and the vagina. The mesenchyme tissue develops into uterine stroma and smooth muscle cells.

1.3.2 Anatomy of the uterus

The uterus is a pear shaped organ in the female reproductive system in which the fertilized eggs, when successfully implanted, result in pregnancy. It is composed of three layers: an outer thin serosal layer; a thick middle layer of smooth muscle; myometrium and an inner mucosal layer; the endometrium (Fig 1).

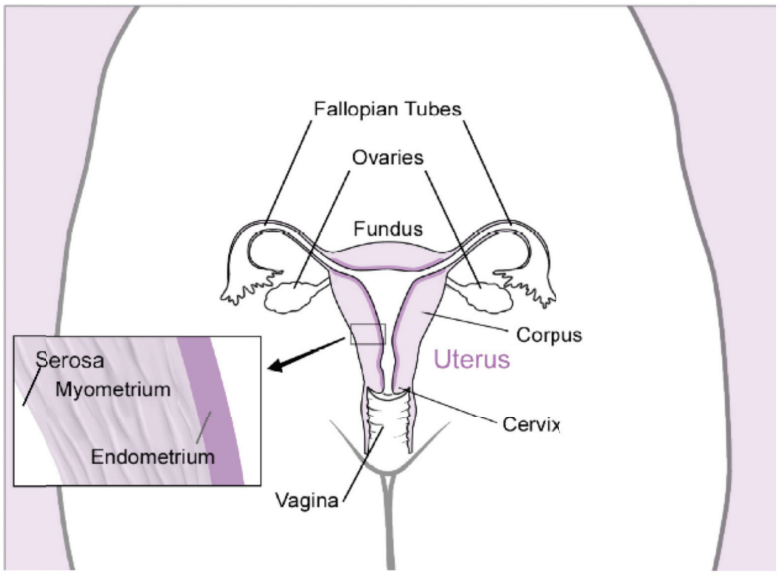


Figure 1. Female reproductive system: Anatomy of uterus (Figure from http://commons.wikimedia.org)

1.3.3 Endometrium

The endometrium is a complex and dynamic tissue undergoing more than 400 cycles of regeneration, differentiation and shedding during a woman's lifetime (Jabbour, et al., 2006). The complex structure of the endometrium is not attained until puberty (Kurman and Blaustein, 2002). Morphologically, the endometrium is classically defined with its two layers: the `Functionalis´ layer- the upper two-thirds and the ` basalis´ layer, the lower one-third (Fig 2). It is the functionalis layer that undergoes cyclical degeneration and proliferation, and is destined to adequately mature for embryo implantation.

The basalis layer is the germinal layer from which renewal occurs following menstrual shedding of the functionalis layer.

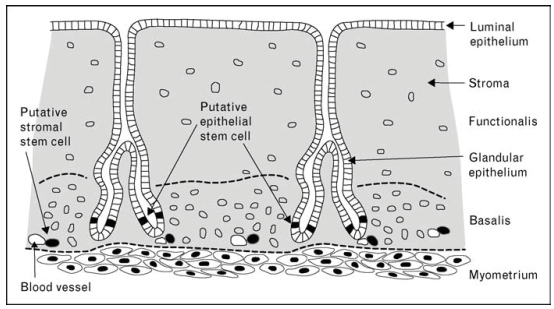


Figure 2. Endometrial morphology. Reproduced with permission (Gargett, et al., 2007).

1.3.3.1 Endometrial cell types

The endometrium constitutes of two major compartments: the glandular or epithelial cell compartment and the stromal compartment

1.3.3.1.1 Epithelium

This layer is interconnected with specialized regions of plasma membrane containing cell junctions and is divided into luminal (surface or superficial) epithelium and glandular epithelium submerged into the stromal compartment (Fig. 2). The luminal epithelium covers the endometrial surface, functioning as a primary barrier of defence against infections, as well as serving as a surface for the attachment of the blastocyst during the implantation process. It is not normally receptive and must transiently acquire this state to allow the initial apposition and attachment of the blastocyst (Dey, et al., 2004). The glandular epithelial cells consist of a single layer of columnar cells with varying morphology, depending on different phase of the menstrual cycle, that secrete glycoproteins, glycogen, cytokines and express cell adhesion molecules during the secretory phase of the menstrual cycle (Cunningham, et al., 2010).

1.3.3.1.2 Stromal compartment

The stromal compartment includes mainly stromal cells, leucocytes, macrophages and endothelial cells that vary in their numbers according to different phases of

menstrual cycle. They produce matrix metalloproteinases (MMPs), Tissue inhibitor metalloproteinases (TIMPs), vascular endothelial growth factor (VEGF) and many other cytokines that are involved in angiogenesis and recruitment of inflammatory mediators in endometrial shedding during menstrual phase. The stromal cell compartment is generally less well studied than the epithelial compartment. Although largely thought to be undergoing independent cyclical changes, in recent years, there is compelling evidence that there exists a paracrine epithelial effect on the stromal cells. Mitoses of stromal cells reach a maximum (about 10 per 1000 cells) around the time of ovulation and this is later reduced to almost zero over the peri-implantation period, with an increase when nearing the menstrual phase (Noyes, et al., 1975). The stromal cells prepare for implantation by increasing their mean nuclear profile and stromal cell density from LH+2 to LH+6. During this period, the cells are less densely packed than at any other point of time, with maximal oedema, thus making it possible for the blastocyst to implant. At this same time, there are numerous pre-decidual and peudo-decidual changes closer to the blood vessels providing the nutritive support by secreting glycogen (More, et al., 1974).

1.3.3.1.3 Decidual changes

Once the embryo invades the luminal epithelium, a decidual response is elicited, characterised by transformation of stromal cells into secretory, epitheloid-like decidual cells, and influx of specialized uterine immune cells and vascular remodelling (Brosens, et al., 2009, Brosens, et al., 2002). In humans, this decidual response is primarily under maternal control and initiated in the mid-secretory phase of each menstrual cycle, irrespective of whether pregnancy has occurred or not. In the absence of pregnancy, spontaneous menstruation occurs, thus signifying that the integrity of decidual endometrium is dependent on the continuous progesterone signalling. Declining progesterone levels incite a non-conducive secretory repertoire of decidual stromal cells, which include expression of pro-inflammatory cytokines, chemokines and matrix metalloproteinases, activating a sequence of events and tissue breakdown resulting in menstruation (Jabbour, et al., 2006, Macklon, et al., 2002).

1.3.3.1.4 Endometrial stem cells

Human endometrium has extensive regenerative capacity, growing more than 5mm in 7 days, and is essential for endometrial maturation during the implantation window. In non-menstruating species, there is endometrial regeneration and apoptosis with every oestrous cycle. The concept of endometrial regeneration mediated by endometrial stem cells (Fig 2) was proposed many years ago and adult stem cells in the endometrium were extensively studied by Garget´s group (Chan, et al., 2004). These stem cells were identified as clonogenic and they possessed tissue regeneration activity (fig 2). Colony-forming cells were purified from epithelial and stromal cells. Proliferative and menstrual phase endometrium expressed a high number of a sub-population of stem cells called side-population cells (Kato, et al., 2007). There have been various markers identified, showing their perivascular location in the functionalis and basalis layers of endometrium. Multipotent stromal cells express markers such as $CD146^{+}PDGF\text{-}R\beta^{+}$ (Schwab and Gargett, 2007). A small population of colony-forming cells that were $EpCAM^{+}$ were identified in epithelial cells of the endometrium (Schwab, et al., 2005). The clinical reports of increased IVF take home baby rates after endometrial biopsies or curettage indicates an active role of endometrial progenitor cells, which otherwise likely remain quiescent (Gargett and Ye, 2012). It is possible that these stem cells may also have an active involvement in the regeneration of endometrium and any functional impairment can be a predictor for infertility disorders/thin endometrium. Recently, stem cells isolated from endometrial biopsies/menstrual blood and human embryonic stem cells have been proposed as promising treatments for infertility disorders, endometriosis, dysfunctional uterine bleeding and Asherman´s syndrome (Deane, et al., 2013).

1.3.4 Heterogeneity in endometrial cell types

It is a known fact that in a given endometrial tissue biopsy, significant morphological variations are observed under microscopic examination. Central to the ovarian steroid responses, the functionalis layer, in comparison to the basalis layer at the upper or fundal part of the uterus, is more responsive due to the higher number of estrogen and progesterone receptors in these areas. Therefore, the study

of endometrial morphology should be more confined to the functionalis from the upper part of the uterus, as inclusion of the basal layer or the isthmic part will introduce unnecessary variance. The variances within the functionalis layer itself, termed microheterogeneity, have been discussed earlier (Dockery, et al., 1988), but the biological significance is yet to be explored.

Therefore, for the stated reasons, endometrial biopsies should ideally be obtained from the upper/fundal part of the uterus and great attention should be paid to exclude the basalis or the isthmic specimens. Such a specimen has revealed reduced variance in analysis of endometrial protein markers (Li, et al., 1991). With concerns to the microheterogeneity, the studies evaluating the gene expression of endometrial stromal and epithelial compartments in this thesis have been addressed.

1.3.5 Endometrial cycle

The endometrial cycle, primarily regulated by the hypothalamo-pituitary-ovarian axis, undergoes a sequence of histological events (the proliferative phase and secretory phase) endowed to fulfil its goal `to receive and nurture an embryo´. In a failure to do so, it succumbs to `the fall – the menstrual phase´, undergoing aseptic inflammatory/apoptotic breakdown and shedding (Fig 3). The cyclical transformation during the reproductive life is an integrated evolutionary cycle of endometrial growth and regression that is repeated some 400 times during the adult stage of the human female. A normal or ideal menstrual cycle varies between 28-35 days. Elegant studies of these events have been described in the literature long ago and are practised even today (Noyes, et al., 1975).

1.3.5.1 Morphological changes during menstrual cycle

The proliferative phase or early follicular phase marks the growth of follicles in the ovary under the influence of follicle stimulating hormone (FSH) from the pituitary gland and estrogen from the ovary. Gradually increasing oestrogen, through negative and positive feedback, results in the development of a dominant follicle in the late follicular phase and eventually leads to ovulation (Fig 3). In the

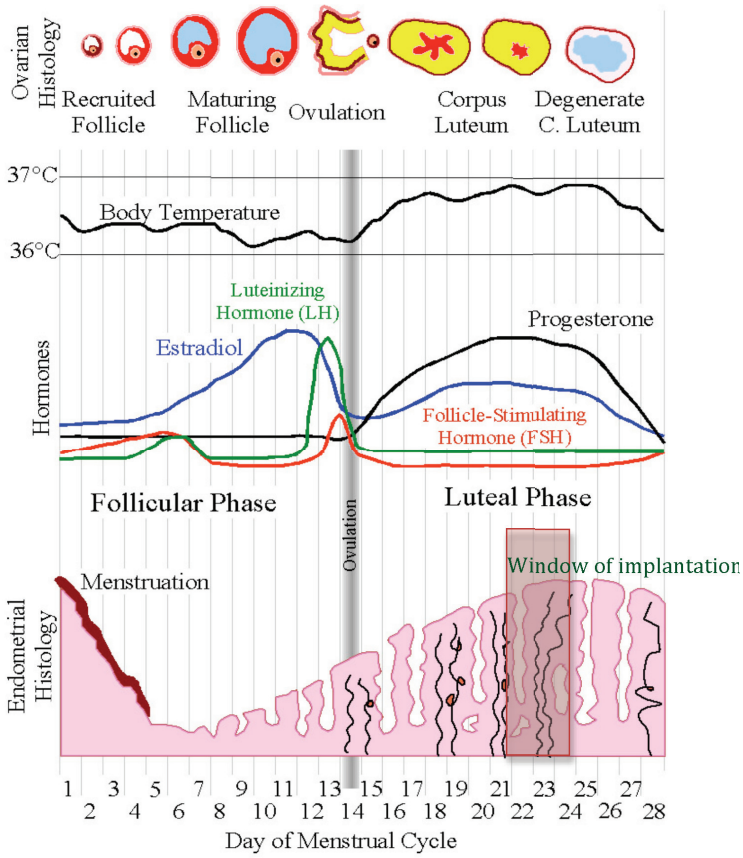


Figure 3: Different phases of menstrual and ovarian cycle in women. Window of implantation ranges from cycle day 21-23 (Figure modified from http://simple.wikipedia.org)

endometrium, menstruation ends in the early follicular phase and renaissance begins in response to estrogen that marks the proliferative phase. Thus an estrogen-dominant phase results in endometrial regeneration characterised by an increase in mitotic activity (increased metabolic activity, cell number and cell size). The early proliferative phase is characterised by the presence of narrow glands, thin surface epithelium and compact stroma. During the mid and late

proliferative phase, the endometrium becomes thick with prominent stroma (King and Critchley, 2010).

