THESIS ON NATURAL AND EXACT SCIENCES B204

Genes Regulated by Estrogen and Progesterone in Human Endometrium

KARIN ROSENSTEIN

TUT
PRESS

TALLINN UNIVERSITY OF TECHNOLOGY
Faculty of Science
Department of Gene Technology

This dissertation was accepted for the defence of the degree of Doctor of Philosophy on April 6th, 2016

Supervisors: **Professor Andres Salumets, PhD**
University of Tartu, Faculty of Medicine,
Institute of Biomedicine and Translational Medicine and
Women's Clinic

Professor Madis Metsis, PhD
Tallinn University,
School of Natural Sciences and Health

Post-doctoral researcher Jaak Simm, PhD
KU Leuven, ESAT-STADIUS
Stadius Centre for Dynamical Systems

Opponents: **Professor John Aplin, PhD**
The University of Manchester
Institute of Human Development

Senior researcher Kersti Lilleväli, PhD
University of Tartu, Faculty of Medicine,
Institute of Biomedicine and Translational Medicine

Defence of the thesis: 20.05.2016

Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.
/Karin Rosenstein/

ISSN 1406-4723
ISBN 978-9949-23-915-3 (publication)
ISBN 978-9949-23-916-0 (PDF)

LOODUS- JA TÄPPISTEADUSED B204

Östradiooli ja progesterooni reguleeritud geenid inimese endomeetriumis

KARIN ROSENSTEIN

TTÜ
KIRJASTUS

TABLE OF CONTENTS

ORIGINAL PUBLICATIONS

The current thesis is based on three original publications:

I. **Tamm K***, Rõõm M*, Salumets A, Metsis M. (2009). Genes targeted by the estrogen and progesterone receptors in the human endometrial cell lines HEC1A and RL95-2. Reproductive biology and endocrinology, 7 (170), 1-12.

*These authors contributed equally to the work.

II. **Tamm-Rosenstein K,** Simm J, Suhorutšenko M, Salumets A, Metsis M, (2013). Changes in the Transcriptome of the Human Endometrial Ishikawa Cancer Cell Line Induced by Estrogen, Progesterone, Tamoxifen and Mifepristone (RU486) as Detected by RNA-Sequencing. PloS ONE, 8, e68907.

III. Altmäe S, **Tamm-Rosenstein K**, Esteban FJ, Simm J, Kolberg L, Peterson H, Metsis M, Haldre K, Horcajadas JA, Salumets A, and Stavreus-Evers A. Endometrial transcriptome analysis indicates superiorty of natural over artifical cycles in recurrent implantation failure patients undergoing frozen embryo transfer. Reproductive BioMedicine Online. In Press.

My personal contribution to the publications:

I. I participated in designing and performing experimental work, data analysis, and in writing of the manuscript.

II. I participated in the designing and performing of all experiments, participated in data analysis, and wrote the manuscript.

III. I participated in *in silico* data analysis of predicting hormone response elements in promoter regions of genes expressed in human endometrium, and compared the results with data retrieved from Ishikawa cell-line. Participated in manuscript writing.

INTRODUCTION

Steroid hormones are important molecules with wide spectrum of roles in human physiology. Sex steroid hormones, estrogen and progesterone, are responsible in development of female secondary sex characteristics in puberty, and are mandatory for female reproduction as they play pivotal role in initiation and maintenance of the pregnancy.

The same hormones have a 'dark side' in human pathology as dysfunctions of estrogen and progesterone actions often lead to problems with fertility and are common causes or consequences of endocrine related cancers. Infertility affects men and women across the world leaving approximately 50 million couples childless (Mascarenhas et al., 2012). Assisted reproductive technologies (ART), especially *in vitro* fertilization (IVF), provide solutions to overcome infertility. The general success rate of IVF procedures has remained around 30% for the past nearly 40 years since the first IVF child was born in 1978. Despite the fact that there are over 5 million IVF children born, there are still questions related to infertility treatments - how the exogenous and endogenous steroid and non-steroid hormones trigger the signalling pathways in female reproductive tissues, including in ovaries and endometrium.

Implantation failure is one of the major causes of not achieving a pregnancy both naturally and via ART cycles. It has been estimated that approximately 20% of women continuously fail to conceive despite of good quality embryos transferred in IVF embryo transfer. The failure of implantation is usually caused by insufficient embryo quality or aberrant development of endometrium, which might be un-receptive on the day of embryo transfer. The development of the endometrium is under the direct control of ovarian steroid hormones, estrogen and progesterone. Estrogen is predominantly involved in oocyte maturation and the proliferation of the endometrial cells in the first part of the menstrual cycle. After ovulation both estrogen and progesterone prepare the endometrial lining and environment for potential embryo implantation. In most of the cases several attempts of IVF are needed to achieve a pregnancy. This is why, a better understanding of the molecules involved in steroid hormone signalling in preparing the endometrium for embryo implantation are needed, in order to develop and apply the most suitable molecular diagnostic techniques for endometrial receptivity evaluation.

In the current thesis the role of estrogen and progesterone in human endometrium was studied at gene expression level. The developments of large-scale microarray and sequencing methods enabled us to analyse all genes expressed at the same time in experimental *in vitro* conditions and in biopsies of endometrial tissue obtained from fertile and infertile women. Both *in vitro* and *in vivo* methods were used including a comprehensive *in silico* analysis to examine global gene expression in response to female steroid hormones in human endometrium.

ABBREVIATIONS

17β-HSD- 17 beta-hydroxy steroid dehydrogenase
AC- artificial cycle
AKT- protein kinase B (PKB), serine/threonine-specific protein kinase
ART- assisted reproductive technology
cAMP- cyclic adenosine monophosphate
ChIP- chromatin immunoprecipitation
ChIP-Seq- chromatin immunoprecipitation combined with massively parallel DNA sequencing
CoA- co-activator
COH- controlled ovarian hyperstimulation
CoR- co-repressor
CYP19- aromatase, also called estrogen synthetase or estrogen synthase
DBD- DNA binding domain
DEG- differentially expressed genes
E1- estrone
E2- estrogen, oestrogen, 17β-estradiol, oestrogen
E3-estriol
ECM- extracellular matrix
EGF- epidermal growth factor
ER46- membrane bound estrogen receptor alpha variant
ERA- endometrial receptivity array
ERE- estrogen response element
ERx- membrane bound estrogen receptor
ERα- estrogen receptor alpha
ERβ- estrogen receptor beta
FET- frozen embryo transfer
FPKM- Fragments Per Kilobase of transcript per Million mapped reads
FSH- follicle stimulating hormone
GnRH- gonadotropin releasing hormone
GO- gene ontology
GPCR/GPER- G protein-coupled estrogen receptor 1, formerly referred to as G protein-coupled receptor 30 (GPR30)
GRE- glucocorticoid response element
HB-EGF- heparin binding epidermal growth factor
hCG- human chorionic gonadotropin
HEC1A- endometrial epithelial cell line, obtained from human endometrial carcinoma
hESCs- human endometrial stromal cells
HPG axis- hypothalamic-pituitary-gonadal axis
HRE- hormone response element
HRT- hormone replacement therapy
ICM- intra cellular mass of blastocyst

ICSI- intracytoplasmic sperm injection
IGF- insulin like growth factor
IL- interleukin
IPA- Ingenuity pathway analysis
IVF- *in vitro* fertilization
JAK/STAT- second messenger system that transmits information from chemical signals outside the cell, through the cell membrane
LBD- ligand binding domain
LH- luteinizing hormone
LHRH- luteinizing hormone–releasing hormone
LIF- leukemia inhibitory factor
mER- membrane bound estrogen receptor
mPR- membrane bound progesterone receptor
NC-natural cycle
NCOA- nuclear coactivators
NCOR- nuclear corepressors
NR- nuclear receptor
p38/ MAPK, ERK1/2- mitogen-activated protein kinases
P4- progesterone, 4-pregnene-3,20-dione
PAQR- progestin and adipoQ receptors
PCOS- polycystic ovary syndrome
PI3K- Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC- Protein kinase C
PLC- phospholipase C
POF- premature ovarian failure
POI- premature ovarian insufficiency
PRA- progesterone receptor A
PRB- progesterone receptor B
PRE- progesterone response element
RIF- recurrent implantation failure
RL95-2- endometrial epithelial cell line, derived from a moderately differentiated endometrial adenosquamous carcinoma tissue
RNA-Seq- RNA sequencing, whole transcriptome analysis by sequencing
RT-PCR- reverse transcription polymerase chain reaction
RU486- mifepristone, synthetic steroidal antiprogesterone
SERM- selective estrogen receptor modulator
SPRM- selective progesterone receptor modulator
SRC- steroid receptor coactivator
TAM- tamoxifen, synthetic steroidal antiestrogen
TGF-β - transforming growth factor beta
TRANSFAC- database containing published data on eukaryotic transcription factors and their experimentally-proven binding sites and regulated genes
uNK cells- uterine natural killer cells
WOI- window of implantation

REVIEW OF LITERATURE

1. The impact of steroid hormones, estrogens and progestogens, on female physiology

Steroid hormones are classified into five groups, based on their receptors: glucocorticoids, mineralocorticoids, androgens, estrogens, and progestogens. Androgens, estrogens, and progestogens form a smaller subclass of steroid hormones called sex hormones because of their major role in reproductive health. Both sexes have all three steroid hormones, although in different levels and showing different dynamics. Estrogens and progestogens are known as hormones responsible for the female phenotype, in contrast to androgens, which predominantly determine the male phenotype. The main target tissues for these sex steroid hormones are uterus, ovary, breast, prostate, and testis but their importance is also well-known for other organs like brain, bone, heart, blood vessels, liver, skin, gut, and bladder (Fauser et al., 2011; Foryst-Ludwig and Kintscher, 2010; Scarpin et al., 2009).

Female physiology depends on the changing levels of estrogens and progesterone across her full lifetime, especially during puberty, pregnancy, and menopause. Female hormones play major role in reproduction, and disturbances of their actions are usually related to reproductive disorders like premature ovarian insufficiency (POI, also referred as premature ovarian failure or POF), polycystic ovary syndrome (PCOS), and endometriosis, which all are common causes of female infertility (Fauser et al., 2011). POI affects 1% of women and is characterized by amenorrhoea with elevated levels of follicle stimulating hormone (FSH) and low concentrations of estrogens (De Vos et al., 2010). PCOS affects approximately 5% to 10% of women in their reproductive age (Ehrmann, 2005). The exact aetiology of PCOS is not known but ovarian dysfunction, insulin resistance, and abnormal luteinizing hormone (LH) levels are associated with exposure to higher levels of androgens during intrauterine life (Eisner et al., 2000). Endometriosis affects approximately 176 million women worldwide (Adamson and Pasta, 2010). In endometriosis, endometrium-like ectopic lesions show increased estrogen activity and response but resistance to progesterone action (Attia et al., 2000; Ryan et al., 1994). On the other hand, the same hormones and their analogues are broadly used for contraceptive purposes to prevent unplanned pregnancies or for early medical abortion (mifepristone or RU486) (Henzl and Loomba, 2003; Weiss, 1993). In addition, hormone replacement therapy (HRT) with estrogen analogues alone or in combination with progesterone is used to overcome the absence of steroid hormones in post-menopause with the beneficial effects on skin and dental structure, in preventing osteoporosis, and reducing the risk of colon cancer incidence (Henzl and Loomba, 2003). However, the attention should be paid to HRT treatment in women with high body mass index (BMI) because of the

increased risk to develop post-menopausal breast cancer (Munsell et al., 2014). Furthermore HRT and smoking have been associated with higher incidence of venous thrombosis (Pomp et al., 2008). Moreover, the changing levels of estrogens and progestogens may sometimes lead to other hormone-related cancers. In addition to 1.7 million new breast cancer cases, there are more than 500,000 cervical cancer cases, and about 200,000 ovarian cancer cases diagnosed worldwide in each year (http://www.wcrf.org/). Previous studies have also shown that disturbed estrogen and progesterone levels play a significant role in developing cognitive and mental disorders, such as, depression and Alzheimer disease (Frye and Walf, 2009; Walf and Frye, 2010). Female steroid hormones have been associated with decreased blood pressure and are therefore used in treatment of cardiovascular diseases (dos Santos et al., 2014).

Taking into account the pleiotropic actions of steroid hormones in female physiology, special care should be taken in clinical treatments and interventions involving estrogens and progestogens especially when there are certain risks factors engaged, like patient's high BMI, smoking, and genetic mutations predisposing to the occurrence of cancers or thrombosis (Roy et al., 2007; Wiegratz and Thaler, 2011).

1.1 Estrogen

Estrogens are synthesized in ovaries, placenta, and adrenal cortex but also, for example, in adipose tissue. There are three major biologically active estrogens found in women: estrone (E1), estrogen (also named 17β-estradiol) (E2), and estriol (E3). The chemical difference of three estrogens comes from the number of hydroxyl groups. E2 is the predominant estrogen during female´s reproductive age. During pregnancy E3 is the most abundant estrogen in female body, and in the beginning of menopause E1 becomes the dominating circulatory estrogen. Estrogens are synthesized from androgens by the enzyme aromatase (Nelson and Bulun, 2001). Ovarian E2 is produced by granulosa cells, which are supporting cells surrounding the oocyte (Figure 1). E2 has a key role in first part of the menstrual cycle where it participates in the maturation of oocyte(s) and induces the proliferation of endometrial cells (Ghosh et al., 1994; Smitz et al., 1993). The average concentration of E2, at the same menstrual cycle, fluctuates approximately four times from 180 pmol/L in early follicular phase to 870 pmol/L at the time of ovulation (40 pg/ml to 250 pg/ml, respectively). Because of its broad spectrum of functions, E2 levels are monitored in many clinical applications. For example, observation of serum E2 concentration during ovarian stimulation in IVF procedures helps to prepare the timing of the treatment and to reduce the risk of ovarian hyperstimulation syndrome (Kwan et al., 2014).

Besides the important role in reproductive system, E2 also participates in many other physiological processes like modulation of bone density, influence on nervous system and cardiovascular function (dos Santos et al., 2014). E2 replacement therapy has been proven to prevent or diminish calcium loss from

bones in post-menopausal women, thus preventing osteoporosis (Venken et al., 2008). In the nervous system both estrogens and androgens have been reported to influence verbal fluency, performance of spatial tasks, verbal memory capacity, and fine motor skills (Kelly and Ronnekleiv, 2008). Additionally, it has been shown that estrogens shape and maintain the distribution of body fat and adipose tissue metabolism (Pallottini et al., 2008).

1.2 Progesterone

Progesterone (pregn-4-ene-3,20-dione) (P4) is another female hormone belonging to group of progestogens. P4 itself is the precursor of the mineralocorticoid aldosterone which can be converted to testosterone and E2 (White, 2009). P4 is predominantly produced in ovaries but it is also secreted by adrenal gland, placenta, and adipose tissue. After ovulation, the *corpus luteum*, which is formed from the ovulated follicle produces the increasing amount of P4. This female hormone is critical in developing the favourable uterine environment to support the embryo implantation. The highest concentration of P4 is around 40 nmol/l (13 ng/ml), which coincides with the optimal time for potential embryo implantation. P4 also promotes uterine growth and suppresses myometrial contractility to prevent abortion or early labour (Graham and Clarke, 1997, 2002). P4 appears to decrease the maternal immune response which helps to avoid rejection of the embryo during early pregnancy (Ragusa et al., 2004). In addition, the mammary gland P4 participates in lobular-alveolar development in preparation for milk secretion postpartum, while suppressing milk protein synthesis before parturition (Macias and Hinck, 2012).

In IVF procedures P4 is commonly used as a luteal phase support to help the preparation of the endometrial lining and to support the embryo implantation and early pregnancy (van der Linden et al., 2015). The use of P4 is advocated, because the ovarian stimulation and follicular puncture used in IVF may lead to endocrinological disturbances with possible sub-optimal P4 level and luteal phase insufficiency. To overcome these problems P4 support is generally applied following the egg retrieval and embryo transfer (van der Linden et al., 2015).

In addition, P4 signalling in brain is exerting beneficial effects in brain injuries (Graham and Clarke, 1997; Schumacher et al., 2007). Increasing evidence supports its significant role in modulation of bone mass as P4 increases the number of osteoblasts, promotes their maturation, and differentiation (Seifert-Klauss et al., 2012). Higher trabecular bone mass in women is needed for the development of foetal skeleton during pregnancy. Moreover, the third trimester of pregnancy, during which 80% of the foetal skeleton is mineralized, coincides with the maximum rate of P4 production in human physiology (Seifert-Klauss and Prior, 2010).

1.3 Steroidogenesis

The original building block of all steroid hormones is cholesterol. Endocrine organs (except placenta) can synthesize cholesterol locally from acetate but can also absorb the circulating cholesterol from blood. Although steroid hormones are relatively insoluble in water, they are made soluble for transport in the bloodstream and other extracellular fluids by binding to specific carrier proteins, from which they dissociate before entering a target cell. This secretory process is called endocrine action, which affects the function of many target tissues in females, like endometrium, mammary gland, brain, bones, liver, and heart. Steroid hormones also act in close proximity to their site of secretion on adjacent cells and tissues as it happens in testes and ovaries, a process called paracrine action. In addition, autocrine action exists when secreted hormones have the effect on the cells where they were originally produced.

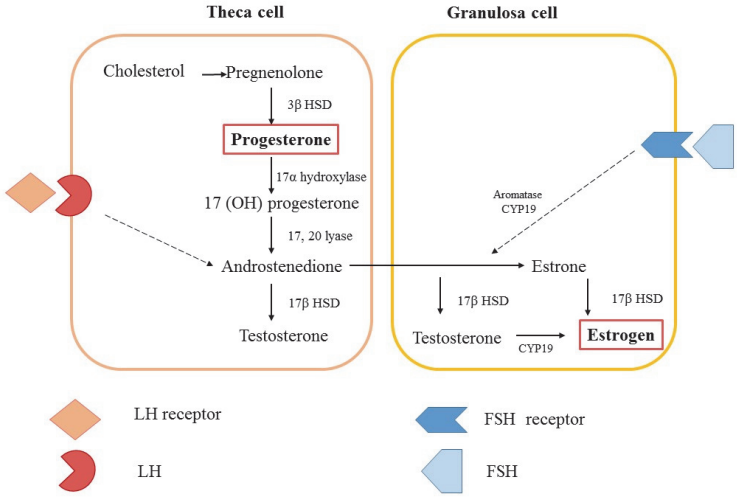

Figure 1. A simplified graph of ovarian steroidogenesis in theca and granulosa cells. Cholesterol enters theca cells where after consecutive enzymatic reactions it is converted to progesterone, and thereafter to androgens (androstenedione and testosterone). Synthesis of androgens is also induced by luteinizing hormone (LH) and its receptor. Androstenedione and testosterone are released from theca cells and diffuse into the nearby granulosa cells. Granulosa cells have a high number of FSH receptors and aromatase - CYP19, which converts androstenedione and testosterone to estrone and estrogen, respectively. The weak estrone can be converted to estrogen by 17 beta-hydroxysteroid dehydrogenase (17β HSD).

Steroidogenesis in the ovary is compartmentalized in a cell-specific manner where two types of cells participate, e.g. theca and granulosa cells.

Developing oocyte is surrounded by layers of granulosa cells followed by outer layers of thecal cells. In theca cells cholesterol is converted to pregnenolone which is a precursor of P4 and is further synthesized to androgens (White, 2009). At the same time theca cells respond to luteinizing hormone (LH), which also induces the production of androgens. Androstenedione and testosterone diffuse into the neighbouring granulosa cells where they are converted predominantly to E2 by aromatase and 17β-HSD types 1 and 7 (Luu-The, 2001; Mindnich et al., 2004). Granulosa cells exhibit receptors of follicle stimulating hormone (FSH) and through FSH binding induce the activity of aromatase - CYP19, thereby increasing the conversion of androgens to E2 (Figure 1).

1.4 E2 and P4 signalling

Steroid hormones have an ability to diffuse directly across the plasma membrane of target cells and mediate their signals through nuclear receptors (NRs) in a steroid hormone action, called genomic signalling (Figure 2A). NRs bind to DNA and act as transcription factors regulating the expression of target genes (O'Malley and Means, 1974).

NRs consist of a ligand-binding domain (LBD) (also called a C-terminal), a highly conserved DNA-binding domain (DBD), and an N-terminal domain. Within N-terminal domain and LBD there are usually at least two transcription activation subdomains (AF1 and AF2) present. LBD and DBD are usually highly conserved, whereas N-terminal domain could be variable (Beato and Klug, 2000). N-terminal domain contains multiple Ser/Thr phosphorylation sites, which could be involved in mediating cross-talk with other signal transduction pathways or interaction with co-regulators (Lange et al., 2000). NRs are usually located in cytoplasm of a steroid hormone target cell. Binding of a steroid hormone to its cognate receptor results in a conformational change in the NR that allows the ligand-receptor complex to bind with high affinity to hormone response elements (HREs) on DNA and regulate transcription of target genes (Khorasanizadeh and Rastinejad, 2001). The binding to HRE could happen directly when ligand-receptor complex binds to DNA and interacts with RNA Polymerase II transcription initiation and co-activator complexes (Klinge, 2000). The other mechanism is called “tethering” when NR does not bind directly to DNA but associates with other transcription factors e.g. AP-1, Sp1, c-Jun, c-Fos, or other proteins which are already attached to DNA (Scholz et al., 1998) (Figure 2A). In the absence of ligand, NRs are held in a multi-subunit complexes containing heat-shock proteins such as Hsp90, SP70, HSP40, Hop, and p23 (Wolf et al., 2008). In response to ligand binding, NR dissociates from the heat-shock proteins, changes its conformation, dimerizes, and binds to specific HRE to regulate transcription of target genes (Nilsson et al., 2001).

Not all effects of E2 and P4 can be explained by classical model of steroid action via NRs. Additional non-transcriptional signalling pathway exists, called non-genomic action, where effects are usually membrane initiated and

involve the activation of several signal transduction pathways like PLC/PKC, p38/MAPK, and JAK/STAT (Figure 2B) (Bjornstrom and Sjoberg, 2002; Falkenstein and Wehling, 2000). Non-genomic action is usually characterized by a shorter lag time (within seconds to minutes) to elicit a biological response following steroid hormone stimulation. Non-genomic effects of steroid hormones could be mediated by classical NRs localized in the plasma membrane, other membrane proteins, or G protein-coupled receptors (GPCRs). Generally, it is hard to determine the line between genomic and non-genomic action of steroid hormones because both could be active at the same time or one can take over others actions. Non-genomic effects of E2 and P4 have been mostly described in relation to nervous and cardiovascular systems where their effects may be transmitted through G protein-coupled estrogen receptor 1 (GPER), include stimulation of cAMP, and Ca2+ mobilization, as well as MAPK, ERK1/2, and PI3K/AKT pathways (Revankar et al., 2005; Thomas et al., 2005).