1.3.5.2 Secretory phase

The secretory phase is marked by the gradual production of increasing amounts of progesterone and estrogen by the corpus luteum. Thus, the progesterone-dominated phase is characterised by the presence of spiral arterioles, the decidualization of the cells, and the polarization of the glandular epithelium with visible presence of luminal secretions in order to prepare itself for the arrival of the embryo. MMPs and other inflammatory mediators aid this process (Gaide Chevronnay, et al., 2012). With the failure of the arrival of the embryo, and with an intrinsic life span of 12 days, the corpus luteum degenerates into an inactive structure called the corpora albicans. This shuts off the progesterone and estrogen secretion, and eliminates the negative feedback signal to the hypothalamo-pituitary gonadotropins.

1.3.5.3 Menstrual phase

As the corpus luteum degenerates and hormone production fails, blood vessels contract, resulting in apoptotic and necrotic changes in the endometrial functionalis zone. Just about 2 days after the corpus luteum ceases to function, or 14 days after ovulation, the endometrium begins to shed. Menstruation ensues for 3-7 days, as the next ovulatory cycle revives.

1.3.5.4 Role of ovarian steroids in endometrium

Estrogen plays an important role in the proliferation of the functional layer of the endometrium after the menses (Fig. 3). It peaks 48 hrs before ovulation and rises along with progesterone during its release from the corpus luteum (Groothius P G et al., 2007). It acts via its nuclear receptors estrogen receptor-α (ER-α) and estrogen receptor-β (ER-β) that are encoded by two different genes in two different chromosomes. They up-regulate the expression of several endometrial proteins like VEGF and lactoferrin (Critchley and Saunders, 2009). Progesterone produced mainly by corpus luteum in the secretory phase acts via its receptors progesterone receptor-A (PR-A) and progesterone receptor-B (PR-B), derived

from the same gene. The protein expression is down-regulated by estrogen. Among the many proteins regulated by progesterone are the IGFBP-1 (Insulin-like growth factor binding protein 1) and glycodelin (pp-14), which play a role in endothelial differentiation and immune response during pregnancy (Young and Lessey, 2010).

1.3.6 Endometrial receptivity/ Implantation window

The endometrium is not primed for implantation for most of the menstrual cycle. It acquires a transient receptive state when capable of receiving a pre-implantation blastocyst, starting approximately from postovulatory day 7 and extending up to day 9, corresponding to cycle day 20 to 24 (fig 3) in an average 28 day cycle. The concept of uterine receptivity was first stated in the literature by Psychoyos (Psychoyos, 1986). It is addressed with different terminologies in the literature: Implantation window (IW), uterine receptivity, and window of receptivity. Thus, a very limited period IW synchronizes implantation with the developing embryo, which serves as a critical mechanism to select against developmentally impaired but potentially invasive embryos. In regard to this concept, a population study evaluating the natural conceptions demonstrated that implantation beyond the normal period of endometrial receptivity had a strong association with early pregnancy loss (Wilcox, et al., 1999). An inadequate/impaired receptive endometrium may itself be a protective mechanism for the natural selection of embryos. More information about endometrial receptivity comes from patients with infertility after IVF treatment. A refractory period after the time of endometrial receptivity may be a natural mechanism to eliminate the developmentally impaired embryos.

1.3.6.1 Hormonal regulation of endometrial receptivity

Progesterone (P) is an essential hormone for the female reproductive system. In 1929, Corner and Allen recognized that low P might cause pregnancy loss (Corner and Allen, 2005). After 20 years, the concept of luteal-phase deficiency was coined by Georgianna-Segar Jones in 1949 (Jones, 1991). In prepubertal females and in the preovulatory phase, the blood levels of P are at the lower limits of

immunoassay sensitivity: <1ng/ml. During the luteal/postovulatory phase, P ranges from 3-15ng/ml. The main source of P is the adrenal gland in the follicular phase and eventually is in the ovary after ovulation (Speroff and Fritz, 2005). Prior to LH surge there is a mild but rapid rise in P levels (Hoff, et al., 1983). After ovulation, all circulating P is produced by the corpus luteum and if pregnancy occurs, this production continues until the 7th week when there is a shift and the placenta takes over. P is vital for the development and maturation of the endometrium and subsequently for the implantation of the embryo (Aitken, 1978). Implantation does take place successfully in ovariectomized monkeys with exogenous administration of P, confirming its critical role in embryo implantation (Ghosh, et al., 1994). It binds to PR-A and PR-B receptors that are distributed in the epithelial, stromal and smooth muscle cells of the human uterus and exerts its effects (Press, et al., 1988).

1.3.6.2 Markers of endometrial receptivity

In humans, the best marker of endometrial receptivity is the implantation of blastocyst itself (Klentzeris, 1997, Younis, et al., 1996). Several emerging factors that determine the implantation window have been unravelled and many are still being identified, but no explicit receptivity marker is yet defined. Some of the important molecular markers suggested in the literature are cell adhesion molecules (integrins, cadherins, selectins, immunoglobulins and mucins), cytokines (LIF, IL-6, IL-11 IL-1), growth factors (VEGF, CSF, TGFβ and prostaglandins.

1.3.6.2.1 Cell adhesion molecules

Integrins are cell adhesion molecules anchored to the plasma membrane. One of the well-characterized markers relevant to endometrial receptivity is αvβ3 integrin (Creus, et al., 1998). This appears in the luminal surface of epithelial cells during the peri-implantation period. Its expression is regulated by HOXA10 (Daftary, et al., 2002) and the low expression of $\alpha_v\beta_3$ integrin is correlated to infertility and success in IVF. MUC1, a carbohydrate glycoprotein, extends from the luminal surface, forming the glycocalyx layer and it is expressed during the IW. Trophinin, L-selectin ligand and heparin-binding growth factor are luminal

endometrial biomarkers with a potential role in embryo attachment (Fukuda, et al., 1995).

1.3.6.2.2 Cytokines and growth factors

The expression of various growth factors and their receptors in the uterus in a temporal and cell specific manner during the peri-implantation period suggests that these factors are imperative for implantation (Carson, et al., 2000, Cross, et al., 1994, Giudice, 1994, Lim, et al., 2002, Norwitz, et al., 2001, Paria, et al., 2002, Tazuke and Giudice, 1996). Cytokines are a group of glycoproteins mediated by specific receptors that modulate a variety of cellular functions, such as cellular proliferation and differentiation and play a major role in inflammatory-like processes in menstrual cycle and are critical for implantation.

1.3.6.2.2.1 Leukaemia inhibitory factor

Leukaemia inhibitory factor (LIF) is involved in the regulation of differentiation, proliferation, and survival of many types of cells, including the blastocyst and uterine endometrium (Hilton, 1992). The action of LIF is mediated by the LIF receptor complex, a heterodimer of the LIF receptor and gp130, a common receptor for the interleukin-6 (IL-6) cytokine family (Heinrich, et al., 2003). Activation of LIF-receptor-gp130 complex activates several signal transduction pathways, with the Janus-Kinase-signal transducer and activator or transcription pathway in the uterus both in mice and humans (Cheng, et al., 2001, Marwood, et al., 2009). LIF is one of the first cytokine shown to be important in human embryo implantation (Stewart et al., 1992). Progesterone is a major regulator of LIF expression and coincides with progesterone dominance. Treatment with antiprogestin, mifepristone immediately after ovulation reduces endometrial glandular LIF (Danielsson, et al., 1997). Uterine expression of LIF increases during the luteal phase in many species, including mice, monkeys and humans (Bhatt, et al., 1991, Charnock-Jones, et al., 1994, Laird, et al., 1997, Shuya, et al., 2011, Yue, et al., 2000). LIF antibodies administered to the uterine lumen in monkeys during the peri-implantation period reduce blastocyst implantation, further supporting its essential role in implantation process (Kojima, et al., 1994, Sengupta, et al., 2006, Shuya, et al., 2011, Yue, et al., 2000). It is expressed in the glandular and luminal epithelial cells of the endometrium in response to the

estrogen and progesterone hormones (Sengupta and Ghosh, 2000). Additionally, it is co-expressed with the expression of pinopodes in the receptive endometrium (Aghajanova *et al.* 2003). There have been conflicting data concerning the levels of LIF in uterine flushings for the prediction of fertile status during the IW. Whilst low levels are suggested as predictors of implantation (Ledee-Bataille et al. 2002), high levels are indicative of inflammation. Interestingly, in unexplained infertility patients, lower levels of LIF are observed (Laird et al. 1997). Thus an optimal level of LIF required for implantation has yet to be studied.

1.3.6.3 Clinical assessment of endometrial receptivity by ultrasound

Endometrial thickness (ET) is the most widely used ultrasound parameter to evaluate endometrial receptivity in infertility clinics. Although, it offers the advantage of being non-invasive and clinically practical, the thickness measured fails to reflect molecular maturation of the receptive endometrium. However, several ultrasonography parameters are proposed as predictors of endometrial receptivity, despite conflicting views.

Endometrial thickness varies over the menstrual cycle, reaching its maximum around ovulation (Fleischer, et al., 1986, Friedler, et al., 1996). Yet, no standardization exists to the exact timing of ultrasonographic measurement that best predicts the occurrence of pregnancy. It has been suggested that endometrial thickness <6mm has high negative predictive value when estimating endometrial receptivity (Gonen and Casper, 1990, Gonen, et al., 1989, Shapiro, et al., 1993). Conversely, ET >6mm has low positive predictive value in endometrial receptivity and cannot predict implantation either (Glissant, et al., 1985, Oliveira, et al., 1997, Welker, et al., 1989, Zollner, et al., 2012).

The echogenecity of the endometrium changes over the menstrual cycle. The proliferative phase is characterized with three hyperechogenic lines (classically defined as triple-line endometrium), representing uterine cavity and the myometrial-endometrial junctions on each side. Few studies, though, favor the triple-line pattern for possibility of pregnancy (Bustillo, et al., 1995, Serafini, et al., 1994, Sher, et al., 1993), conflicting views have been reported (Merce, et al., 2008, Puerto, et al., 2003). The other parameters relevant to endometrial receptivity are endometrial blood flow based on pulsatility and resistance indices

measured by color Doppler ultrasound. (Friedler, et al., 1996, Nastri, et al., 2012). However, conflicting results raise suspicion towards their significance in routine clinical practice (Alcazar, 2006).

1.3.6.4 Transcriptomics studies on Endometrial Receptivity

Microarray technology has been used to study the gene profile of the endometrium during different phases during the past decade. Ponnampalam et al. compared the gene expression profiles across the menstrual cycle and found that 425 genes were differentially regulated during this time (Ponnampalam, et al., 2004). Talbi et al., carried out similar studies on four different phases of the endometrium and found that in a Principle Component analysis (PCA) on the transcriptome, different biological processes take place during the different phases, so a specific transcriptome profile exists (Talbi, et al., 2006). The gene expression profiles during the endometrial receptive phase have shown huge variance in their results due to endometrial biopsy from different patient population, processing and additionally microarray analysis (Borthwick, et al., 2003, Carson, et al., 2002, Haouzi, et al., 2012, Kao, et al., 2002, Mirkin, et al., 2005, Riesewijk, et al., 2003). The consensus molecules derived from different microarray studies on endometrial receptivity are the structural protein osteopontin, transporter apolipoprotein D and the signalling molecules Dickkopf/DKK1 (Achache and Revel, 2006). Each of the above studies revealed many candidate genes that defined the receptive endometrium; however, they failed to define a set of genes that could be used as markers for endometrial receptivity. It is difficult to derive a conclusion for endometrial receptivity genes using samples from women with unexplained infertility, as the samples have heterogeneous molecular profiles, due to different idiopathic reasons. Nonetheless, a recently described endometrial receptivity array (ERA) has shown that genomic array could be a promising diagnostic tool for endometrial receptivity (Diaz-Gimeno, et al., 2013).