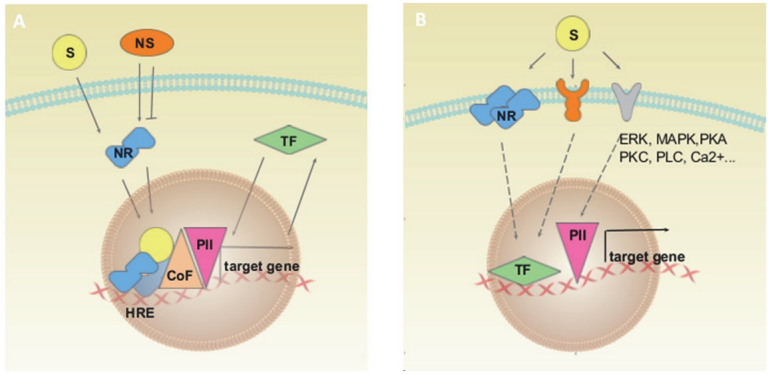

Figure 2. Genomic **(A)** and non-genomic **(B)** signalling of steroid hormones. **A.** Steroid receptors bound to their respective steroid hormones attach to cognate HREs on DNA. Alternatively, nuclear receptors can bind other proteins (CoF or TF) rather than DNA. Non-steroidal ligands can also act through nuclear steroid receptors. **B.** Membrane-associated steroid receptors, either isoforms of classical receptors, or unrelated transmembrane receptors, recognize steroid hormones and initiate a cytoplasmic signalling cascade. CoF = co-factor; HRE = hormone response element; NR = nuclear steroid receptor; NS = non-steroid; PII = RNA polymerase II; S = steroid hormone. The figure was generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity).

1.4.1 Estrogen receptors

In humans, there are two major forms of E2 receptors (ERs), ERα and ERβ, which are encoded by separate genes on different chromosomes, chromosome 6 and 14, respectively (Enmark et al., 1997; Green et al., 1986; Kuiper and Gustafsson, 1997). ER subtypes are highly conserved in their DBD (97%) and LBD (55%) regions and show only minor differences in DNA-binding affinity in response to E2. Although ERα and ERβ are co-expressed in many target tissues, they exhibit different tissue-specific expression patterns and are likely functionally distinct (Edwards, 2005a). With high similarity in their DBDs, both receptors interact with the same conserved estrogen response element (ERE) (5'-GGTCAnnnTGACC-3') as homodimers or α/β heterodimers (Klinge et al., 2004). However, it has been noted that ERβ holds low trans-acting capability on ERE-containing E2 target genes, and α/β heterodimers are less efficient than ERα homodimers in promoting target genes activity (Zhao et al., 2007). Studies have shown that both ERα and ERβ coding genes have alternatively spliced transcripts. Two main splice variants *ERa46* and *ERa66* have been reported for ERα (Penot et al., 2005; Wang et al., 2005). Both truncated isoforms lack transcriptional activation and are more likely to have inhibitory effects on ERα and ERβ, or participate in non-genomic E2 signalling. For ERβ also shorter isoforms have been identified but their exact functional role is not fully understood (Moore et al., 1998).

ERs have also been localized to cytoplasmic membrane while being bound to G-protein coupled receptors (Warner and Gustafsson, 2006). Membrane bound ERs (mERs) include ER46, ERx and G protein-coupled estrogen receptor 1 (GPER/GPR30) (Li et al., 2003; Thomas et al., 2005; Toran-Allerand et al., 2002).

It is important to point out, that in addition to steroid hormones there exist endogenous, environmental or pharmaceutical non-steroidal ligands like, lipoxin A4 (LXA4), bisphenol A and clomiphene citrate, that also bind steroid hormone receptors and can modulate transcription of target genes (Li et al., 2012; Russell et al., 2011). Selective E2 and P4 receptor modulators will be discussed in further chapters.

1.4.2 Progesterone receptors

The genomic P4 signalling is also mediated by two receptors, PRA and PRB. Though, they are transcribed from the same gene on chromosome 10, using different promoters and resulting in the translation of an additional 164 amino acids at the N-terminus of PRB (Kastner et al., 1990; Wen et al., 1994). Despite the similar DNA-binding ability of progesterone receptors (PRs), PRB is a stronger transcriptional activator in most cell types, while PRA often acts as a dominant negative repressor for PRB activity (Tung et al., 1993; Vegeto et al., 1993). Both P4 receptors bind to the same DNA sequence, as well as to DNA

sequence recognising the glucocorticoid receptor (GR), since its core DBDs exhibit 90% amino acid sequence identity with PRs (Smith et al., 1997; Takimoto et al., 2003). The classical sequence of a GRE/PRE is 5'-GGTACAnnnTGTYCT-3', where the right half of the palindrome is more conserved (Schauer et al., 1989). *In vitro* studies have revealed two additional truncated PR isoforms: PRC and PRM. PRC isoform lacks the first zinc-finger of the DBD causing it to lose its DNA-binding ability but retain the ligand binding ability. The main function of the PRC could be to antagonize PRA and PRB activity (Wei and Miner, 1994). In addition, PRM isoform, which arises from splicing of an in-frame start codon, could also play a role in silencing of main PR action (Saner et al., 2003). However, *in vivo* role of these receptors has been questioned due to their low expression levels (Samalecos and Gellersen, 2008).

In addition, PRs have also been found on membranes, where they participate in non-genomic signalling of hormones. For example, in sperm cells they are responsible for the rapid activation of Ca^{2+} (influx) and Cl^{-} (efflux) channels which lead to acrosome reaction (Tesarik and Mendoza, 1993). As chromatin of a sperm cell is very tightly packed, genomic action is not an option. Moreover, membrane bound PRs have been found on the surface of osteoblasts, granulosa cells, and oocytes (Bagowski et al., 2001; Grosse et al., 2000; Machelon et al., 1996). Membrane bound PRs (mPR) belong to a highly conserved family called progestin and adipoQ receptors (PAQR) (Zhu et al., 2003). There are three members of mPRs identified: α, β, γ (Zhu et al., 2003; Tokumoto et al., 2006). Firstly, it was postulated that mPRs are classical G protein-coupled receptors with seven transmembrane domains, but later it was found that mPRs' amino-terminus is in cytoplasm and carboxy-terminus resides on cell surface (Tang et al., 2005). Upon P4 binding mPRs modulate signal transduction cascades including ERK1/2, p38 MAPKs, inhibition of cAMP production, and stimulation of intracellular Ca2+ levels (Ashley et al., 2006; Hanna et al., 2006; Thomas et al., 2007).

1.4.3 Selective ER and PR modulators

Selective steroid hormone receptor modulators are implemented in clinical settings to treat hormone responsive cancers and ovulatory dysfunction, prevent osteoporosis, and are taken for contraceptive purposes. ER and PR modulators have an ability to interact with respective receptors, and change the conformation of the NRs leading to alterations in E2 and P4 signalling (Berrodin et al., 2009; Chang et al., 2010).

In general, most selective estrogen receptor modulators (SERMs) have an E2 agonist activity in bone and antagonist activity in the breast, while the activity in the uterus varies among the molecules. The anti-estrogenic action of SERMs results from the inappropriate folding of an ERα or ERβ complex, which therefore cannot recruit the CoA molecules needed or alternatively recruit the

unexpected CoR molecules. This conformational change produces anti-estrogenic action at certain locations, like breast, opposing the E2-like effects in the uterus if an excess of CoA molecules is present. The first SERM available for clinical use was tamoxifen (TAM), which has been used as a highly effective drug for the prevention and treatment of breast cancer in premenopausal and postmenopausal women (Fisher et al., 2005; Fisher et al., 1998). This compound binds with high affinity to ER, thereby blocking the action of native E2. Subsequently it inhibits or modifies the interaction of ER with DNA, which impedes the transcriptional activation of target genes (Berry, 2005). One of the most significant side effects of the treatment with the TAM appears to be its proliferative effect on the endometrial cells (Bergman et al., 2000; Buzdar and Hortobagyi, 1998). Thus, the use of TAM is accompanied in a significant 3.3 fold increase in endometrial cancer risk (Fisher et al., 2005). As a consequence, the repression of the cell proliferation in breast tissue with TAM could lead to uncontrolled endometrial cell proliferation. Uterine hyperplasia, polyps, carcinomas, and sarcomas are the most frequent endometrial pathologies associated with the use of TAM (Cohen, 2004). Another well-studied anti-estrogen, Raloxifene, with E2-antagonistic effect similar to TAM, is reported to have smaller or negligible proliferative effect on uterine cells (Fugere et al., 2000). The most known „pure“ anti-estrogen is fulvestrant (also known as ICI 182780) as it blocks ER activity by forcing it to change receptor's conformation, which is then recognized as being misfolded and inducing its rapid degradation (Wakeling and Bowler, 1991; Wu et al., 2005)

Selective PR modulators (SPRMs) are developed in order to antagonize processes activated by P4. Mifepristone (RU-486) acts as a P4 antagonist by competing with endogenous P4 for receptor binding, and has 3 primary pharmacological effects: endometrial, gonadotropic, and adrenocortical (Goldberg et al., 1998). It has 2 to 10 times higher binding affinity to PRs compared to P4 (Brogden et al., 1993). Because PRs are found primarily in reproductive organs, RU-486 exerts its principal effect on the uterus. More precisely, it blocks the effect of natural P4 on the endometrium and decidua. While P4 is supposed to support the pregnancy, anti-P4 leads to degeneration and shedding of the endometrial lining, thereby preventing or disrupting implantation of the conceptus (Weiss, 1993). Therefore, the mifepristone is the accepted drug for the termination of the pregnancy at least in some countries.

2. Cyclic changes of ovarian steroid hormones

To understand the regulative hormonal changes in female reproductive physiology we need to look into tightly coordinated action of hypothalamus, pituitary gland, and ovaries. This signalling is called hypothalamic-pituitary-gonadal (HPG) axis and it is activated in puberty. HPG axis is responsible for coordinating the ovarian and uterine e.g. menstrual cycle both occurring concurrently usually within 28 days (Figure 3). Ovarian cycle is responsible for

the maturation of the oocyte(s) and ovulation, and it is regulated by gonadotropic hormones FSH and LH released form the anterior pituitary gland and ovarian E2. At the same time menstrual cycle is responsible for the development of the endometrial lining for potential embryo implantation and is predominantly regulated by ovarian steroid hormones E2 and P4 (Figure 3).

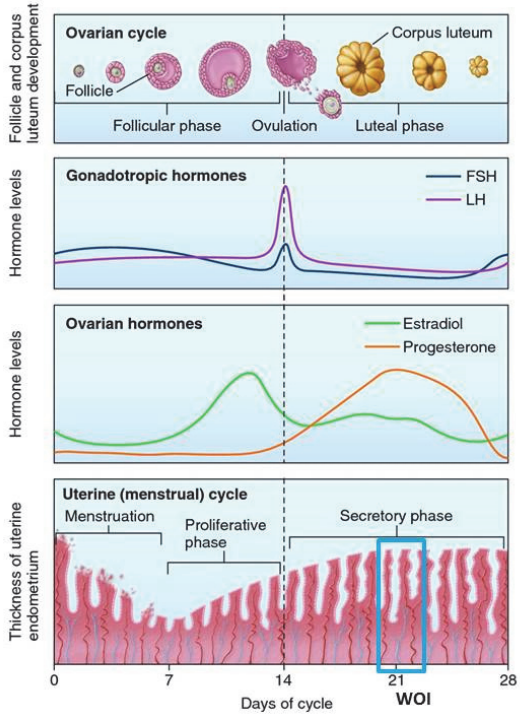

Figure 3. Ovarian cycle and uterine (menstrual) cycle. Ovarian cycle (above) is divided into follicular phase, ovulation and luteal phase. The maturation of the oocyte is regulated by gonadotropins FSH and LH but also induced by ovarian E2. Ovarian hormones E2 and P4 regulate the development of uterine lining - endometrium. Uterine cycle (below) is divided into three phases: menstruation, proliferative and secretory phases. Endometrium is most receptive for embryo implantation between days 20-23, during window of implantation (WOI) (light blue square). Figure adapted from http://biology-forums.com.

2.1 Ovarian cycle and the role of steroid hormones

Estonian-German scientist Karl Ernst von Baer discovered in 1827 that the mammalian oocyte is enclosed within the ovarian follicle. Ovarian follicle is fluid filled sac that in addition to oocyte contains different somatic cells (cumulus, granulosa and theca cells) which surround the oocyte and support oocyte maturation. Follicular development starts already in girl's foetal life when primordial follicles enter to their first meiotic division but stay arrested in first prophase until puberty (Baker, 1963). At 20 weeks of gestation the number

of follicles is estimated to be 7 million and is thereafter gradually declined. At birth there are approximately one million follicles and at the time of puberty around 400,000 follicles remain, and during woman´s reproductive life about 400 oocytes are ovulated (Hansen et al., 2008). Follicular development and oocyte maturation continues during the puberty with the activation of HPG axis when every month subset of primary follicles becomes responsive to gonadotropins (Craig et al., 2007). Hypothalamic pulsatile secretion of gonadotropin releasing hormone (GnRH) controls the synthesis and secretion of gonadotropins, FSH and LH, from the pituitary gland. FSH and LH are thereafter secreted into the blood circulation and are attached to their respective receptors on follicular granulosa and theca cells where they stimulate the production of ovarian steroid hormones (Hillier et al., 1981). Ovarian cycle can be divided into follicular phase (days 1-14), and luteal phase (days 14-28) with ovulation in between (on day 14) (Figure 3). During the early follicular phase (days 1-5) low levels of E2 produced by theca cells have negative feedback over FSH and LH by inhibiting GnRH secretion. Therefore, follicles start to compete for the remaining levels of the FSH and follicle with the highest number of FSH receptors on its granulosa cells becomes dominant. Hereafter granulosa cells of the dominant follicle produce increasing levels of E2 and at the same time thecal cells provide additional E2 from androgens. During pre-ovulatory period over 90% of the E2 comes from dominant follicle with the highest level on the day before ovulation (Baird and Fraser, 1974). Subsequently granulosa cells of the dominant follicle form LH receptors and become more LH responsive instead of FSH (Filicori et al., 2002). At the same time high levels of E2 have positive feedback to the hypothalamus and pituitary gland to trigger the surge of LH necessary for final maturation, meiosis and ovulation of the oocyte (Baerwald et al., 2012). Ovulation takes place usually within 24 hours of the LH surge (Bomsel-Helmreich et al., 1979). After ovulation, the disrupted empty follicle forms *corpus luteum*, which starts to secrete increasing amounts of P4 and lower levels of E2. From that point onwards, the P4 becomes the dominant hormone during the luteal phase lasting from day 14 to 28 (Figure 3). In case of embryo implantation and pregnancy, the *corpus luteum* will continue to grow and will be the main source of P4 during the first trimester of pregnancy. Later, it will slowly regress and placenta takes over the role of its hormonal biosynthesis for the maintenance of the pregnancy. Without embryo implantation *corpus luteum* degenerates in 10-12 days after ovulation and new selection of follicles become responsive to gonadotropins during the next ovarian cycle (Baerwald et al., 2012).

2.2 Endometrium and menstrual cycle

The inner tissue lining of the uterus – the endometrium, plays fundamental role in establishing, supporting and maintaining the pregnancy. This dynamic tissue

undergoes approximately 400 rounds of regeneration, differentiation, and degeneration during woman´s reproductive years (Kyo et al., 2011).

The endometrial tissue is divided into two major compartments: basal and functional layer. Basal layer is adjacent to the myometrium and generally remains intact from cycle to cycle. Basal layer is the source of the putative endometrial stem cells responsible for restoring the functional layer after shedding (Maruyama et al., 2010). Functional layer consists of two types of epithelium, luminal and glandular epithelium. Luminal epithelium is the upper lining of the endometrial surface. During the endometrial development epithelial cells invade to stromal compartments and form tubular glands across the tissue to constitute glandular epithelium. Underneath epithelial cells is stromal connective tissue that involves mesenchymal cells with fibroblastic appearance, endothelial cells and leukocytes (Gellersen and Brosens, 2003). The monthly development of functional endometrium is under the direct control of steroid hormones, E2 and P4, going through proliferation, differentiation and tissue shedding (Tabibzadeh, 1998). Aforementioned cyclic changes in human endometrium are called menstrual cycle consisting of three phases: proliferative, secretory and menstrual phase.

Proliferative phase (days 5-14) is characterized by active tissue proliferation, anti-apoptotic processes, and stimulated angiogenesis where ovarian E2 is the main key player (Ghosh et al., 1994; Smitz et al., 1993) (Figure 3). In addition, E2 is stimulating the expression of its own receptors (ERs) and also PRs which is crucial for P4 action in secretory phase (Chauchereau et al., 1992). After ovulation rising levels of P4 start to inhibit proliferative activity of E2 and initiate changes in endometrial tissue specific to secretory phase. Initiated changes in endometrium are important in preparing it for embryo implantation. Second part of the menstrual cycle can be subdivided into early-, mid-, and late secretory phase, with potential embryo implantation in the mid-secretory phase (Lim et al., 2002; Martin et al., 2002) (Figure 3). Secretory transformation of human endometrium involves changes in uterine glands, reprogramming of stromal cells, vascular remodelling and influx of the uterine natural killer (uNK) cells - the whole process known as decidualization (Dunn et al., 2003; King, 2000). In humans decidualization process starts after ovulation in response to P4 rise and unlike in rodents it is independent form blastocyst signal (Gellersen et al., 2007). Molecular reprogramming of endometrial stromal cells depends on the induction of P4 and cyclic adenosine monophosphate (cAMP) signalling pathways (Gellersen et al., 2007). Upon decidualization endometrial stromal cells enlarge and transform into cells with larger nuclei, abundant cytoplasm with glycogen accumulation and lipid droplets and secrete proteins of extra cellular matrix (ECM) but also various cytokines, growth factors, and neuropeptides (Dimitriadis et al., 2005; Gellersen and Brosens, 2003).

In case of oocyte fertilization decidualization culminates with embryo implantation during mid-luteal phase. Embryo implantation can happen in each

menstrual cycle during very restricted period, between days 20-23, and this period is called "Window of Implantation" (WOI) (Figure 3) (Giudice, 1999; Harper, 1992). In the absence of embryo implantation declining levels of steroid hormones cause degeneration of the endometrial tissue in menstrual phase (days 1-4) where P4 plays the key role in the decision of cell survival or death prior to the menstruation (Brosens and Gellersen, 2006).

2.2.1 The molecular biomarkers of receptive endometrium

Sixty five years ago Noyes and colleagues described histological changes of the endometrium throughout the menstrual cycle, where particular features of endometrial histology were correlated to respective phases of the menstrual cycle (Noyes, 1956). Since then Noyes criteria have been used as a "golden standard" and main reference in endometrial evaluation. Later on the scanning electron microscopy revealed the appearance of bulging structures from the apical surface of luminal epithelial cells, termed pinopods, during the mid-secretory phase (Nikas et al., 1995). Pinopode detection has also been used as a morphological marker for endometrial receptivity as their appearance corresponds with putative WOI although their exact role remains questionable (Aghajanova et al., 2003; Quinn and Casper, 2009; Stavreus-Evers et al., 2001)

The evaluation of ERs and PRs has also suggested for assessment of endometrial receptivity. Generally, E2 up-regulates the expression of both ERs and PRs in human endometrium while P4 has a suppressive effect on both receptor types (Lessey et al., 1988). Both ERs are expressed at high levels during the proliferative phase, ERα more intensively compared to ERβ. In secretory phase ERα level continuously declines both in epithelial and stromal compartments while ERβ expression remains in stroma also during secretory phase (Critchley and Saunders, 2009; Mylonas et al., 2007). PRs are also highly expressed in endometrial cells during proliferative phase but are reduced from epithelium in secretory phase. Although the expression of PRA subtype remains in the stoma during secretory phase and early pregnancy (Wang et al., 1998).

With the arrival of microarray and whole genome sequencing technologies, the search for molecular biomarkers for receptive state endometrium has continued and wide range of genes have been found differentially expressed in human endometrium during the WOI (Altmae et al., 2010; Carson et al., 2002; Diaz-Gimeno et al., 2011; Diaz-Gimeno et al., 2013; Garrido-Gomez et al., 2013; Haouzi et al., 2009a; Haouzi et al., 2009b; Horcajadas et al., 2004; Hu et al., 2014; Kao et al., 2002; Krikun et al., 2005; Mirkin et al., 2005; Punyadeera et al., 2005; Riesewijk et al., 2003). Each new study has identified potential biomarkers for receptive endometrium but the number of common genes between publications has remained relatively low. However, some common genes and pathways can be highlighted, such as leukemia inhibitory factor (LIF) pathway molecules, which are involved in the

maintenance of the receptive state of human endometrium as shown in numerous studies (Aghajanova et al., 2009; Cullinan et al., 1996; Steck et al., 2004; Stewart, 1994). Although, there were big expectations on the use of recombinant human LIF (r-LIF) in ART, it did not improve implantation and pregnancy outcomes in IVF procedures in women with unexplained implantation failure (Brinsden et al., 2009). Differential expression of interleukins (IL-6 and IL-11) has also been shown in human endometrium at the time of WOI (Cork et al., 2001; Dimitriadis et al., 2010a; Dimitriadis et al., 2010b; Dimitriadis et al., 2000). Two integrins, α4β1 and avβ3, have been raised to the status of endometrial receptivity markers, and have been used in clinical practice to evaluate the endometrial quality (Lessey, 1997; Lessey and Castelbaum, 2002; Lessey et al., 2000). Growth factors and their respective receptors expressed both in endometrium and on the surface of the embryo, have been shown to play crucial role in implantation process (Kabir-Salmani et al., 2004). Over the past research, the most attention has been given on factors like transforming growth factor beta (TGF-β), epidermal growth factor (EGF), heparin binding epidermal growth factor (HB-EGF), and insulin like growth factor (IGF) (Jones et al., 2006; Lessey et al., 2002; Stavreus-Evers et al., 2002).

Based on the previous research, a molecular tool, endometrial receptivity array (ERA) was developed helping to evaluate the transcriptomic signature for human endometrial receptivity (Diaz-Gimeno et al 2011). ERA consists of 238 genes whose expressions have been shown to differ from pre-receptive to the receptive stage of the endometrium in fertile women. This test is used clinically and is believed to be more accurate method for evaluating the correct developmental stage of human endometrium, rather than the histological assessment (Diaz-Gimeno et al 2011). However, in a clinical perspective, it is crucial to identify factors, which regulate the expression of genes in human endometrium at the time of WOI. As the steroid hormones – E2 and P4 are the main regulators of genome activity in endometrial tissue, the special attention should be paid on analysing the effects of E2 and P4 on the expression of WOI-specific genes.

3. Implantation from embryo side

Several events are indispensable for successful embryo implantation. Pre-implantation development of embryo starts with successful fertilization of an oocyte, which naturally takes place in Fallopian tube. Normally fertilized oocyte (zygote) has two pronuclei at 17-18 h post-fertilization, originating from the oocyte and sperm cell. During its transportation towards the uterus, the zygote begins to divide. In normally developing human embryo, there are three cell divisions finished, resulting in a 8-cell-stage embryo on day 3, when the embryonic genome is activated (Niakan et al., 2012). By day 4 there are usually 8-16 blastomeres, which start to adhere to each other and form a morula. From that stage onward, the embryonic cells begin to differentiate and form a

blastocyst by accumulating the fluid between the blastomeres and forming a fluid-filled cavity – the blastocoel. By day 5 or 6 blastocyst arrives to the uterus. The developed blastocyst has two cell types, an inner cell mass (ICM) and a surrounding cell layer, referred as trophectoderm. ICM comprises pluripotent cells that are able to give rise to all cells of the foetus. The trophectoderm, surrounding the blastocoel, forms upon implantation the extra-embryonic cells and tissues, like placental cytotrophoblasts, syncytiotrophoblasts, and extravillous trophoblasts (Niakan et al., 2012). Before the embryo can attach to endometrium, it needs to be hatched from the surrounding glycoprotein layer called *zona pellucida* (Figure 4). It has been observed that increased zona thickness could hinder embryo hatching and has been associated with lower implantation rates (Cohen et al., 1989).