1.3.6.5 Tools to study hormonal regulation of endometrial receptivity

1.3.6.5.1 Mifepristone

Mifepristone (RU486) is an orally active steroidal antiprogestin, executing its biological actions by binding with the PR. It also binds strongly to the glucocorticoid receptor and, to a lesser extent, the androgen receptor but not to the mineralocorticoid receptor (Raynaud, et al., 1985). Mifepristone has both agonistic and antagonistic actions. The effects depend on the dose and phase of the menstrual cycle administered, exhibiting either direct action on the ovary or through the HPO axis. Endometrial development during the implantation period is not affected after treatment with mifepristone in the follicular phase (Swahn, et al., 1988). However, low dose (1mg) continuous administration for a period of 1 month has shown disturbed endometrial development with prolonged follicular phase and LH surge (Batista, et al., 1992). Once weekly administration (5mg) has shown to inhibit endometrial maturation without inhibiting ovulation (Gemzell-Danielsson, et al., 1996). Given immediately after ovulation, 200mg of mifepristone inhibits the secretory development of the endometrium, without disturbing the menstrual cycle or the secretion of ovarian steroids (Gemzell-Danielsson, et al., 1996, Swahn, et al., 1990). It has been shown that this highly potent antiprogestin, when administered on LH+2 in the above dosage and treatment, inhibits embryo implantation and pregnancy in humans (Gemzell-Danielsson, et al., 1993). Thus, this action was further explored for its clinical usage and has been proposed to be a promising fertility-regulating agent (Baird, 1993).

1.3.6.5.2 Levonorgestrel

Levonorgestrel (LNG), a progestin classified under second-generation oral contraceptives, is the active isomer of norgestrel. Norgetsrel is a racemic mixture of d-norgestrel and l-norgestrel (Levonorgestrel). The contraceptive effects are due to the combined action on the cervical mucosa, endometrium, Fallopian tubes and ovary. The cervical mucous becomes thick and impermeable to sperm transport and presumably, to the progestational influence on the peristalsis and secretion of the Fallopian tube. At a dose of 400 μg of LNG before the LH peak,

the gonadotropin surge was suppressed with decreased estrogen and progesterone levels and cervical mucus score when compared with post LH surge administration (Spona, et al., 1975). Single administration of the same dose of LNG, 3-10 hours after intercourse decreased the number of sperms recovered from the uterine cavity due to the alkalinisation of the intrauterine fluid and resulted in immobilization of the sperm as well as increased viscosity of the cervical mucus (Kesseru, et al., 1974).

1.3.6.5.3 PEGylated LIF antagonist

A potent LIF receptor antagonist (LA) was developed by mutating key amino acids of human LIF that interact with gp 130 and the LIF receptor (Fairlie, et al., 2004). Since LA itself has a short half-life in serum, conjugation with polyethylene glycol (PEG) increases serum half-life. PEGylation also protects LA from proteolytic cleavage with good renal clearance (Harris, et al., 2001, White, et al., 2007). This molecule PEGLA, when administered parenterally and vaginally, was localized to epithelial cells of the uterine endometrium with reduced STAT3 phosphorylation, and effectively blocked implantation in mice (Menkhorst, et al., White, et al., 2007). However, no study has been done to see its effect on human embryo implantation.

1.4 FERTLIZATION AND HUMAN EMBRYO TRANSPORT

Fertilization of the ovum occurs in the ampulla part of the Fallopian tube and the fertilized oocyte enters the uterine cavity about 72-96 hours after ovulation as a morula (Croxatto et al., 1978). The embryo undergoes a series of cell divisions and genomic activation, compaction and cavitation to form a blastocyst (Fig 4). Within 24 hours of fertilization, the zygote undergoes cleavage to form the blastomere. In the early morula (16-cell stage), the cells get polarized and differentiate into two different populations: the inner cell mass forms the embryo proper and the outer cell mass develop into the trophoblast and eventually the placenta. The inner cell mass gives rise to the embryo and all the organs. Approximately 24 hours later, fluid accumulates in the intercellular spaces of the inner cell mass, which increases the hydrostatic pressure, and the blastocyst cavity

is formed as it transforms into the blastocyst. The glycoprotein surrounding the embryo (zona pellucida) is lysed by components in the uterine fluid and the blastocyst hatches for implantation (Edwards 1994).

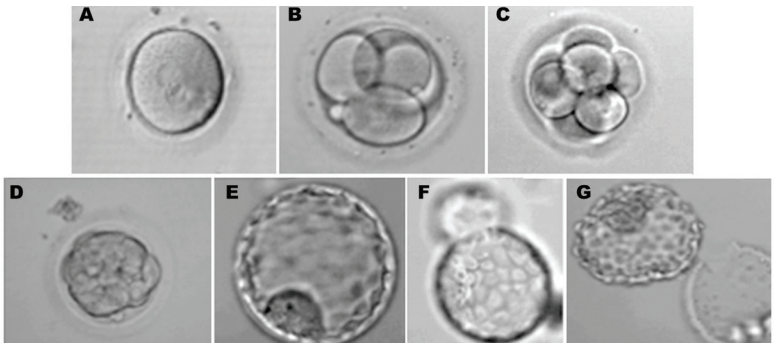


Figure 4: Different developmental stages of human embryo. A) Fertilized oocyte with 2PN stage. B) Embryo at 4-cell stage. C) Embryo at 8-cell stage. D) Morula. E) Expanded blastocyst. F) Hatching blastocyst. G) Hatched blastocyst

1.4.1 *In vitro* development of the Embryo

Embryo development *in vitro* is invariably slower than that *in vivo* and the viability of *in vitro* grown embryos decreases progressively as the time in culture is increased. Even under most favourable conditions, embryos take at least 20% longer to develop as evidenced in murine studies (Quinn, et al., 1985).

1.4.2 Embryo grading

Since the inception of IVF, scientists and clinicians have developed various methodologies to assess the quality of the embryo depending upon the morphology and cleavage rates (Antczak and Van Blerkom, 1999, Sakkas, et al., 2001, Shoukir, et al., 1997, Tesarik and Greco, 1999, Toner, 2002, Van Royen, et al., 1999). Due to limitations of morphologic grading, scientists have further evolved methodologies to assess the reproductive potential of a given embryo by its nutrient secretion/consumption, thus signifying the metabolic differences in *in vitro* cultures in IVF/ICSI treatments (Brison, et al., 2004, Conaghan, et al., 1993,

Gardner, et al., 2001, Houghton, et al., 2002). Gardner and colleagues observed that nutrient use correlated with the highest morphologic grade of embryo (Gardner, et al., 2001). However, the use of these techniques has been limited due to the high cost, sensitivity of the technique and need of high staff expertise. Recently, a newer technique that is non-invasive, rapid, consistent and clinically applicable by assessing the metabolomics profile of the embryo-spent media using Raman spectroscopy has been proposed (Scott, et al., 2008).

In most IVF clinics embryos are graded (Table 1) according to the standard method, Gardner and Schoolcraft´s classification (Gardner and Schoolcraft, 1999). In routine clinical practise, blastocysts with a minimum grade of 3BB are used for embryo transfer.

Table 1: Grading of blastocysts: Gardner and Schoolcraft´s classification

Grade	
1	Blastocoele cavity less than 50% of the volume of the blastocyst
2	Blastocoele is more than 50% of the volume of the blastocyst
3	Full blastocyst- blastocoele fills the entire volume of the blastocyst and a clear inner cell mass (ICM) and trophectoderm (Troph) is discernible
4	Expanded blastocyst- blastocoele volume exceeds that of the early embryo and there is zona thinning
5	Hatching blastocyst
6	Hatched blastocyst that has completely escaped from the zona

Grading of blastocyst: Grading of ICM and trophectoderm is done only from Grade 3 blastocyst onwards:

Grade	ICM
A	Many tightly packed cells
B	Several cells, loosely packed
C	Very few cells

Trophectoderm grading

Grade	Trophectoderm
A	Many cells forming a cohesive epithelium
B	Few cells forming a loose epithelium
C	Very few large cells

1.5 HUMAN EMBRYO IMPLANTATION

1.5.1 Implantation

Implantation of the human blastocyst begins after the hatching process, with the apposition phase, where the blastocyst comes in close contact with the endometrial surface epithelium. At this point, embryonic signals initiate the process leading to endometrial preparation for development of placenta. Progesterone is important for the regulation of a large number of these factors. It has been suggested that the uterine response during implantation is pro-inflammatory in nature and has an immunological bias towards counteracting the `trauma´ inflicted by implanting blastocyst (Finn, 1986).

1.5.2 Embryo-endometrial dialog

With the advancement of research, it is quite evident that there exists an 'embryo-endometrial dialogue' based on molecular notes. This symbiotic molecular exchange between the endometrium and the blastocysts leads to successful implantation (Fazleabas, et al., 2004, Perrier d'Hauterive, 2004). Human chorionic gonadotropin hormone (hCG) is one of the earliest embryonic signals sensed by the endometrium. The mRNA of hCG is expressed as early as the 8-cell stage of the human embryo and it is well known that the blastocyst produces hCG (Bonduelle, et al., 1988, Lopata and Hay, 1989). The expression of endometrial epithelial trophinin, a cell adhesion molecule, is increased by hCG in the presence of IL-1 beta (Sugihara, et al., 2008). The endometrial luminal and glandular epithelium express receptors for hCG-R. Another interesting factor is histamine releasing factor (HRF), which is expressed in human embryos (Cocchiara, et al., 1986). It is proposed that embryonic HRF may be involved in the uptake of histamine by endometrial epithelial cells stimulating the formation of decidua to support implantation (Noskova, et al., 2006). *In vitro* experiments have shown that hCG stimulates the production of LIF, VEGF and MMP-9 by the epithelial cells (Tsampalas, et al., 2010). Recently, with the help of microarray and bio-informatics tool, Altmäe et al. have shown a battery of molecules and molecular network involved in embryo endometrial interaction (Altmae, et al., 2012). This study reveals the cytokine and cytokine-receptor interactions involved in

implantation. The major pathways were osteopontin, leukaemia inhibitory factor, and leptin pathways.

1.5.3 Markers of embryo implantation

The definitive marker of implantation in humans is the implantation of an embryo itself. But given that the implantation cannot be observed directly, the best indirect marker of implantation is hCG (Hearn, et al., 1991). The early signalling starts with the expression mRNA reported at the eight-cell stage (Bonduelle, et al., 1988) as mentioned earlier. The exponential rise in maternal serum or urine may not mark the early steps in implantation process, but they do mark the point at which the conceptus has successfully invaded the maternal tissue.

1.5.4 Natural selection of embryos

Charles Darwin, who propounded the theory of natural selection, made us think differently about the evolution of human race. It is believed that humans have the capability of selecting good quality embryos for implantation and to develop them into healthy foetuses, leading to normal healthy baby. This is understood from the fact that poor quality embryos fail to implant (Van den Abbeel, et al., 2013) and 90% of the karyotypically abnormal pregnancies terminate in recurrent first trimester miscarriages (Quenby, et al., 2002). It is highly possible that endometrial stromal cells have a potential role in sensing the good quality embryos leading to successful pregnancy. And to the best of Nature's rescue, a majority (93%) of the karyotypically normal pregnancies continue, a remarkable but yet unexplained phenomenon. Does this suggest a potential signal from the pre-implantation competent blastocyst? Recently it has been shown that decidualization of endometrial stromal cells serve as sensors for selection of embryo quality that ultimately lead to successful implantation and pregnancy (Teklenburg, et al., 2010). Additionally, analysis of mid-secretory endometrial biopsies from women with recurrent pregnancy loss displayed decreased expression of the decidual marker prolactin and increased levels of prokineticin-1, a cytokine that promotes implantation (Salker, et al., 2010). Thus, the authors conclude that impaired

endometrial decidualization although facilitates implantation, yet terminates in pregnancy loss by disrupting the maternal-embryonic responses.

1.5.5 Challenges in studying embryo implantation

For centuries, research in understanding the conception and fertilization has intrigued scientists globally. In humans, in *vivo* embryo-implantation studies are ethically and technically difficult or nearly impossible. Most of the information is gathered either from animal models or *in vitro* models. The first *in vitro* mammalian study was described almost 50 years ago using rabbit endometrial strips (Glenister, 1961) and led to some basic observations: 1) necessity of removal of zona pellucida to assist extracorporeal implantation, 2) and the inevitable need for endometrium in the progestational phase of development, indicating that endometrial differentiation is a critical prerequisite for successful implantation. Essentially, blastocyst implantation occurs only when the endometrium is receptive and fails to implant in non-receptive endometrium in the uterus. Nevertheless, how could we explain ectopic pregnancies and abdominal pregnancies? The mechanisms underlying these implantations need more research. However, its been suggested that blastocysts can attach to any surface covered by simple squamous or columnar epithelia derived from mesoderm (Glenister, 1966). So far there is no single model where the results can be directly extrapolated to human embryo implantation due to the inherent species differences. Besides, the type of implantation varies among species where one needs to pay attention while selecting the model.