Implantation of human embryo starts 6-9 days after ovulation. Implantation starts with the apposition of the embryo onto the endometrial surface, while blastocyst anchors itself into mucus, rolls on the epithelial lining and orientates its ICM toward decidua. In initiating the first connection between blastocyst and endometrium the roles of mucins and pinopods seem to be important. Thereafter stronger attachment and adhesion of the blastocyst begins with reciprocal signalling and communication between trophectoderm and receptive endometrium where different integrins, cadherins from both sides and endometrial prostaglandins are involved. Next, the blastocyst penetrates through the endometrial lining and enters into the stromal compartment. Blastocyst invasion to endometrial tissue requires degradation of the components of ECM (like cadherins, laminins and collagens) by serine endopeptidases and metalloproteinases (Aplin and Kimber, 2004; Bentin-Ley and Lopata, 2000).

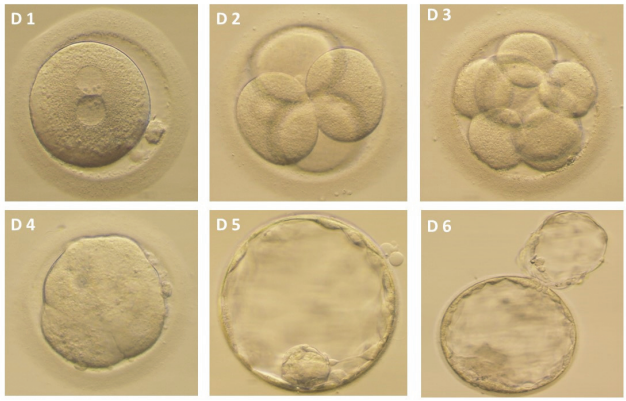

Figure 4. Stages of human pre-implantation embryo development from day 1 (D 1) to day 6 (D 6), following fertilization. Two pronuclei can be visualised after 17-18h post-fertilization on D 1. By day 2 (D 2), two cell divisions have taken place forming a 4-cell embryo. On day 3 (D 3), an 8-cell embryo can be seen. On day 4 (D 4), blastomeres fuse together, in a process called compaction, and result in morula. The blastocyst, which has formed by day 5 (D 5), consists of an inner cell mass and trophectoderm. On day 6 (D 6), the blastocyst 'hatches' from the *zona pellucida* and is then ready to implant into endometrium. Phase-contrast images of human embryos from Nova Vita Clinic.

Generally there is a correlation between good embryo quality and successful implantation. However, previous studies have shown that 30-60% of human pre-implantation embryos may carry chromosomal abnormalities, which may even exist in embryos with apparently good morphological quality (Alfarawati et al., 2011; Bahce et al., 1999; Sandalinas et al., 2001). Genetically aberrant embryos do not usually implant or the pregnancy is aborted in a very early stage. Chromosomal abnormalities in early cleavage-stage embryos are usually caused by the meiotic defects in oocytes or alternatively derived from the mitotic errors in embryos, leading to full chromosomal aneuploidies and sub-chromosomal gains and losses, respectively (Vanneste et al., 2009). The increased maternal age is one the strongest factors predisposing to the meiotic chromosomal errors, and aneuploidies in oocytes and preimplantation embryos (Balasch and Gratacos, 2012). Furthermore, the chromosomal aberrations could also be inherited, as for example, increased frequency of female chromosomal abnormalities, such as translocations, mosaicism, inversions, deletions, and chromosomal breakages, has been observed in young women with several implantation failures (Raziel et al., 2002; Tarlatzis et al., 2000).

4. Infertility and ART

Infertility is defined as the failure to achieve a clinical pregnancy after at least 12 months of regular unprotected sexual intercourse (Zegers-Hochschild et al., 2009). As infertility is a very personal and delicate matter it is difficult to estimate how many couples exactly suffer from this problem, but it has been estimated to affect between 8 and 12% of reproductive-aged couples worldwide (Ombelet et al., 2008). Approximately 20-30% of couple`s infertility cases are explained by male problems, 20-35% by female problems, and 25-40% of infertility cases are caused by the problems in both partners. In 10-20% of the cases, the cause of infertility remains unexplained (ESHRE “Art fact sheet” 2014). Numerous factors from both female and male side have been associated with the couples’ infertility, including ovulation defects, spermatogenic failure, parental age, endometrial dysfunction, obesity, and infections, in addition to specific genetic problems (Venkatesh et al., 2014).

The first report of establishing full-term pregnancy using *in vitro* fertilization (IVF), embryo culture and transfer happened in 1978 and is still the most striking and inspiring landmark in development of assisted reproductive technologies (ART) (Steptoe and Edwards, 1978). Today the estimated number of IVF children born worldwide has already crossed five million (Ferraretti et al., 2013). According to Estonian Birth Register 355 children were born as a result of IVF on 2014, which makes 2.6 % of all births (Report of National Institute for Health Development 2015). ART typically involves controlled ovarian stimulation to obtain multiple oocytes, which are fertilized in laboratory conditions (Griesinger et al., 2005). In a classical IVF procedure, oocytes and

sperm cells are co-incubated together in a culture dish. In case of severe male infertility, intracytoplasmic sperm injection (ICSI) is applied, where a single spermatozoon is injected into the oocyte cytoplasm using micromanipulation (Palermo et al., 1992). After successful fertilization human embryos are monitored under the microscope and embryos are cultured for the following 2-6 days in laboratory conditions. Embryo(s) with the best morphological quality and correct developmental stage is (are) selected for transfer to the uterus.

The general success rate of IVF procedures has stayed around 30% for almost four decades (Voullaire et al., 2002). Cumulative pregnancy rate after six consecutive IVF cycles remain below 75%, leaving a substantial proportion of couples childless even despite of numerous IVF cycles (Alsalili et al., 1995). One of the last barriers for ART success, seems to be the implantation process, as apparently good quality embryos are not always implanted (Edwards, 2006). Embryo implantation is a complex process, which depends on the quality of the embryo and the receptivity of the endometrium. In addition, both processes need to be synchronized and happen in certain limited time period. It is therefore estimated that inadequate uterine receptivity is the cause for almost 2/3 of implantation failures, whereas the embryo itself is responsible for only 1/3 of failures (Ledee-Bataille et al., 2002).

4.1 Recurrent implantation failure

In every IVF clinic there is a certain group of women who repeatedly fail to achieve pregnancy in spite of the good quality embryos transferred. In many cases the reason why embryo(s) do not implant remains unexplained. Failure to conceive after repeated attempts of IVF treatment is often referred as recurrent implantation failure (RIF), also classified as unexplained or idiopathic infertility. Most commonly it means that there have been 2-6 unsuccessful IVF attempts per couple with good quality embryos (Polanski et al., 2014; Tan et al., 2005). Usually women with RIF are otherwise healthy with good ovarian reserve, patent Fallopian tubes, and normal serum hormonal levels (Polanski et al., 2014).

In addition to possible aforementioned embryonic defects, like chromosomal aberrations, various uterine pathologies can also cause RIF, such as thin endometrium, altered steroid hormone balance, changed expression of adhesive molecules and immunological factors in endometrium (Margalioth et al., 2006). Compelling evidence confirms that the endometrial receptivity is altered among the patients with RIF (Aghajanova et al., 2008; Ledee et al., 2011).

Several methods have been proposed to overcome RIF (Margalioth et al., 2006). For instance, treatment of intrauterine pathologies found in hysteroscopic evaluation, which potentially may lead to improved pregnancy outcome (Demirol and Gurgan, 2004; Donnez and Jadoul, 2002). Medications, like low-dose aspirin and vaginal sildenafil, seem to improve the uterine blood

flow, which may boost the endometrial development at least in some cases (Sher and Fisch, 2002; Weckstein et al., 1997). Furthermore, vaginal administration of micronized E2, or antifibrotic treatment with pentoxifylline and high-dose vitamin E, have shown positive effect in certain cases (Ledee-Bataille et al., 2002; Tourgeman et al., 2001). Interestingly, it has been speculated that endometrial scratching may cause a pseudo-decidual reaction that enhances implantation rate (Barash et al., 2003; Friedler et al., 1993), which still is a debated subject. Other studies have shown that there is no difference in outcome after induced endometrial injury, and suggest to avoid this procedure because of the other risks associated (Dain et al., 2014; Yeung et al., 2014). Because of the obvious involvement of the immunological factors in implantation, immunotherapy with intravenous immunoglobulins (IVIG) has also been applied in cases of repeated IVF failure (Coulam et al., 2006; Elram et al., 2005). Still, the usage of these proposed methods are controversial and the exact effectiveness lacks proper evidence. Therefore, the understanding the steroid-hormone responsive gene expression in endometrium would help to devise the novel treatments to overcome the implantation failure in ART.

4.2 Endometrial development in IVF

In contemporary IVF treatment, hormone-based drugs are used to stimulate ovaries during the follicular phase, including clomiphene citrate, urinary and recombinant gonadotrophins (LH and FSH), and GnRH agonists and antagonists (Edwards, 2005b). The purpose of stimulation is to produce multiple follicles to grow simultaneously, which means that many more oocytes can be retrieved, leading to increased number of embryos available for transfer. Protocols using GnRH agonists temporarily down-regulate the patient's own pituitary activity by supressing the GnRH receptors, while exogenously administered FSH is used to induce the folliculogenesis and the production of ovarian steroid hormones. Alternatively, the ovarian stimulation with FSH might be accompanied with the use of GnRH antagonists, which compete with the endogenous GnRHs at their pituitary binding sites and prevent the natural LH surge. The ovulation is triggered by administration of chorionic gonadotropins (hCG) in both GnRH agonists and antagonists schemes. Although there is evidence that GnRH antagonists decrease the FSH dose required for ovarian stimulation, they may also slightly lower the number of eggs retrieved and embryos obtained in one IVF cycle (Edwards, 2005b).

The general aim to increase the number of oocytes and embryos in IVF has neglected the detrimental influence of supraphysiological levels of ovarian steroid hormones on the endometrium (Simon, 2008). There is growing body of evidence that endometrial function in stimulated IVF cycles is adversely affected by high levels of E2, and premature secretion of P4 results in dysregulation of the endometrial receptivity (Devroey et al., 2004; Papanikolaou et al., 2005). The application of ovarian stimulating drugs often results in shorter secretory

phase of the endometrium, which is therefore no longer synchronized with embryo development for successful implantation (Lass et al., 1998). Ovarian stimulation may alter the intrauterine milieu of cytokines, chemokines, and growth factors as detected from endometrial secretions aspirated before embryo transfer (Boomsma et al., 2010). The formation of pinopodes has also been shifted to day 17 or 18, when compared to day 20 in a natural cycle (Stavreus-Evers et al., 2001). Elevated concentrations of E2 and subtle increase in P4 in the late follicular/proliferative phase during IVF may also lead to modulated steroid hormone receptor profiles in endometrium (Papanikolaou et al., 2005). While histological study has proven down-regulation of ERs, PRs and pinopodes in stimulated cycles compared to natural cycles (Develioglu et al., 1999). Indeed, reduced production of serum E2 is related to better pregnancy rates, which explains the fact that there are better IVF results recorded for donor oocyte recipients who have only received P4 support prior to embryo transfer (Check et al., 1995). There are several approaches developed to restore the length of luteal phase by stimulating corpus luteum with hCG, or by supplementing the luteal phase with steroids, such as E2 and P4 (Smitz et al., 1992). Milder stimulation protocols with lowed drug doses have also been developed for IVF treatments, which are believed to improve the pregnancy results (Nargund and Frydman, 2007; Pennings and Ombelet, 2007; Ubaldi et al., 2007).