Based on different types of implantation process, more specifically, the blastocyst-uterine cell interactions, implantation has been classified into three broad categories: centric, eccentric and interstitial (Wimsatt, 1975). In centric implantation (rabbits, dogs, cow, pigs, sheep and many marsupials) the blastocyst grows large and forms ample surface contact to fuse with the luminal epithelium without penetrating through it. In eccentric implantation (mice, rats, and hamsters), the luminal epithelium forms an invagination to surround the blastocyst. In interstitial implantation (humans and guinea pigs), the trophoblast passes through the luminal epithelium to invade the endometrial stroma and

become embedded into the wall of the uterus. Thus, every animal model has its own unique mechanistic and molecular variations where scientists have been careful in selecting the models depending on the process of implantation phase to be studied (Lee and DeMayo, 2004). However, regardless of the type of implantation process, the main purpose of the implantation, central to every species, is to bring the maternal vascular supply in contact with the developing embryonic blood vessels.

1.5.6 Models to study embryo implantation

1.5.6.1 Animal models

Animal models (mostly murine models) have been the first-hand models to investigate the early steps of embryo implantation. Mice and rats are good models to understand the physiological aspects of ovarian steroids on the uterus and to study the mechanism of decidualization, as the decidual response can be reproduced. The decidual site is easily discernable, even in the absence of trophoblast attachment. In certain rodents, there is a delayed implantation, where the uterus remains in a quiescent state with dormant blastocysts and is easily stimulated with parenteral estrogen supplementation. The decidual response can also be artificially stimulated either by intraluminal oil infusion or scratching, suggesting that the decidual response is an intrinsic ability of uterine stromal cells (Dey, et al., 2004). The fact that mouse genetics can be easily altered, either by overexpressing or ablating genes, making mice an excellent choice for a model system. However, due to the extremely rapid eccentric implantation with apposition, attachment and invagination of the uterine epithelium (within 6 hours), they are not good models for understanding the mechanisms of early steps of human embryo implantation.

Rabbit models are considered to be good models, due to their precisely timed ovulation (10 hours after mating) and well-documented time of apposition (at 6.5 days) with centric or fusion type of implantation. The fact that the blastocysts adhere solely to the epithelial apices, as well as the ability of the trophoblasts to attach to in vitro cultured epithelium, make rabbits the model of choice to elucidate the apical cell adhesion (Hoffman, et al., 1998).

Guinea pigs, like humans, are examples of interstitial implantation, and useful models for explaining the mechanism of transepithelial penetration, where the blastocysts intrude directly into the endometrial stroma (Enders, 2000). Pig models are good to study the early phases of implantation due to their prolonged apposition and attachment phase (Carson, et al., 2000). The pleiotropic effects of ovarian steroids are also studied in unique ewe model; ovine uterine gland knockout animal by prolonged progesterone exposure into the neonatal ewes (Spencer, et al., 1999). This model has been most useful for identifying genes expressed by endometrial epithelium (Gray, et al., 2002).

Although implantation studies in non-primate models show high fidelity to human implantation and have distinct advantages in terms of economic costs and ability to be genetically modified, the knowledge in understanding the physiological mechanisms of implantation is limited. In this regard non-human primate models are good models to study both the early (rhesus monkey and marmoset) and late implantation events (macaque) as the trophoblast adheres to the endometrium adjacent with the ICM, similar to humans (Einspanier, et al., 2006, Enders and Blankenship, 1999, Enders, et al., 1983, Enders and Lopata, 1999, Fazleabas, et al., 2004). However, such studies are expensive, time consuming and impractical for obtaining large numbers of early implantation stages due to relatively low fertility. However, non-human primates are good models to study the contraceptive potential of drugs (Sengupta, et al., 2006).

Animal models have their own limitations due to failure to translate the information completely to understand human embryo implantation (Lee and DeMayo, 2004) .

1.5.6.2 In vitro models

In vitro implantation models have been realistic in understanding the molecular cross talk/signalling pathways through the manipulation of cells in culture models that mimic the *in vivo* scenario.

1.5.6.2.1 Monolayer co-cultures

Considering the facts above, a co-culture of purified set of cells from human endometrium facilitated a better viability and diffusion into the cells. Blastocysts

cultured on monolayer (2D) co-cultures (Fig 5B), mimicked a mini-organ to study the implantation events (Landgren, et al., 1996). Traditional monolayer cell

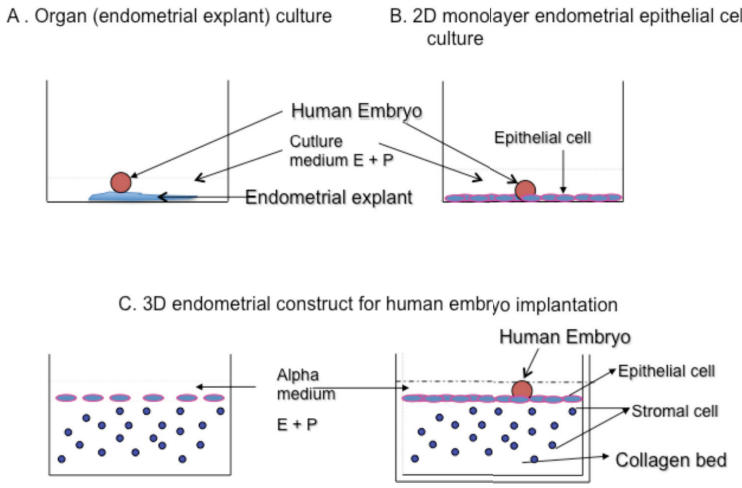


Figure 5: In vitro models to study human embryo implantation

culture produce cells which have a flattened, squamous morphology irrespective of the original type and constitute a single specific type of cells representing the receptive endometrium. By growing cells in a matrigel, it is possible to produce cells that are morphologically similar to the *in vivo* endometrium (Glasser, et al., 1988, Rinehart, et al., 1988). There were further modifications with coculture of endometrial cells with trophoblast cells revealing that trophoblast cells induced secretory changes in the endometrial cells and upon contact with the stromal cells showed contact necrosis signifying the activation of proteases (MMPs) in the endometrium/trophoblast (Kliman, et al., 1990). A few simpler co-culture models with fibroblast cells were also used to study the implantation (Wetzels, et al., 1992, Wetzels, et al., 1995). Over time, monolayer cultures have yielded valuable information of implantation events (Carver, et al., 2003, Fujiwara, et al., 2002, Kliman, et al., 1990, Lindenberg, et al., 1985, Mercader, et al., 2003). These culture models also provide the opportunity to identify the specific cell factors by gene silencing techniques. However, monolayer cultures do not mirror the 3 dimensional *in vivo* state, due to cultures grown on artificial plastic surfaces, and

they have limitations as relevant biological properties are lost, including cellular communications, differentiation and interaction with ECM (Arnold, et al., 2001, Bentin-Ley, et al., 1994, Eritja, et al., 2010)

1.5.6.2.2 Three-dimensional embryo-endometrial co-cultures

It is a basic biological assumption that cells which have the appropriate cell morphology are likely to behave and function in a more realistic way than cells which have an altered structure. Taking this assumption, tissue culture models have been developed which are anatomically and functionally closer to the female reproductive system (Bentin-Ley, et al., 2000, Bentin-Ley, et al., 1994, Mardon, et al., 2007, Teklenburg and Macklon, 2009). The co-culture of both endometrial stromal (ESC) and epithelial cells (EEC) along with an embryo overcomes the limitations of monolayer models and facilitates a better study of the cellular interactions between EECs and ESCs, besides the direct actions with the embryo itself. Fresh human endometrial tissue biopsies collected in appropriate phase of menstrual cycle are subjected to mechanical and enzymatic cell isolation techniques to obtain a pure population of epithelial and stromal cells. These cells are overlaid on a artificial extracellular matrix (matrigel) coated onto a millicell insert, are placed in multiwall tissue culture plate, and are grown for several weeks by changing the medium every 2-3 days. These co-cultures (3 dimensional) of endometrial stromal and epithelial cells along with the embryo overlaid mimic the *in vivo* system exhibiting inter/intracellular interactions and the embryo (Fig 5c). The therapeutic use of co-cultures using autologous endometrial cells to improve the implantation potential of the embryo has been a remarkable advancement in combating the clinical problems of recurrent implantation failure (Barmat, et al., 1999, Rubio, et al., 2000).

The organ explant cultures (Fig 5A) have also been valuable to understand the early stages of embryo implantation (Glenister, 1962, Glenister, 1961, Glenister and Cooling, 1969). Nonetheless, organ explant cultures have their limitations of tissue necrosis due to poor diffusion of culture media and tedious maintenance of the cultures.

These models, although showing some variances, produce epithelial cell confluence and correspond to the cellular morphology and functional

characteristics as in the *in vivo* biopsy (Bongso, et al., 1991, Glasser, et al., 1988, Rinehart, et al., 1988). Thus implantation models from a simple, basic monolayer culture model to complex culture models have served the purpose of helping to increase our understanding the implantation process. Is there a need for a more complex model? Tecklenberg et al., in their review article, state that a more complex model mimicking the *in vivo* system more closely may not necessarily be a ``better´´ model as their complexity may increase the artifactual errors (Teklenburg and Macklon, 2009).

Advantages and limitations of implantation models

- Although monolayer cultures/cell populations do not reflect the *in vivo* endometrial cells populations in totality, these models are easier to be executed and allow study of extensive cellular components.
- 2D and 3D cell culture models do serve a better purpose but are limited due to their robust and complex stringent protocols, demanding advanced scientific expertise.
- Animal models are the best near mimics of the *in vivo* human embryo implantation process, but the knowledge acquired cannot be translated completely due to species differences.
- The only source of human embryos is from IVF, which may not be representative of the natural embryo pool.
- Culture systems are generally difficult to be sustained beyond 10 days due to spontaneous cell death.
- A unique opportunity to study the blastocyst and its endometrial interactions beyond day 6, which otherwise is difficult to pursue *in vivo*.
- Failed implantations in *in vitro* models are difficult to be attributed to the virtual causal factor: endometrial or embryonic origin because of the differential intrinsic properties.

1.6 RESEARCH INVOLVING HUMAN EMBRYO

The term embryo research broadly includes novel or experimental in utero or ex utero interventions for therapeutic research intended to benefit mother, embryo, or

both. Much of the research involving early-stage embryos is inextricably aimed at improving fertility in human reproduction. Additional focus is also on improving the knowledge and understanding of early embryonic development, birth defects, stem cells, genetic expression and contraceptive development. Such research provides the essential basics for many of the techniques of assisted reproduction, and relies on the ex vivo, live embryos from donor couples that are not intended for transfer into a woman´s uterus.

1.6.1 Source of embryos

Embryos for research are routinely obtained from fertility clinics, which are explicitly donated for research - generally cryopreserved embryos that remain following completion of in vitro fertilization (IVF) treatment that are no longer wanted for transfer by the intended couples who produced them (also called ``spare embryos´´). Around 400,000 embryos were cryopreserved in 2003, in the United States and only 2.8% (roughly 11,000) were donated for research. In the UK, a total of 52,000 were cryopreserved (Boulton, 1996).

1.6.2 Ethics and Human embryo research

The ethical concern related to human embryo research has long been debated since the inception *of vitro* fertilization programs. The use of embryos requires special considerations to limit the research to important issues that cannot be addressed in other ways and to conduct the research within the boundaries established to reflect appreciation for the special nature of human embryos. The 1994 National Institutes of Health (NIH) Human Embryo Research Panel proposed a limit of 14 days after fertilization for the use of embryos for research (Committee on Research and Bioethics, 2001) and Sweden also has the same guidelines.

1.7 THE HIATUS

From the literature, we know that progesterone leads the endometrium to the receptive state. Over decades, there has been an attempt to decipher the molecular notes of progesterone mediated receptive endometrium. Much of the progress has been made in the last few years with the advancement of the technology. However, the knowledge of endometrial receptivity is still rudimentary. Thus, a novel approach incorporating large scale screening of genes is needed to identify candidate markers for endometrial receptivity. Also, a lack of a model to study the process of progesterone regulated human embryo implantation makes it far more difficult to understand the intriguing molecular mechanisms involved in blastocyst implantation process.

2 AIMS OF THE THESIS

The general aim of the current thesis was to increase knowledge about the factors important for endometrial receptivity and to develop a tool to understand human embryo implantation process.

Specific aims

1. To establish an *in vitro* model to study the process of human embryo implantation and test the model for progesterone regulation.
2. To investigate the role of LIF in the human blastocyst implantation process.
3. To characterize the progesterone regulated gene expression in the endometrial glandular compartment during implantation window.
4. To investigate the gene expression in the endometrial stromal compartment during the receptive period, regulated by progesterone.