4.2.1 Endometrial preparation for frozen embryo transfer

IVF procedure often results excess number of good quality embryos, which can be used in following months/years for frozen embryo transfers (FETs). Hence cryopreservation is extensively practiced as a safer and cost-effective method to store the embryos for future use and to increase the cumulative pregnancy rate per single IVF cycle. Freezing option also gives more flexibility in oocyte/embryo donation programs (Glujovsky et al., 2010). Furthermore, due to advances in embryo freezing and thawing methods, many IVF clinics are working towards single embryo transfer to eliminate the risk of multiple pregnancies. Clinical pregnancy rates after FET have become comparable to fresh embryo(s) transfers and are in some countries over 30% (Ferraretti et al., 2012; Ferraretti et al., 2013). Another major beneficial aspect of FETs is that endometrium is no longer affected by the high doses of drugs used for oocyte stimulation, as compared to the conditions in fresh embryo transfer (Glujovsky et al., 2010).

In general, FET protocols are simpler compared to whole IVF treatment and are needed only to prepare the endometrium to receive the thawed embryo(s). The crucial aspect in FET is the timing because the development of an embryo and endometrium needs to be synchronized. Protocols used in FET include natural cycle (NC), artificial cycle (AC), and exogenously regulated cycle with GnRH agonists (Hill et al., 2010). In NC-FET the preparation of the endometrium is achieved by endogenous sex steroids produced by a developing

follicle and the timing of embryo transfer is determined by detecting the spontaneous LH surge. Therefore, in NC-FET, LH levels need to be regularly monitored in either blood or urine. To overcome the daily LH monitoring, hCG is often used to trigger the ovulation. In this case the term 'modified NC-FET' is used. In both natural and modified NC cycles, thawing and transferring of the embryo(s) should be performed 3-5 days after ovulation depending on the stage of the embryo at freezing (Nawroth and Ludwig, 2005; Paulson, 2011).

In AC-FET cycle, E2 and P4 based drugs are administered to mimic the endocrine exposure of the endometrium in the normal menstrual cycle. Initially, E2 is given in order to cause proliferation of the endometrial cells, while suppressing the development of the dominant follicle. This is continued until the endometrium is attained the 7-9 mm thickness, when P4 administration is started to initiate the secretory changes in the endometrium (El-Toukhy et al., 2008). The timing of embryo thawing and transfer is planned according to the start of the progesterone supplementation. Sometimes the administration of E2 and P4 does not guarantee the complete pituitary suppression, and a dominant follicle may still be selected. If the follicle undergoes spontaneous luteinization then the endometrium may be exposed to premature P4, which may result in incorrect timing of thawing and transferring of the embryo. For that reason co-treatment with GnRH agonists may be used to down-regulate the pituitary gland and to prevent the follicular growth, referred as AC-FET with GnRH-FET. Both of these AC-FET approaches require medications, and are therefore less 'physiological'. To the contrary these cycles are easier to plan, when compared to NCs, which makes them a popular choice among many clinicians. However, how the genes are regulated in endometrial tissue in NC- and AC-FET cycles has remained largely unknown.

AIMS OF THE STUDY

The general aim of this thesis was to evaluate how ovarian steroid hormones – E2 and P4 regulate gene expression in human endometrium and endometrial cell lines. More precise purpose of this study was to identify the hormone responsive genes and their hormone response elements (HREs), which play crucial role in receptive endometrium in embryo implantation, and whose dysregulation could be the reason for implantation failure. In addition, we focussed on identifying E2 and P4 responsive genes, which are essential in preparing the endometrium for frozen embryo transfers, as potential diagnostic biomarkers.

The specific aims of the current thesis were:

1. To investigate whether the pre-selected 'endometrial receptivity' genes from the public databases are directly regulated by E2 and P4 via respective receptors in human endometrial epithelial cell lines HEC1A and RL95-2.

2. To reveal the E2 and P4 dependent transcriptome in Ishikawa endometrial epithelial cell line.

- To compare E2- and P4-dependent genes in Ishikawa cell line with those in endometrial biopsies collected during the receptive mid-secretory phase from women with recurrent implantation failure.

- To evaluate the agonistic and antagonistic activities of steroid receptor modulators, TAM and RU486, on the whole transcriptome of Ishikawa cells and the involvement of target-genes in reproductive diseases.

3. To compare the endometrial gene expression profile in women with recurrent implantation failure with that from fertile women in natural cycle and in artificial cycle with E2 and P4 supplementation, commonly used to prepare the endometrium for frozen embryo transfers.

- To perform functional analysis of E2 and P4 responsive genes and their HREs involved in natural menstrual cycle and in artificially prepared endometrium for frozen embryo transfers

- To compare the *in vitro* data obtained from endometrial cell lines with results retrieved from endometrial tissue samples.

MATERIALS AND METHODS

Following methods were used during the study:

- *In vitro* cell cultures of human endometrial cell lines: HEC1A, RL95-2, and Ishikawa cell lines (publications I and II);
- Handling of human endometrial biopsy samples (publications II and III);
- Steroid hormone and their analogue treatments of cell cultures (publications I and II);
- Western blot for ERs and PRs in HEC1A and RL95-2 (unpublished data);
- Chromatin isolation and immunoprecipitation with antibodies specific to ERs and PRs (ChIP) (publication I);
- ChIP followed by RT-qPCR (publication I);
- RNA isolation, cDNA synthesis and RT-PCR (publications I and II);
- Quantitative real-time (qRT)-PCR (publication I);
- Sample and library preparation for multiplex RNA-sequencing (publication II);
- High-throughput sequencing data analysis using Bowtie, TopHat, Cufflinks, and MACS (publication II);
- Total RNA isolation and microarray hybridisation (publication III);
- Array data analyses (publication III);
- *In silico* analysis to predict EREs and PREs in promoter regions of hormone responsive genes, using eukaryotic transcription factor binding site database - TRANFAC (publication III);
- Ingenuity Pathway Analysis (IPA) and other functional analysis programs to evaluate the functionality of the identified hormone responsive genes (publications II and III).

RESULTS AND DISCUSSION

5. E2 and P4 dependent nuclear receptor binding on target genes in HEC1A and RL95-2 cells (publication I)

In order to achieve consistent and reproducible experimental data related to the action of E2 and P4 on genome activity in embryo implantation, the human endometrial cell lines were used in initial experiments. The use of cell lines helped us to avoid the use of *in vivo* endometrial biopsy samples, which might show substantial inter-individual variations. The inconsistency of results can be due to differences in biopsy collection day, age, general health, the genetic variations and other possible diseases of the patient or medications used.

HEC1A and RL95-2 cell lines are both known as endometrial epithelial cell lines. RL95-2 is derived from a moderately differentiated endometrial adenosquamous carcinoma tissue (John et al., 1993; Kuramoto et al., 1972; Way et al., 1983), and HEC1A is obtained from human endometrial carcinoma (Harduf et al., 2007; Thie et al., 1995). In addition, HEC1A and RL95-2 cell lines have been described as models of the non-receptive and receptive endometrium, respectively, according to their adhesiveness to JAR trophoblast spheroids. Thus, they have been suggested to be used experimentally as *in vitro* implantation models (Rohde and Carson, 1993).

Our interest was to investigate whether RL95-2 and HEC1A cells act as models of receptive and non-receptive endometrium by analysing their responsiveness to E2 and P4 treatments. The purpose of the study was to test whether the pre-selected 'endometrial receptivity genes' (n=382) (Carson et al., 2002; Kao et al., 2002; Krikun et al., 2005; Mirkin et al., 2005; Punyadeera et al., 2005) were directly regulated by E2 and/or P4 by testing the binding ability of ERs and PRs to the promoter regions of selected 'endometrial receptivity' genes. After short steroid treatment with E2 and P4 (45 min), chromatin of the cells was fixed and precipitated with antibodies specific for ERs and PRs. Promoter regions of 'endometrial receptivity genes' were analysed with RT-qPCR using specific primers designed for genomic regions in front of the pre-selected genes.

As a main outcome of the first study, we demonstrated that E2 and P4 regulate a distinct group of genes engaged in endometrial receptivity in HEC1A and RL95-2 cells. HEC1A cell line was more responsive to E2 treatment as promoters of 137 genes (35.9 % of studied genes) were bound to ERs compared to 35 ER targets (9.2 %) identified in RL95-2. The prevalence of ER targets was significantly higher in HEC1A cells compared to RL95-2 cells (p-value less than 10^{-16}). In contrast, P4 treatment resulted in significantly higher number of 83 PR targets (21.7 %) in RL95-2 cells and only 7 target genes (1.8 %) in HEC1A cells (p-value less than 10^{-16}) (Figure 5).

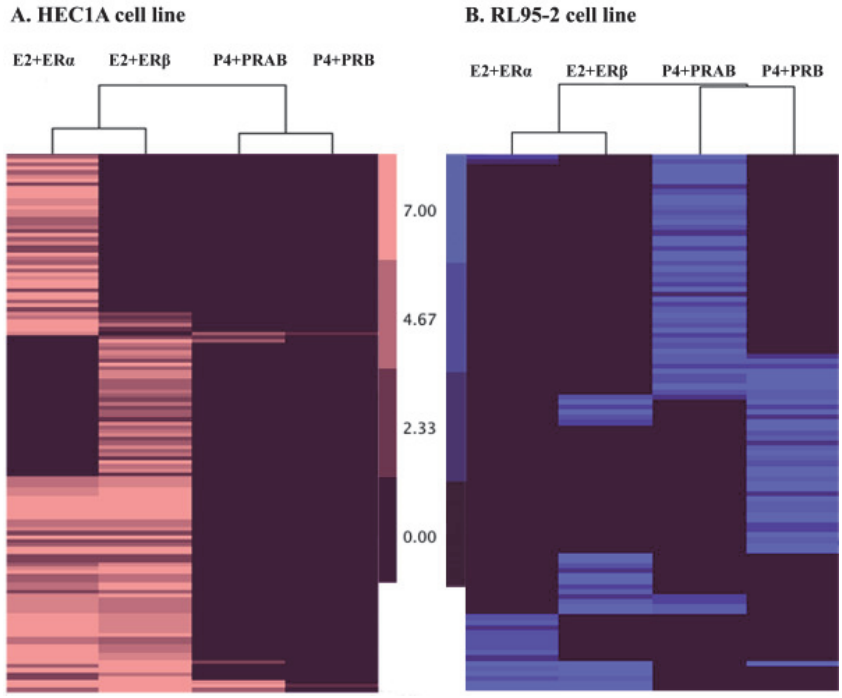

Figure 5. The distinct target genes for ERs and PRs in HEC1A and RL95-2 cells. Target genes revealed from ChIP-qPCR experiments were clustered with Cluster 3.0 program and visualized using Java TreeView software. **A.** HEC1A cell line. E2+ERα: 101 ERα target genes after E2 treatment; E2+ERβ: 96 ERβ target genes after E2 treatment; P4+PRAB: 7 PRAB target genes after P4 treatment; and P4+PRB: 2 PRB target genes after P4 treatment. **B.** RL95-2 cell line. E2+ERα: 17 ERα target genes after E2 treatment; E2+ERβ: 24 ERβ target genes after E2 treatment; P4+PRAB: 52 PRAB target genes after P4 treatment; and P4+PRB: 40 PRB target genes after P4 treatment (Figure from publication I).