3 MATERIALS AND METHODS

More detailed description of the materials and methods are given in the individual original articles (Paper I-IV)

3.1 SUBJECTS

Healthy fertile women aged 22-40 years with normal menstrual cycles (28-32 days) volunteered for the endometrial biopsies in all the studies. None had used any hormonal contraception or intrauterine device for a minimum of 3 months prior to the study. The subjects were recommended to use barrier contraception, if not sterilised. A routine gynaecological examination was performed to rule out any pathological clinical condition. In paper III-IV every subject was her own control after treatment with mifepristone 200mg on LH+2. The first endometrial biopsy was collected from the non-treatment cycle and later the second biopsy after treatment (progesterone was depleted).

3.2 ETHICAL PERMITS

The Ethics committee of Karolinska University Hospital approved all the studies. Written informed consent was obtained from all the participating subjects, either for the use of supernumerary cryopreserved embryos or endometrial samples. All patients who donated the embryos were investigated according to the standard clinic protocols, but the outcome of these routine investigations was not taken in account in either the recruitment into this study or in the analysis of the data.

3.3 BLASTOCYSTS

Supernumerary human embryos or those cryopreserved for 5 years were used which were collected from the IVF clinic, Fertilitetscentrum Stockholm. Blastocysts with minimum grade 3BB (Gardner and Schoolcraft, 1999) were used (Paper I & II). Paper I: 46 embryos and Paper II: 69 embryos were taken and among these, 51 embryos, which survived the thawing were used in the study (Paper II, Fig 1 and supplementary Table 1).

3.4 BLASTOCYST COLLECTION AND CULTURE

Ovarian stimulation, ovulation induction, oocyte retrieval and IVF/ICSI were performed according to standard clinical protocols. Supernumerary, good quality embryos were subsequently cryopreserved using RapidVit Blast (Vitrolife, Sweden). After collapsing them by inserting a partial zona dissection (PZD) needle, they were then transferred into prewarmed Vitri 1 Blast for 5 minutes at 37^{o}C. This was followed by incubation in Vitri 2 solution for 2 minutes followed by incubation in a 20μL droplet of Vitri 3 Blast for 45 seconds. Finally the blastocysts were placed in the cryoloop and immersed into liquid nitrogen and stored in liquid nitrogen until use.

On the day of overlaying the blastocysts to the endometrial cultures, vitrified blastocysts were thawed using RapidWarm™ Blast (Vitrolife). The blastocysts were removed from the cryodevice and placed along with the cryodevice into the solution: Warm 1™ Blast, supplied along with the kit. The detached blastocyst from the loop was allowed to stand for 2 minutes in the same solution. Latter it was transferred into Warm 2™ Blast followed by Warm 3™ Blast and incubated for 3 and 5 minutes respectively. The thawed blastocysts were allowed to expand in culture medium for 3 hours before using them in the study. Blastocysts that recovered the thawing and met the standards of clinical embryo transfer quality were chosen for the study. These randomly selected and thawed blastocysts with minimum grade of 3BB as per Gardner's classification that had at least 90% of cells survived and gained 90% of their cell contour were used further in the study.

3.5 MEDICATION

In Paper III-IV, volunteers were treated with single dose of 200mg of mifepristone on LH +2 and endometrial biopsy obtained on LH+7. A control biopsy without any pre-treatment was obtained on LH+7 in another menstrual cycle. Thus every woman was her own control, thus minimising the heterogeneity. These samples were called paired samples.

3.6 ENDOMETRIAL BIOPSIES

Endometrial biopsies were collected on one of the days LH+4 or 5 (Paper I-II) and on LH +7 (Paper III-IV) according to the urinary LH peak detected by rapid self-test (Clearplan, Searle Unipath Ltd, Bedford, UK). The samples were obtained using a Randall curette from the upper or fundal part of the endometrial cavity without prior cervical dilatation or local anaesthesia.

The tissue biopsy obtained was collected in HAM-F12 (Paper I-II) and either taken for cell isolation or snap frozen in RNAlater® (Paper III-IV). A small sample of biopsy was taken in formalin for morphological analysis.

3.6.1 Endometrial cell isolation

The endometrial tissues were minced with a scalpel and incubated with pancreatin–trypsin EDTA (0.05 g/ml of trypsin–EDTA solution, Sigma-Aldrich, Stockholm, Sweden) followed by adding collagenase (150 IU ml^{-1}; Sigma-Aldrich, Stockholm, Sweden). The mixture of cells was filtered through 40 micron mesh cell strainer (Falcon; BD Biosciences, Belgium) that allowed single stromal cells to pass through and the glandular tubules were restrained in the filter. The cells were collected in different eppendorf tubes and stored in -80 freezers until further use.

3.6.2 Three dimensional embryo-endometrial cell culture model

Endometrial three-dimensional stromal and epithelial co-cultures mimicking endometrium were prepared as described earlier (Bentin-Ley, et al., 1994, Bentin-Ley, et al., 1999) with minor modifications. The standardization of the three-dimensional cell culture had challenges such as delay in the formation of collagen gel and leakage of collagen solution from the construct. These were taken care of by slightly increasing the pH to 7.6 of the initial collagen, used for the formation of the base gel. Stromal cells ($0.5X10^7$cells/ml) in the culture insert (Millipore Billerica, MA, USA) were embedded in 200 µl of purified sterile bovine collagen (3mg/ml) in solution (PureCol, Inamed, Fremont, CA, USA). After forming the gel, murine basement membrane material (Matrigel; BD Biosciences, Belgium) was coated on its top by adding 200 µl of matrigel followed by its removal with

polished Pasture pipette and air-drying it for 5 minutes. Epithelial glands were seeded on the top of matrigel to cover ~70% of the gel surface and cultured with modified alpha-medium by adding 4 ml Amniomax C100, penicillin–streptomycin (2000 IU), 0.2 ml L-glutamine (200 mmol/L, Life Technology, Invitrogen AB, Sweden), 5 ml foetal calf serum (FCS; In vitrogen, Sweden AB, Stockholm, Sweden), 0.5 g BSA (Sigma-Aldrich, Stockholm, Sweden) to 100 ml of the medium. The final concentration of estrogen and progesterone were 0.3nmol/L and 900nmol/L. The cells were cultured in an incubator at 37^0C with 5% CO_2 in air. After two days of culture, the inserts were washed to remove the unbound cells and fresh medium was added. During standardization, we faced problems of maintaining the constructs as the gels contracted after couple of days of culture. This was tackled by decreasing the amount of epithelial cells layered on to the culture construct. Latterly, in study II; this was further modified by layering a single cell suspension of epithelial cells on to the top of the construct. The epithelial cells layered as tubules (Paper I) on the top of the endometrial construct were spread out and grew over the surface gradually. However, with time, some of the epithelial cells formed clumps, which made it difficult to differentiate between embryos. Thus, we stained embryo attachment section with the marker for trophoblast cells (cytokeratin 7), to identify the embryo in the section (Paper I, Fig 6). In study II, this was taken care of by replacing with single cell suspension of endometrial epithelial cells onto the top of the construct that made it better in identification of embryo attachment.

3.6.2.1 Treatment, embryo viability and attachment

After 5 to 6 days of culture when the epithelial cell layer was confluent, all cultures were treated with progesterone (10^{-5}M) along with or without mifepristone (10^{-5}M) or levonorgestrel (10^{-5}M) in 5 µl of ethanol as base (Paper I). In Paper II, LIF and PEGLA were used in the cultures, and described in detail in the methodology section in Paper II. Only vehicle (PBS) was added in the control group. Modified alpha medium was used for the culture of embryo – endometrial construct and the medium was changed every second day. Human blastocysts were placed on the epithelial cell layer at the onset of treatment. A professional embryologist performed the thawing of human blastocysts, as they are very

sensitive and precious. Embryo pipettes (size, 145 μm; Vitrolife) were used to handle the blastocysts while transferring them onto the construct. After exposing the embryos along with the cell culture system with the above treatment for five days, the embryos were checked for any attachment with the culture matrix by washing thoroughly with PBS. The embryo attachment to the cultures was recorded by light microscopy. They were fixed in 4% formalin and were used to prepare paraffin blocks.

3.6.2.2 Dosage of progesterone, mifepristone, LIF and PEGLA

The progesterone concentration used in the study was 10^{-5}M, which was used in a previously published article by (Petersen, et al., 2005). Equimolar antiprogestin mifepristone or SPRM levonorgesterel was added to block the progesterone receptor in the cells. The dose of rhLIF was initially calculated as 35 pg/ml taking the median level of LIF present in the uterine secretion (Mikolajczyk, et al., 2007, Mikolajczyk, et al., 2006). However, this dose was reduced to 10pg/ml of rhLIF as the embryo attachment with the former dose showed a reduction in the embryo attachment (30%) as shown in Fig 6 (unpublished data).

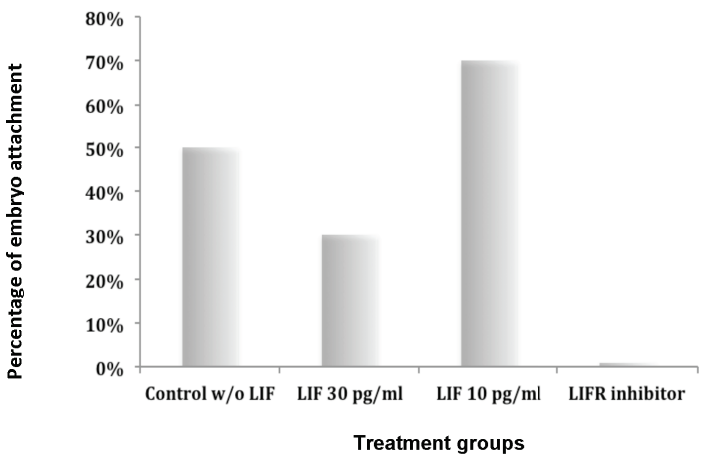


Figure 6: Effect of different doses of LIF on blastocysts attachment *in vitro*. A lower percentage of embryo attachment was seen with 35 pg/ml of rhLIF compared to the cultures without addition of LIF and 10 pg/ml of rhLIF.

PEGLA was used in the concentration of 800 pg/ml, a dose determined from previous experiments, based on the bioassay. It was calculated using the following data: IC_{50} of PEGLA for rhLIF at 12 pM was 5.5-6.8 nM PEGLA and the MW of PEGLA and rhLIF are 62 kD and 19.7 kD respectively (White, et al., 2007). Thus, in order to completely inhibit the effect of 10 pg/ml LIF, we took 800 pg/ml of PEGLA.

3.6.3 Immunohistochemistry

Immunohistochemistry (IHC) was used to determine the expression of progesterone regulated endometrial receptivity markers, LIF, LIFR, and gp 130. The relative quantity of protein was detected by a semiquantitative method. In general, IHC staining technique enables us 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 chromogen substrate. The enzymatic activation of the chromogen results in a visible reaction product at the antigenic site reaction. A Zeiss Axiovert microscope 200M (Ziess, Göttingen, Germany) was used to analyse the IHC stained sections.

3.6.3.1 Scoring system

Two independent blinded observers evaluated the staining using a semi-quantitative method (IRS scoring) as described previously (Mylonas, et al., 2004). In case of any discrepancy, a third observer evaluated the slides. The score was calculated as follows: IRS=SI x PP, where SI is the staining intensity and PP is the percentage of positively stained cells (Table 2)

Table. 2: Immunohistochemistry scoring matrix

Intensity of Staining	Percentage of Stained cells
0 = No Staining	0 = 0 %
1 = Weak Staining	1 = <10 %
2 = Moderate Staining	2 = 11- 50 %,
3 = Strong Staining	3 = 51- 80 %
	4 = > 81 %

3.6.4 Immunofluorescence (Paper II)

Human embryos treated with LIF and PEGLA (n=4 each) were examined for the expression of phospho-Akt by immunofluorescence as explained in the methodology section of paper II. A primary Rabbit polyclonal antibody against phosphor-Akt (Ser 473) (Cell signalling, MA, USA) was used. Replacing the primary antibody with isotype-matched rabbit IgG, a negative control was performed.

3.6.5 Laser Capture Microdissection

The frozen tissue was sectioned at 10 µm thickness using a crytome (Reichert Jung Cryocut 1800, Leica) and fixed to a membrane slide (Membrane Slide NF 1.0 PEN, Carl Zeiss Microimaging GmbH, Germany) with 70% ethanol at -20^{o}C for 30 minutes. Prior to use, the slides were irradiated in UV for 30 minutes to make them hydrophilic. The tissue sections were stained using a staining solution (Histogene, Applied Biosystems, Life Technologies, Carlsbad, CA). The slides were then washed and dehydrated stepwise in 70% ethanol, 95% ethanol, absolute ethanol and xylene for 1 minute each and then air-dried. The slides were then placed in an airtight box and placed at -70°C till they were used for laser micro dissection. The slides were brought to room temperature before the start of laser microdissection process. The Laser capture microdissection of the stromal cells or glandular epithelial cells was performed individually using PALM Laser-Micro Beam System (Carl Zeiss Microimaging GmbH, Germany). The tissues were examined at 40x objective and the stromal and gland cells were marked using the PALM Robosoftware. A pulsed 355-nm diode laser was used to cut and catapult (Carl Zeiss Microimaging GmbH, Germany) the sample and captures it on to a cap of a MicroTube 500 containing 35µl of the extraction buffer (Arcturus, PicoPure). The tube was fixed in a PALMTM Robostage & RoboCap mover (Carl Zeiss Microimaging GmbH, Germany) which makes it easier to robotically move the cap containing the extraction buffer closer to the sample for the catapult capture of the dissected section. Approximately 200 cells were dissected from the glands or stromal cells in approximately 15-20 sections into RNA extraction buffer. The samples were then frozen at -80°C until further use. All the above

steps were carried out in a strictly RNase free environment to maintain the integrity of the RNA for the microarray experiments.

3.6.6 RNA extraction (Paper III & IV)

The RNA extraction was carried out using the Arcturus PicoPure Frozen RNA Isolation Kit (Applied Biosystems Life Technologies, Carlsbad, CA) according to the manufacturer's protocol from the glands and stroma, obtained after laser dissection of glands or stroma. The concentration of the isolated RNA was measured spectrophotometrically using Nanodrop 1000 (Thermo Scientific, Wilmington, DE). The quality control on RNA was performed using Agilent RNA 6000 Pico Kit (Agilent Technologies, Inc., Santa Clara, CA) and Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). The RIN value obtained as a result of this method determines the quality of RNA.

3.6.7 Microarray (Paper III & IV)

The microarray was performed at the core facility for Bioinformatics and Expression Analysis, located at the Department of Biosciences and Nutrition at Novum, KI. The RNA was amplified using NuGEN Ovation pico WTA System™ (NuGEN Technologies Inc., San Carlos, CA). The cDNA (amplified complementary mRNA) was then used to generate the sense target cDNA using NuGen WT-Ovation™ Exon Module version 1.0 (NuGEN Technologies Inc., San Carlos, CA). Fragmentation, biotin labeling and target preparation of the ST cDNA were performed using the NuGEN Encore™ Biotin Module (NuGEN Technologies Inc., San Carlos, CA). The hybridization of the ST cDNA was performed on the Affymetrix GeneChip® Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA) and subsequently; the arrays were washed and stained in Affymetrix Fluidics Station 450 (Affymetrix, Santa Clara, CA). The above processes were carried out according to manufacturer's protocol. Instrument handling and image processing were performed using the Affymetrix GeneChip Command Console Software (AGCC).

3.6.7.1 Data Analysis

The initial analysis of the data was performed with Affymetrix Expression Console™ software. The summarization algorithm used was Probe Logarithmic Intensity Error Estimation (PLIER). The background correction was done using perfect match GC composition-based background correction and the normalization was performed by the Global Median method.

3.6.7.1.1 The Stringent method

The data was filtered to remove background noise before any statistical analysis was performed. Samples with hybridization signal above 40 in all 4 samples in at least one group, were picked up for further analysis. With this data, a ratio was made between each pair (control & treatment) and an up-regulation/down-regulation (U/D) call was made for each pair. Only those gene probes that had greater than 4U or greater than 4D were considered for the next step of analysis. Fold change (FC) was calculated and a paired t-test was performed for each gene. The q-value was calculated from the p-values using the method suggested by Storey and Tibshirani (Storey and Tibshirani, 2003) with the value of lambda (tuning parameter) set to zero, which produces the estimate implicit in the Benjamini and Hochberg (Klipper-Aurbach, et al., 1995) methodology. The interpretation of the q-value is similar to that of FDR. Genes with fold change greater than or equal to 2 and q-value less than 0.1 were considered as significantly regulated genes and genes with fold change greater than or equal to 2 and unadjusted p-value less than 0.05 as potentially differentially regulated genes.

3.6.7.1.2 The Lenient method

The data was filtered to remove background noise. Samples with expression data above 40 in 3 out of 4 samples in at least one group, was picked up for further analysis. With this data, a ratio was made between each pair (control & treatment) and an up-regulation/down-regulation (U/D) call was made for each pair. Only those gene probes that had greater than 3U or greater than 3D were considered for the next step of analysis. Fold change was calculated and those genes that had a fold change greater than 2 were used for the Ingenuity Pathway Analysis (IPA).

3.6.7.1.3 Ingenuity Pathway analysis (IPA)

Ingenuity Pathway Analysis (Ingenuity System, www.ingenuity.com) was used to perform pathway analysis using the gene set from the stringent and lenient analysis methods.

IPA is a web-based application that performs analysis on a given set of data to suggest the pathways that are regulated by the given data. IPA works using it's own database called the Ingenuity Knowledge base, which is manually curated using information from various other databases (Entrez Gene, RefSeq, OMIM, GWAS Database, Gene Ontology, etc.) containing scientific information and updated regularly by the program developers.

3.6.8 Real Time RT-PCR

Complementary DNA (cDNA) was synthesized from 1µg RNA obtained from laser dissection of endometrial glands using Paradise kit (Bucher Biotec AG). Real time PCR was performed to determine the relative quantity of ENPP3 using TaqMan® Gene Expression Assays (Applied Biosystems) and 7300 Real Time PCR System (Applied Biosystems) detection system. Eight paired endometrial samples were used for this study. The target gene expression levels were tested with three internal controls 18 S, GAPDH and beta-actin. The data were analyzed using comparative Ct method, where Ct is the cycle number at which the fluorescence first exceeds the threshold. The relative fold change (ΔCt) was calculated by subtracting the value of the endogenous control from the Ct value of the target gene. The mean ΔΔCts, which is the difference in the expression levels between the two groups, was calculated. From this the fold change in the expression was calculated as $2.^{-\Delta\Delta Ct}$

3.7 STATISTICS

3.7.1 Paper I

Fisher's exact test was done to study the significance in blastocyst attachment rate between the control and treatment groups.

3.7.2 Paper II

Nonparametric statistical evaluation, Mann-Whitney's test was performed to compare the difference between the two groups of immunohistochemical analysis in the endometrial construct. The results are presented as mean $\pm$ standard deviation (SD). The Kruskal-Wallis test was performed to find any statistically significant difference in the age of the patients, grade of blastocyst used, and the day of embryo vitrification in different study. Chi-square test was performed to evaluate the difference between the groups in embryo attachment rates. A P-value of ≤ 0.05 was considered statistically significant.

3.7.3 Paper III and IV

The paired t-test for the microarray data was performed using Microsoft Excel 2010. The software R was used for calculating the q value using the package q value. The IRS score of IHC data was statistically analysed by performing the non-parametric Mann-Whitney test to compare control and treatment group. Results with p-value less than 0.05 was considered statistically significant. For real-time PCR, matched t-test was done.

All statistical tests, if not specifically mentioned were performed using the software Statistica.

4 RESULTS

4.1 PAPER I

An *in vitro* 3 dimensional human endometrial construct expressing progesterone regulated receptivity marker to study human implantation process was developed.

4.1.1 Development of 3-dimensional endometrial construct

Endometrial stromal and epithelial cells were isolated from the endometrial biopsies (n=20). Out of these 18 were used for the cell culture, as two biopsies did not yield sufficient number of cells. Eleven of these cultures were used in this study. The cross-sections of the co-culture stained with haematoxylin showed stromal cells embedded into collagen matrices with surface covered with epithelial cell layer (Fig 1, paper I). One of the cultures was contaminated with fungi and the rest were used in establishing the fine technique.

4.1.2 Expression of endometrial receptivity markers

The epithelial cells in the endometrial constructs were positive for estrogen receptors, progesterone receptors and androgen receptor, as seen by immunohistochemistry. The immunoreactivity was mainly confined to the nucleus. The stromal cells were mostly negative. The expression of interlukin-1β was very strong in the epithelial cells, whereas the stromal cells were slightly positive. Both epithelial and stromal cells were positive for LIF and VEGF. The expression of cell adhesion molecule integrin $\alpha_v\beta_3$ was detected only on the surface of the epithelial cells by immunofluorescence. Only epithelial cells expressed muc-1 on the apical side. Stromal cells were negative for these factors. Representative micrographs are given in fig 2, paper I.

4.1.3 Blastocyst implantation rate

We found 59% of the blastocysts attached to the matrix in the control group treated with the vehicle, where as in the levonorgestrel treated group, 43% of the embryos attached to the construct. In the mifepristone treated group, none of the 15 embryos attached to the constructs (table 3). The embryos in the control group expanded and contracted the gel after attachment to the surface of the endometrial construct (see fig 5 in Paper I).

Table 3: Attachment of blastocysts in different treatment groups; control, levonorgesterol and mifepristone groups. Antiprogestin mifepristone inhibited blastocyst attachment significantly ($p<0.01$).

Treatment	Number of blastocysts used	Number of blastocysts attached	Percentage of blastocyst attachment
Control (Vehicle: 5 ul Ethanol/5000 μl medium)	17	10	59
Levonorgesterol (10^{-5} M)	14	6	43
Mifepristone (10^{-5} M)	15	0	0**

Interestingly, two embryos which failed to attach, one in control and the other in levonorgestrerol group left a hollow on the surface of the culture (see figure 4 in paper 1).

4.2 PAPER II

The role of LIF in human embryo implantation using PEGLA was studied in the *in vitro* human embryo implantation model.

The cultures exposed to LIF at a lower level, 10 pg/ml had a significantly higher rate of human blastocyst attachment (70%, $p<0.01$) compared to 35pg/ml of LIF (30%), as represented in figure 6. None of the blastocysts exposed to PEGLA, attached to the endometrial constructs ($p<0.01$). The endometrial cultures were positive for LIF receptor (LIFR) and gp130 as detected by immunohistochemistry. The expression and accumulation of LIF in the epithelial cells was lower in the cell cultures exposed to PEGLA ($p>0.05$) as studied by immunohistochemistry and real time PCR respectively. In addition, reduced expression of gp130 in epithelial cells was observed in the PEGLA treated group when compared with control group.

Interestingly, the blastocysts, which were exposed to PEGLA, were degenerated in the culture. This led to further investigation of the effect of LIF inhibition on the blastocysts. We found that LIF blockade decreased (1.7 fold) the

expression of cell survival factor AKT1 in the embryos as studied by real time PCR and immunofluorescence (Fig 4, paper II).

Both the real-time PCR and the immunofluorescence showed an increased blockade of LIF with increased activity of Caspase-3 (cleaved caspase 3; n=4) compared to the control group of blastocysts exposed to vehicle alone. The details of embryos used in this study are given in paper II supplementary table 1.

4.3 PAPER III

Progesterone regulated endometrial epithelial cell marker/s for receptivity was explored.

A total of 42 genes were differentially regulated with a q value higher than 0.1. The top 10 up-regulated genes with fold change (FC) > 4.5 were SNORA 2, MPZL2, ENPP3, MT1G, SCGB1D4, MOGAT1, SCGB1D2, SGK1, RPSA and PHF8. The top 10 molecules which had statistical significance ($p<0.05$) were ENPP3 (-83.2), ARL15 (-5.99), HBB (-5.23), SFRP4 (-4.66), TSPAN6 (-3.79), BCL11A (3.71), DNAJC19 (-3.56), DEXI (-3.34), DIO2 (-3.3) and RPSA (4.75). Molecules belonging to cellular movement, genetic disorder and hematological diseases had 12/27 molecules differentially expressed. Nine out of 19 molecules, belonging to cellular development, cell death; cellular growth and proliferation top the list (Fig 7). The major canonical pathway altered in this study belongs to energy metabolism; the oxidative phosphorylation pathway and mitochondrial dysfunction. The details of differentially regulated canonical pathways are given in figure 8.

4.3.1 ENPP3

The expression of ENPP3 was significantly reduced in treatment group (progesterone depleted by mifepristone treatment) as studied by microarray. This was reconfirmed by real time PCR and the protein accumulation in the glands was almost undetectable. This was further confirmed by examining endometrium in different phases of menstrual cycle, which showed increased expression in endometrium glands during the progesterone dominated luteal phase (figure 4, Paper III).