These results corroborated the view about the cell line specific genomic actions of steroid hormones in human endometrium. In addition, mRNA expression of selected 20 genes showed that steroid hormones have different and often opposite effect on gene expression level in studies cell lines (Tamm et al., 2009). In summary, the first publication supported the opinion of HEC1A as a better model of the non-receptive endometrium where E2 exerts its primary role in cellular and tissue proliferation. On the contrary, RL95-2 cell line acts as a model of receptive endometrium where P4 dominantly controls the expression of target genes in secretory phase of the endometrium.

6. Global changes of transcriptome of the Ishikawa cells in response to steroid hormones and their receptor modulators (publication II)

The aim of the second publication was to identify the E2 and P4 dependent transcriptome in endometrial epithelial Ishikawa cell line. Ishikawa cells are derived from a well-differentiated adenocarcinoma of the human endometrial epithelium that expresses functional steroid hormone receptors for E2 and P4, and is probably the best available model for studying the response of the endometrial epithelium to E2 and P4 (Croxtall et al., 1990; Lessey et al., 1996a; Nishida et al., 1985).

In order to study E2 and P4 action in endometrium, high-throughput RNA-sequencing (RNA-Seq) was applied to reveal hormone responsive mRNA changes of the entire transcriptome. In addition, genome-wide effects of selective steroid hormone receptor modulators, TAM and RU486, were studied in order to evaluate their agonistic or antagonistic activities in an endometrial context. RNA-Seq allows analysing complete set of transcripts in an untreated sample and in specific conditions, such as, following the response to hormonal stimuli.

In sequencing, the resulting reads (FPKMs - Fragments Per Kilobase of transcript per Million mapped reads) aligned to a reference human genome show us a snapshot of molecular responses following E2 and P4 treatments in Ishikawa cells. Altogether, more than 16,000 known genes were expressed in response to female steroid hormones and respective antagonists – tamoxifen as ER modulator and RU486 as PR modulator. Over 2,500 genes showed significant changes in their expression levels in response to steroid hormones or after the treatment with respective nuclear receptor modulators in Ishikawa cells.

The transcriptome data of Ishikawa cells revealed 59 genes with remarkably high expression (FPKM values of >1,000 after hormone/modulator treatments), including known housekeeping genes, but also genes, which have not been mentioned in endometrial context before, including:

- ✓ E2 and P4 responsive genes: *PSAP*, *ATP5G2*, *ATP5H*, *GNB2L1* and *FTL*;
- ✓ TAM responsive genes: *S100A2*, *S100A6*, *HSP90AA1, HSPA8*, *PKM2* and *FTL*.

Glycoprotein prosaposin coding gene *PSAP* is known to be up-regulated in the endometrium during GnRH antagonist-treated cycles and also contains predicted ERE site in its promoter region (Mirkin et al., 2004; Zhang et al., 2010). The presence of PSAP in the endometrial epithelial cells can be involved in glycosphingolipid metabolism or transport in the uterine environment, which appears to be steroid hormone dependent (Spencer et al., 1995). Other genes with elevated mRNA expression in response to E2 and P4 included ATP synthase subunits *ATP5G2*, *ATP5H*, and cell proliferation-inducing gene,

GNB2L1. The expression of aforementioned genes has not described in endometrium before.

Moreover, numerous genes exhibited high expression level in response to TAM treatment. For example, genes encoding the S100 calcium binding proteins A2 (*S100A2*) and A6 (*S100A6*), heat shock protein 90 kDa alpha (*HSP90AA1*), and heat shock protein family A member 8 *HSPA8*, as well as pyruvate kinase in muscle (*PKM2*). Among aforementioned TAM-responsive genes only *S100A2* has been previously related to TAM treatment, albeit in breast cancer tissue (Golouh et al., 2008).

6.1. Significant gene expression changes in the Ishikawa cell line after E2 and P4 treatments (publication II)

Genome-wide analysis of Ishikawa transcriptome revealed significant change in the expression of 1,691 and 1,692 genes following E2 and P4 treatments, respectively. These included 1,061 genes common to both groups, likely regulated by both hormones. According to IPA classification 82 biomarkers for E2 and 93 biomarkers for P4 were emphasized among hormone responsive genes previously mentioned in the context of female reproductive endocrinology. For instance, potential biomarkers among E2 and P4 responsive genes included:

- ✓ E2 responsive genes related to the endometrium and embryo implantation: *MUC1, HMGCR, MDK, PRDM2, PXN* and *SLIT2;*
- ✓ E2 and P4 regulated genes linked to endometrial receptivity or endometriosis: *ARG2, ANXA1, AR, BMPR2, CDKN1C, CXCL16, EGFR, FGFR1, HMGA1, IGFR2, IL1R1, JAG1, MCAM, NCOA3, NOTCH1, PDCD4, PGR, RACGAP1, TMSB10* and *TNC;*
- ✓ P4 responsive genes common to endometrium or early pregnancy: *CTNNA1, ERBB3, FGFR2, IGFBP5, IKBKB, IL6ST, KCNMA1, NOTCH3, S100A4, STAT3, TCF7L2, TGFB1* and *TGFBR3.*

The first physical interaction between developing blastocyst and maternal endometrium depends on the expression of specific cell adhesion receptors and ECM-ligands from both endometrial and embryonal origin (Aplin, 2006). Epithelial cells of the endometrium are the first to form contact with embryo, and they initiate molecular communication between endometrium and implanting embryo. Among E2 and P4 responsive biomarkers, various ligands and components of ECM were present, including *MUC1*, *JAG1, IGFBP5,* and *MCAM.*

Glycoproteins mucins have been broadly studied in context of human endometrium (Aplin et al., 1994; DeLoia et al., 1998; Lurie et al., 1988). However, data regarding mucin 1 (MUC1) and its role in human endometrium is inconsistent. Some studies report an increase in its expression during the

receptive phase (Acosta et al., 2000; Aplin et al., 1998), while others have shown its decrease from pinopodes at the time of embryo implantation (Horne et al., 2005; Horne et al., 2002). Controversial expression of MUC1 during the luteal phase can be explained by its disappearance only at the site of embryo implantation. More recent study has found that there is no significant change in *MUC1* and other family member *MUC16* mRNA expression during different stages of cycling endometrium in relation to the epithelial marker, cytokeratin-18 (*KRT18*) (Dharmaraj et al., 2014). Thus, the apparent rise in MUC1 and MUC16 reported previously probably coincides with increased epithelial content of the endometrium during the secretory phase (Dharmaraj et al., 2014). In our study with Ishikawa cells, mRNA level of *MUC1* increased significantly after E2 treatment and epithelial marker *KRT18* was also induced by E2, albeit not significantly. MUC1 expression has previously shown to be up-regulated by P4 treatment in HEC1A cells in result for longer hormonal treatment (48h) (Horne et al 2006). The different effects of the steroid hormones of the *MUC1* expression can be explained with shorter treatment time and applying RNA-Seq in publication II.

The mRNA expression of cell surface protein jagged 1 (JAG1) and its known receptor notch 1 were both affected by steroid hormones in Ishikawa cells. E2 significantly supressed the expression of both *JAG1* and *NOTCH1*, while P4 significantly down-regulated only *NOTCH1*. Notch signalling pathway is known to be activated in many developmental processes including cell-to-cell communication and tissue differentiation. The expression of *NOTCH1* and *4* and ligands *JAG1* and Delta-like ligand 4 (*DLL4*) has previously been detected in human endometrium epithelia and stroma (Mikhailik et al., 2009). Moreover, in epithelial cells show significantly higher expression of *JAG1* compared to stromal cells (Mikhailik et al., 2009). In the current study we demonstrated that both the *NOTCH1* and *JAG1* genes are down-regulated by steroid hormones in Ishikawa cells.

Insulin-like growth factor binding protein-5 (*IGFBP5*) was down-regulated by P4 in Ishikawa cells. This was in concordance with earlier reports showing that *IGFBP5* gene expression is supressed by P4 during natural menstrual cycle (Giudice et al., 1991; Zhou et al., 1994) and inhibited in endometrial stromal cells when decidualization process was induced by cAMP (Tierney et al., 2003). IGFBPs are involved in various cellular processes, like cellular growth regulation and induction of apoptosis in endometrial cells where they participate in interactions between stromal and glandular compartments and in communication between embryo and receptive endometrium (Mohan and Baylink, 2002; White et al., 2005). Significant decrease in *IGFBP-5* mRNA expression level has also been previously noted in Ishikawa cells after E2 and P4 treatment followed by GnRH analogue leuprolide acetate (Zhang et al., 2010). Opposite effect on *IGFBP-5* expression has been seen when using GnRH antagonists during controlled ovarian stimulation where it was up-regulated in human endometrium (Macklon et al., 2008; Mirkin et al., 2004). This means that

controversial expression of *IGFBP-5* following GnRH agonist and antagonist treatments deserves further attention.

The spatiotemporal expression of growth factors and their respective receptors has also gained broad attention in implantation studies (Guzeloglu-Kayisli et al., 2009; Whitman and Melton, 1989). In second sub-study numerous growth factor receptors were present among steroid responsive genes identified in Ishikawa cells, like *EGFR, ERBB3, FGFR1, FGFR2, IL1R1, TGFBR* and *BMPR2.*

Epidermal growth factor (EGF) family members have been the focus of reproductive biology studies for a long time (Tamada et al., 1991). Three members of the ERBB family receptor tyrosine kinases, *EGFR*, *ERBB3,* and *ERBB4,* had E2 and P4 responsive expression patterns in Ishikawa cells. In this context, *EGFR* was significantly supressed by both hormones, while *ERBB3* was down-regulated only by P4. To the contrary, *ERBB4* was up-regulated by both E2 and P4 in Ishikawa cells. The role of EGF family members has been demonstrated in mice where ablation of *Erbb3* gene leads to the reduction in litter size. *Egfr* seems to be more critical regulator of endometrial decidualization while its deletion leads to subfertility in mice and its suppression in human endometrial stromal cells causes reduction of decidual markers, *IGFBP1* and *PRL* (Large et al., 2014).

Transforming growth factor beta (TGFβ) family of cytokines participate in cell proliferation, differentiation, and ECM turnover. They are considered to be major regulators of endometrial differentiation in preparation for implantation and pregnancy (Jones et al., 2006). From TGFβ family receptors, *TGFBR3* was up-regulated by both hormones but significantly only by P4 in Ishikawa cells. The encoded receptor is a membrane proteoglycan that often functions as a co-receptor with other TGF-β receptor superfamily members. TGFβ type I and II receptors are expressed by endometrial cells, and thus TGFBR3 may also be important for enhancing TGFβ signalling and action in the endometrium (Jones et al., 2002; Schilling and Yeh, 2000). Similarly, the expression of *TGFβ1* was induced by both E2 and P4 treatments, but only P4 up-regulated it significantly in Ishikawa cells. TGFβ has previously been localized both in stromal and epithelial endometrial cells (Godkin and Dore, 1998; Gold et al., 1994).

Three genes coding for members of fibroblast growth factor receptor (FGFR) family, *FGFR1-3*, showed significant hormone responsive action in Ishikawa cells, where *FGFR1* was induced by both E2 and P4, but *FGFR2* was down-regulated by P4 and *FGFR3* induced by E2. Based on published data, *FGFR1* is down-regulated when proliferative versus mid-secretory phase endometrium is compared (Borthwick et al., 2003; Carson et al., 2002; Kao et al., 2002; Talbi et al., 2006). Different *FGFR1* expression pattern in Ishikawa cells compared to previous data is most likely related to the cancer origin of the Ishikawa cells. Furthermore, the expression of *FGFR1* was also significantly induced by TAM, representing its agonistic activity in endometrial cells. In contrast, the expression of *FGFR2* was significantly decreased after P4 treatment

of Ishikawa cells. FGFR2 seems to play a crucial role in the structure of luminal epithelium as conditional knockout of *Fgfr2* in mice leads to aberrant development of luminal endometrial epithelium (Filant et al., 2014). Therefore, our studies contributed to the understanding how FGF receptors are regulated in endometrial epithelial cells in response to steroid hormones.