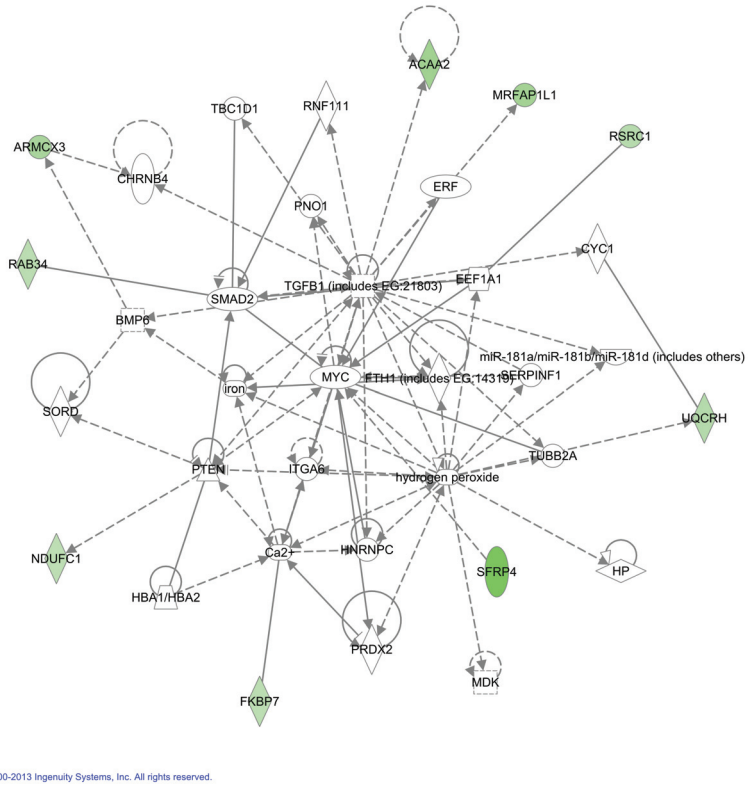
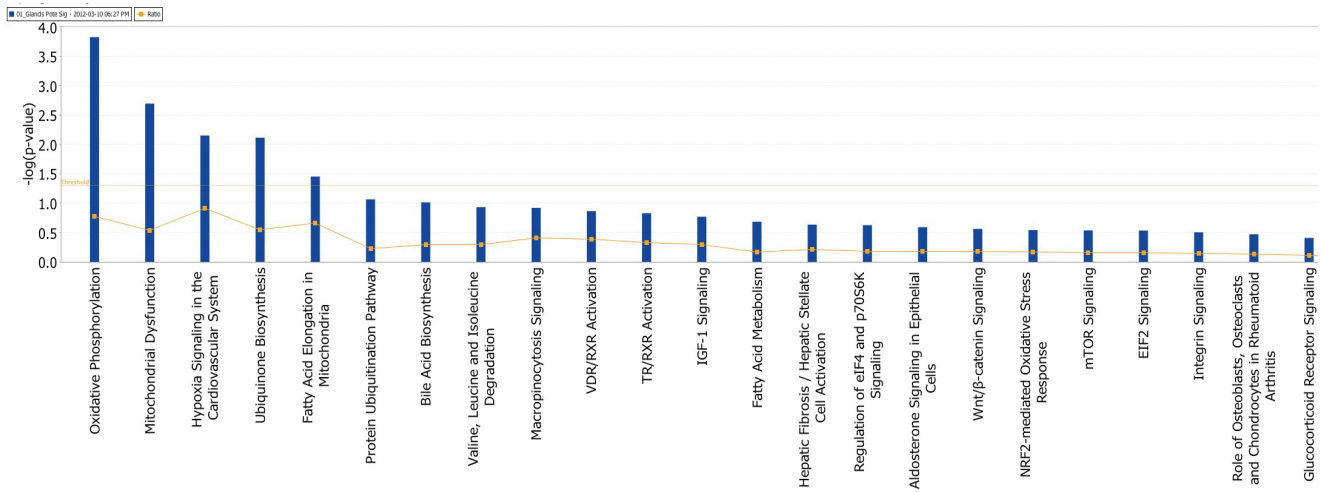


Figure 7: Top network of molecule regulated by progesterone and involved in cellular development, cell death, cellular growth and proliferation

Figure 8. The top progesterone regulated canonical pathways in the glands as analysed by IPA. The y-axis displays the –log of p-value that is calculated by Fisher's exact test right tailed. The points in the bar indicates the ratio which is calculated as the number of genes in a given pathway the meet the cut-off criteria, divided by the total number of genes that make up the pathway. The threshold is drawn at p = 0.05.



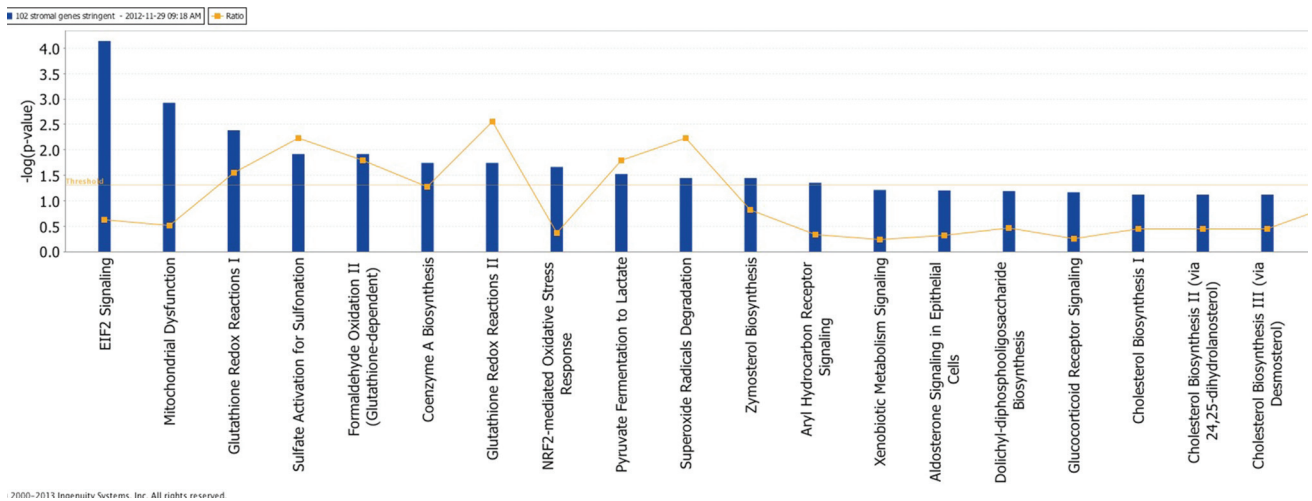
4.4 PAPER IV

Progesterone-mediated gene expression in the human endometrial stromal compartment during the implantation window was investigated.

Microarray data analysis showed 2 annotated genes namely, ATP6V0E1 and HMGN5 regulated more than 2 folds in the stroma (FC > 2; $p<0.05$; $q< 0.1$) with progesterone inhibition. We also report additional 121 annotated genes in the stroma as potentially differentially regulated genes (FC > 2 and unadjusted $p < 0.05$).

Eukaryotic translation initiation Factor 2 (EIF2) pathway was the most significant pathways with the highest threshold value of about 4.5 fold, differentially regulated by progesterone dominance. Figure 9 shows the major canonical pathways differentially regulated by progesterone inhibition in stromal compartment. Real-time PCR analysis showed an up-regulation in the gene expression (fold change; p-value) levels of SFRP4 (6.73; p=0.005), CTSC (2.261; p=0.041), SMARCA 1 (1.66; p=0.021), CPM (16.37; p=0.028), and HMGN5 (1.82; p=0.029). Both MT1G (-333; p= 0.003) and MT2A (-4.67; p=0.034) showed a significant down regulation with progesterone inhibition. CPM was the most significantly up-regulated gene following mifepristone treatment, with a fold change of 16.37, whereas MT1G was the most down-regulated gene (-333 folds) after mifepristone treatment. The protein accumulation of the genes studied by immunohistochemistry showed a similar trend as seen by real-time PCR (table 2, paper IV). Genes SCGB2A2, STC1, and SOD2 with FC of -4.78, -23.26, and -2.12, respectively, had a down regulation trend with the inhibition of progesterone.

Figure 9. The top progesterone regulated canonical pathways in the stromal compartment as analysed by IPA. The y-axis displays the –log of p-value that is calculated by Fisher's exact test right tailed. The faint points indicates the ratio which is calculated as the number of genes in a given pathway the meet the cut-off criteria, divided by the total number of genes that make up the pathway. The threshold is drawn at p = 0.05.



5 DISCUSSION

5.1 DEVELOPMENT OF 3D EMBRYO-ENDOMETRIAL MODEL

In vitro embryo-endometrial co-culture models have given a wealth of information in improving our understanding of the human embryo implantation process and have been considerably good alternatives to *in vivo* animal models (Cha, et al., 2012, Lee and DeMayo, 2004, Lindenberg, et al., 1985). Recently modifications in the culture models have been reported, by replacing the fibrin matrix with human plasma which has the advantage of less cost and better tolerance to cells (Wang, et al., 2012). However, a model that is closely reflecting the *in vivo* molecular and histological features in human embryo implantation is essential to improve our understanding of the early steps of the implantation process. The culture system with stromal cells embedded in a collagen matrix facilitates the cells to grow in a 3D cellular structure with endocrine and paracrine communications, as shown by earlier studies (Arnold, et al., 2001). The endometrial construct developed in this study expresses endometrial receptivity markers such as αvβ3, LIF, IL-1β, MUC-1 and VEGF. To our knowledge, this is the best 3D model tested for its progesterone regulation, which was successfully developed using endometrial stromal and epithelial cells from healthy fertile women. The blastocyst attachment on the endometrial constructs contracted the surface confirming their attachment. Additionally, the attached blastocysts were positive for cytokeratin 7, thus confirming the group of cells attached to endometrial construct was embryonic trophoblast cells. In two cultures where the blastocysts failed to attach, they showed epithelial cell displacement which could be possibly due to the degradation of collagen IV in basement membrane by MMPs produced by the trophoblast cells (Puistola, et al., 1989).

Earlier reported models were not tested for their progesterone regulation. In our study, the model was tested for its progesterone regulation with a potent antiprogestin-mifepristone, which is clinically used for various gynaecological conditions (Spitz, 2010). A well-studied gestagen: levonorgesterol was used as positive control for the model to test the progesterone regulation. Thus, the present study is also valuable in testing and understanding the mechanism of action of different fertility regulating substances (Lalitkumar, et al., 2007, Meng,

et al., 2009, Petersen, et al., 2005). This specific application of the model was tested in study II (Lalitkumar, et al., 2013).

5.2 LIF, PEGLA AND EMBRYO IMPLANTATION

The role of LIF in human embryo implantation using a potent LIF inhibitor was tested. To our knowledge, this is the first study to investigate the essential role of LIF in human embryo implantation process using *in vitro* culture model. Our study revealed that LIF is required in optimal levels for human embryo attachment (Fig 6) and the probable levels of endometrial LIF could be <10pg/ml during the receptive state of endometrium and early stages of implantation process. Our study is limited in quoting the exact range of endometrial LIF, as we could not carry out experiments with the linear dose exposure of LIF due to a limited number of embryos and economic costs. The fact that parenteral administration of LIF did not show any significant increase in women with unexplained infertility highlights the need to investigate the local endometrial LIF levels required for human embryo implantation (Brinsden, et al., 2009). Our experiments lead us to more intriguing questions about the mechanism of action of LIF in human blastocysts, as well as at the embryo-endometrial interface. Thus, this study showed that LIF inhibition by potent LIF inhibitor, PEGLA, prevented human blastocyst attachment in *in vitro* endometrial construct. *In vitro* human blastocysts culture with PEGLA showed degenerative changes. A further functional study delineating the molecular basis of effects of PEGLA in human blastocysts showed activation of caspase-3 and AKT pathway signifying the potential mechanism of cell survival factor AKT pathway and apoptosis marker caspase -3.

Our group has reported earlier that human blastocyst express both LIFR and gp 130 through which LIF signal transduction takes place (Wanggren, et al., 2007). It is possible that both LIFR and gp 130 are used for signal transduction by the endogenous LIF present in human blastocyst, thus promoting its development (Dimitriadis, et al., 2005, Hilton, et al., 1991, Sharkey, et al., 1999). Wang et al. reported that SHP2 bound to gp130 triggers extracellular signal-regulated kinase (ERK) signalling (Wang and Tournier, 2006). Therefore, our study is in parallel with these findings, where RT-PCR and immunofluorescence

showed compromised expression of AKT-1 in the human blastocyst. Additionally, there has been supporting evidence that withdrawal of LIF increases apoptosis of embryonic stem cells (Duval, et al., 2000, Majumder, et al., 2012) and STAT3 - deficient mice display degeneration of embryos (Takeda, et al., 1997). This study highlights the fact that inhibition of LIF by PEGLA in human blastocysts decreases cell survival factors by inhibiting AKT pathway and promotes apoptosis by increasing activation of caspase-3. Studies in mice have shown effective inhibition of blastocyst implantation with treatment of PEGLA (White, et al., 2007). Hence, PEGLA could be used as a potential endometrial contraceptive agent. Additionally, LIF in optimal amounts could be a potential fertility-regulating agent. Since there have been conflicting results about the optimal levels of LIF in uterine fluid secretions, it would be worthwhile to identify the ideal range of LIF essential during the implantation window (Laird, et al., 1997, Ledee-Bataille, et al., 2002, Mikolajczyk, et al., 2006). This could probably lead us into further investigations of the dose and route of administration of LIF to improve pregnancy rates in women with unexplained infertility. This study is limited to the study of the role of only one single cytokine, LIF, whereas *in vivo*, it is the cumulative effect of different cytokines acting on the embryo and the endometrium, essential for successful implantation.

5.3 ENDOMETRIAL RECEPTIVITY

The compartmentalized genetic profile in receptive endometrium was studied and compared with non-receptive endometrium using potent antiprogestin-mifepristone treatment *in vivo*. This study (III) reveals a novel molecule, ENPP3, which could serve as potential endometrial receptivity marker. It was significantly up-regulated during the receptive endometrial phase and more to support its progesterone regulated expression, it was down-regulated in progesterone depleted endometrium (non-receptive endometrium). The role of ENPP3 was more compelling by seeing its predominant distribution during the different phases of menstrual cycle as confirmed by immunohistochemistry (Fig 4, Paper III).

ENPP3 is involved in the purinergic cell-signalling mechansims (Bollen, et al., 2000). It has broad substrate specificity acting on ATP, ADP, ADP-

ribose, diadosine polyphosphates and release AMP (Vollmayer, et al., 2003). Additionally, ENPP3 is involved in recycling of extracellular nucleotides, regulating levels of PPi (anion pyrophosphate), and cell motility activation (Goding, et al., 2003). The exact role of this molecule in endometrial receptivity is not known completely. A large clinical study looking into the expression of ENPP3 in infertility patients may pave the road for its application in ART. Such a tool is necessary to minimize the loss of good quality embryos being transferred into non-receptive endometrium, thereby reducing the economic costs. Additionally, understanding the mechanism of ENPP3 in leading the endometrium to receptive state, one could use factors that could influence the expression of this molecule to regulate fertility.

The relevant network of molecules as analysed by IPA from this study are involved in cellular movement, cell death and cellular growth and proliferation. We observed 9 out of 19 molecules known to be involved in the above functions that were differentially regulated. Most of these molecules had either a direct or indirect relation with TGF-beta (Luo, et al., 2005) or to proto-oncogene MYC (He, et al., 2005). TGF-beta, produced by endometrial epithelial cells is elevated by progesterone and involved in decidualization of stromal cells essential for embryo implantation (Kim, et al., 2005).

The progesterone-mediated gene expression in the human endometrial stromal compartment during the implantation window showed alterations in tissue morphology pathways. One of the important members of this pathway is vimentin (VIM). It is a major subunit protein acting on intermediate filaments in mesenchymal cells, and is required for cellular and vascular integrity, mechanotransduction of sheer stress and wound repair (Eriksson, et al., 2009, Goldman, et al., 1996). Another member of this pathway, glucocorticoid regulated kinase 1 (SGK 1) is up-regulated with mifepristone treatment and shows increased expression of SGK 1 transcripts in endometrium of infertile women compared to fertile women (Feroze-Zaidi, et al., 2007), emphasizing its important role in endometrial receptivity. Additionally, we observed the major differentially regulated canonical pathway: the eukaryotic initiation factors (EIF2) signalling.

These are basically GTP-binding proteins comprised of multiple subunits that escort the initiation of specific form of met-tRNA onto the ribosome (Kimball, et al., 1992, Pavitt and Ron, 2012). More details of other differentially regulated major canonical pathways that belong to mitochondrial dysfunction and X-box binding protein 1 are highlighted in paper IV. Thus, the gene expression profiling of compartmentalised endometrium as studied by microarray, followed by ingenuity pathway analysis and reconfirmed by RT-PCR and immunohistochemistry, gives us the specific molecules involved in the intricate molecular changes during the implantation window.

6 CONCLUSIONS

This thesis has addressed some of the major issues involved in the process of human embryo implantation. One of the crucial milestones of this work is that it has overcome one of the major hurdles in understanding the *in vivo* process of human embryo implantation, as it is ethically and technically not possible to conduct such studies in pregnant women. Here, we have developed a progesterone-regulated *in vitro* model to study the process of human embryo implantation, paving the way to decipher the 'embryo-endometrial dialogue'. The model developed and tested in the first part of the study was applied to study the role of LIF in the human embryo implantation process. To our knowledge, this study is the first to report that embryonic LIF is needed for blastocyst survival, which is essential for successful implantation. We also showed that an optimal level of LIF is a prerequisite for successful implantation. At the endometrial level, this study reveals the compartmentalized expression of receptivity molecules. The major finding is the progesterone regulated epithelial cell marker, ENPP3. The role of ENPP3 and other stromal and epithelial molecules derived from this study on the embryo implantation process could be tested using the endometrial construct developed in paper 1.

7 LIMITATIONS OF THE STUDY

This study has the following limitations:

- Embryo-endometrial cultures were limited only to study the attachment phase of implantation process.

- A single cytokine study was carried out where in *in vivo* a complex array of many cytokines and growth factors are involved in embryo-endometrial cross talk.

- Although our studies yielded a better understanding of the genomic profile in endometrial epithelial and stromal compartment, variations central to the IPA are difficult to eliminate.

- The embryos used in this study are not the best quality of embryos.

8 FUTURE PROSPECTS

Embryo implantation is a process in continuum with reciprocal interactions between the blastocyst and maternal endometrium. Although many individual molecules, cytokines and signalling pathways have been reported, the central unifying theme that delineates the embryo-uterine cross talk still needs to be explored. Progesterone regulated *in vitro* endometrial construct, the model developed in this study could be used to explore the cumulative effects of different cytokines on the implantation process. A better understanding of the molecular cues of implantation might be gained by improvements in *in vitro* model by incorporating other endometrial cells and epithelial glands into the stromal matrices.

The functional role of ENPP3, a marker identified in this study needs to be explored to understand its physiological role in endometrial receptivity. Furthermore, the expression level of ENPP3 in fertile and infertile endometrium needs to be studied for its clinical application including fertility regulation.

Reconfirmation of other differentially expressed stromal and epithelial molecular markers derived from studies III and IV need to be explored for their functional role as well as clinical application, specifically in modulating endometrial receptivity, both in the filed of ART as well as fertility regulation. Developing non-invasive methods to assess the molecular markers of receptive endometrium in a desired menstrual cycle would be one of the challenges, for which the uterine fluid could be a good target.

However, more aggressive regulation of endometrium to a receptive state should be handled with caution which otherwise may result in implanting poor quality embryos and eventually may result in ripple effects of implantation failure.

9 GENERAL SUMMARY

An *in vitro* 3 dimensional embryo-endometrial implantation model mimicking the in vivo system was developed to study the early phases of human embryo implantation process. This model expressed progesterone regulated endometrial receptivity markers. The contraceptive mechanism of action of levonorgestrel and mifepristone was explored to identify any direct embryo lethal effects using this *in vitro* model. Both, levonorgestrel and mifepristone altered the molecular profile of the receptive endometrium making it hostile for the embryo implantation process, *in vitro*. The role of an important maternal cytokine, LIF, essential in early phases of embryo implantation was investigated using a potent LIF inhibitor PEGLA. Our study showed that LIF is required in certain optimum levels (<10 pg/ml) during the early phases of embryo attachment in *in vitro* model. The role of embryonic LIF was emphasized as embryo degenerated when cultured with PEGLA, thus highlighting that cell survival factor caspase 3 and apoptosis marker AKT1 was activated.

The genetic profiling of the receptive endometrium in fertile women during the implantation window was studied and compared with non-receptive endometrium treated with antiprogestin-mifepristone. We found an important molecule, ENPP3, significantly up-regulated in receptive endometrium of healthy fertile women and further confirmed by its down-regulated in non-receptive endometrium when treated with antiprogestin. Thus we believe this molecule could be a potential marker for endometrial receptivity. There were certain major canonical pathways that were differentially regulated in the stromal cells and epithelial cells that could also be of importance in understanding the molecular changes required for endometrial receptivity.

Therefore, this study emphasizes the need for in vitro implantation models to study human embryo implantation and opens up new avenues in managing infertility. PEGLA could be a potential non-steroidal fertility-regulating agent.

10 SVENSK SAMMANFATTNING

Bakgrund: Ofrivillig barnlöshet **(**Infertilitet) är ett vanligt gynekologiskt problem som berör cirka 10-15% av alla kvinnor i reproduktiv ålder. Trots framsteg inom assisterad befruktning (ART) når implantationsnivån idag inte högre än 28-30%. Misslyckad implantation är en av huvudorsakerna till oförklarad barnlöshet. Förståelse för mekanismerna bakom fertiliteten kan förbättra diagnostik och behandling vid oförklarad barnlöshet Denna kunskap skulle dessutom kunna användas till utveckling av nya preventivmedel.

Fokus: Att förstå endometriets receptivitet och den humana implantationsprocessen genom experimentell och translationell forskning med hjälp av *in vivo*-och *in vitro*-metoder.

Artikel I beskriver utvecklingen av en tredimensionell *in vitro*-embryo-endometrie-cellkulturmodell som uttrycker receptivitetsmarkörerna ER-α, ER-β, PR-(A+B), PR-B, VEGF, LIF, IL-1β, COX-2, αVβ3 och MUC1. Modellens humana embryoimplantation testades funktionellt gällande progesteronregleringen med hjälp av antiprogesteronet mifepristone och gestagenet levonorgestrel. Inget av de 15 embryon i odlingen som exponerats för mifepristone fäste vid endometrieodlingen, medan 10/17 från kontrollgruppen och 6/14 i levonorgestrolgruppen gjorde det. Modellen användes vidare i ***Artikel II*** för att studera effekten av Leukaemia inhibitory factor (LIF) på human implantation under användande av en potent LIF-hämmare, PEGLA-pegylated LIF antagonist. Inhibering av LIF med PEGLA hindrar blastocysten från att fästa till *in vitro*-systemet och nedreglerar uttrycket av AKT samt triggar apoptos i den inre cellmassan. Detta studerades med hjälp av immunofluorescence och real-time PCR. ***Artikel III och IV*** undersöks progesteronberoende humana receptivitetsmarkörer i endometriet under implantationsfönstret i stroma och epitel med hjälp av laser capture mikrodissektion och microarray. Uttrycket av ENPP3 på både mRNA- och proteinnivå i endometriets epitel var inte detekterbart i gruppen där progesteronet hade hämmats. Detta visades med microarray, real-time PCR och immunohistokemi. De huvudsakliga canonical vägarna som ändrades i epitelet var den oxidativa fosforyleringen och den mitokondriella "dysfunctional pathway". I stromat återfanns 101 potentiellt differentiellt reglerade gener (FC > 2; p-value < 0,05). Analys med real-time PCR visade signifikanta skillnader i uttrycket av SFRP4 (p=0.005), CTSC (p=0.04), SMARCA 1 (p=0.02), CPM (p=0.03), and HMGN5 (p=0.03), MT1G (p= 0.003) and MT2A (p=0.03) med progesteron. Den huvudsakliga canonical vägen som var differentiellt reglerad med progesteron i stromaceller var, enligt analys med IPA, EIF2-signaleringen och mitokondriella dysfunctional pathway.

KONKLUSION: I studien etablerades en tredimensionell *in vitro* embryo-endometriell cellkulturmodell för att studera den humana embryoimplantationsprocessen. Modellen kan användas för att studera den dialog mellan endometrium och embryo som leder till en framgångsrik implantation och även till att utveckla nya preventivmedel. Modellen kastar nytt ljus på vilken roll LIF har i den humana embryo implantationsprocessen. Den nya progesteronreglerade receptivitetsmakrören ENPP3 identifierades i endometriets epitel. Dess betydelse för ART behöver studeras ytterligare. Den nya kunskapen om det vävnadsspecifika uttrycket av progesteronreglerade gener i endometriets körtel-och stromavävnad skulle ytterligare kunna bidra till förståelsen av den molekylära mekanismen bakom endometriets receptivitet, öka fertiliteten hos kvinnor och även till att utveckla nya preventivmedel med endometriet som målorgan.

Nyckelord: *3D*-embryo-endometrie cellkulturmodell*, endometrial receptivitet, humana blastocyst implantation, LIF, PEGLA, AKT, apoptosis, stromal celler, glandular epithelium, ENPP3.*

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