One of the earliest signals from embryo during implantation, along with the secretion of human chorionic gonadotropin (hCG), is secretion of interleukin (IL) 1, which appears to induce molecular signals in endometrium needed for embryo implantation (Krussel et al., 2003). In response to embryonic IL1 the endometrial cells up-regulate the expression of its receptor *IL1R1* (Bellehumeur et al., 2009; Krussel et al., 2003). In second sub-study *IL1R1* was significantly induced by both E2 and P4 in Ishikawa cells indicating also its hormone-dependent expression pattern, which is supposed to help the embryo-induced mechanisms in elevating the IL1R1 expression.

Gene coding for bone morphogenetic protein receptor type 2 (BMPR2) showed significant down-regulation by both E2 and P4 hormones. Important role of Bmbr2 in implantation process has been shown in mice where conditional deletion of *Bmpr2* in the uterus resulted in abnormalities in decidualization that lead to abnormal vascular development, trophoblast defects, and a deficiency of uterine natural killer cells (Nagashima et al., 2013). Thus, our studies corroborate the importance of steroid hormones in regulation of the *BMPR2* gene.

6.2 Comparison of E2- and P4-dependent genes in Ishikawa cell line with those expressed in endometrial biopsies from women with recurrent implantation failure (publication II)

According to RNA-Seq data, 1,671 (98.8%) of E2-responsive genes, and 1,668 (98.6%) of P4-responsive genes, identified in the Ishikawa cell line were also detected in two human endometrial biopsy samples obtained from women with RIF during the mid-secretory phase. Even though the tissue samples in the second sub-study were only used for comparative purposes, the expression of nearly all *in vitro* identified E2- and P4-responsive genes in human endometrium give us an idea that the majority of E2- and P4-responsive genes found in Ishikawa cells are indeed also present in endometrial tissue. Moreover, the fact, that the majority of the E2- and P4-responsive genes have not been mentioned in endometrial context before, refers to the importance of steroid hormones in functioning of the endometrial tissue, but also points to the need for further extensive studies. Some of the biomarkers affected by steroid hormones both in Ishikawa cells and endometrial tissue are as follow (Figure 6):

- ✓ E2 biomarkers: *MUC1, DLGAP1, APOE, HMGCR, MDK, EZH2, SLIT2,* and *RARB;*

- ✓ P4 biomarkers: *ELN, TGFBR3, BMBR1B, IL6ST, BRCA1, DEPDC1,* and *TOP2A.*

Among these genes, for instance, mRNA of *APOE* was up-regulated by both E2 and P4, albeit significantly only by E2 in Ishikawa cells. The expression of *APOE* has been detected before in epithelial and stromal compartments of secretory phase endometrium, where its expression was not altered by P4 (Germeyer et al., 2013). Further studies are needed to clarify the exact role of APOE and the effects of steroid hormones on its gene expression in endometrial tissue.

IL6 signal transducer (IL6ST also known as GP130) is known as a common receptor for all IL-6 family members and acts as a co-receptor for LIF (de Ruijter-Villani et al., 2015). In second sub-study the expression of *IL6ST* was significantly induced by P4 in Ishikawa cells indicating its possible role in human endometrium during secretory phase, and supporting the strong existing evidence about the importance of LIF-pathway in endometrial receptivity.

The expression of retinoic acid receptor (RAR) beta acts as tumour suppressor by inhibiting cell proliferation and angiogenesis, and inducing cell differentiation and apoptosis. This is why *RARB* is often described to be silenced in epithelial carcinogenesis (Morriss-Kay, 1992). In our second sub-study the expression of *RARB* was significantly up-regulated by short E2 treatment in Ishikawa cells, which is in accordance with its possible tumour suppressor activity. It is very likely that *RARB* expression in endometrium is required for transition of the tissue from the proliferative to the secretory phase, which is accompanied by inhibition of the cellular divisions and obtaining the secretory cellular phenotype.

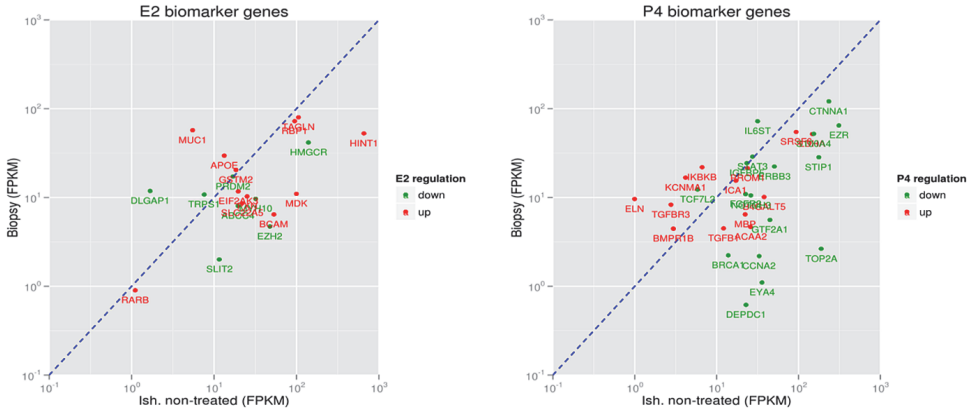

Figure 6. Selection of endometrial specific biomarkers found in Ishikawa cells after 12 h E2 (left) and P4 (right) treatment and their relative abundance in two human endometrial biopsy samples (average of two samples) at the time of embryo implantation. Red genes are up-regulated in E2 and P4 treated Ishikawa cells compared to non-treated cells; green

genes down-regulated. Genes situated on the left side of the diagonal line show higher relative abundance (FPKM) in human endometrial biopsy samples compared to non-treated Ishikawa cells. Genes situated on the right side of the diagonal line show lower relative abundance (FPKM) in human endometrial biopsy sample compared to non-treated Ishikawa cells (Figure from publication II).

6.3 TAM and RU486 regulated genes related to reproductive system diseases in Ishikawa cells (publication II)

An additional objective of the second sub-study was to examine the molecular effects of TAM and RU486 on endometrial Ishikawa cells. TAM and RU486 are classical NR modulators, which bind to ERs and PRs, thereby blocking the action of native E2 and P4, respectively (Berrodin et al., 2009; Buzdar and Hortobagyi, 1998). As such, TAM and RU486 can be considered as E2 and P4 antagonists. However, TAM and RU486 can also affect the gene expression in the same direction similar to E2 and P4, advocating their classification as steroid hormone agonists. As TAM and RU486 are both in clinical use, the knowledge on tissue-specific agonistic and antagonistic effects on endometrial cells is critical to ensure effective and safe use of steroid hormone receptor modulators in patients' care. Therefore, the goal of this sub-study was to profile the antagonistic and agonistic effects of TAM and RU486 on endometrial transcriptome, using the Ishikawa cells.

Following to TAM treatment 1,013 genes were found to be significantly changed compared to non-treated Ishikawa cells. Interestingly only 22.1% (n=224) of genes were significantly regulated by both E2 and TAM and among them the majority of the genes (n=162, 72.3%) were expressed in same direction being either up- (n=61) or down-regulated (n=101), and exhibiting agonistic activity. Significant antagonistic activity of TAM was seen only for 6.1% (n=62) of E2 and TAM regulated genes. Moreover, most of the TAM regulated genes (n=789, 77.9%) were not significantly regulated by E2 at all, suggesting that TAM regulates gene expression in Ishikawa cells also independently from E2 and ERs.

Due to its known E2-antagonistic activity, TAM is often used in breast cancer therapy. However, one of the most troublesome side effects of breast cancer treatment with TAM appears to be its proliferative effect on the endometrium (Bergman et al., 2000; Buzdar and Hortobagyi, 1998). Because of that, TAM has been associated with endometrial pathologies, like hyperplasia, polyps, carcinomas, and sarcomas (Cohen, 2004; Tamm-Rosenstein et al., 2013).

In our study, among the differentially expressed TAM-specific genes 168 were associated with various diseases of the reproductive system, including uterine, ovarian, and cervical cancers, as well as genital tumours, amenorrhea, metrorrhagia and polycystic ovary syndrome according to IPA classification. For instance, genes related to uterine, ovarian, and cervical cancers, like *FGFR1, KIAA0664, MALAT1, OLFM1, TMSB4X, TPM2, JAG2, PAX8* and *SRSF5* were

both up-regulated by TAM and E2 (Figure 7) (Guo et al., 2010; Wong et al., 2007).

In addition, cyclin D1 (*CCND1*) was significantly up-regulated after TAM treatment in Ishikawa cells (Figure 7). CCND1 was also the central molecule of the network associated with regulation of DNA replication, recombination and repair, as well as cell cycle progression, according to IPA. Aberrant expression of *CCND1* has been associated with the development of several human cancers, including parathyroid adenoma, breast cancer, colon cancer, lymphoma, melanoma, and prostate cancer but also in endometrial carcinomas (Cao et al., 2002; Fu et al., 2004; Machin et al., 2002). Thus, our results clearly emphasise the TAM-induced expression of *CCND1* as likely culprit of TAM-activated endometrial cancer.

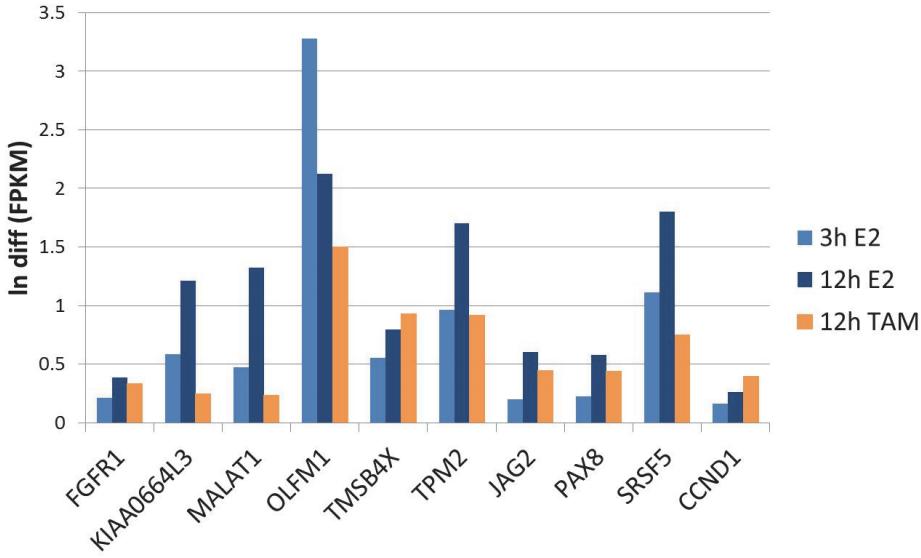

Figure 7. Agonistic activity of TAM on selected E2-responsive genes in Ishikawa cells. E2 and TAM agonistic activities were revealed on genes including *FGFR1, KIAA0664, MALAT1, OLFM1, TMSB4X, TPM2, JAG2, PAX8, SRSF5* and *CCND1*. Light blue shows 3 h treatment of E2, dark blue 12 h treatment of E2, and orange denotes 12 h TAM treatment of Ishikawa cells compared to non-treated Ishikawa cells. FPKM values are compared to non-treated Ishikawa cells and showed in lndiff scale.

Similarly to TAM, RU486 exhibited both agonistic and antagonistic activities for P4 in Ishikawa cells. RU486, which is generally known for its anti-progesterone activity, affected significantly the expression of 546 genes in Ishikawa cells. According to our results, the majority of the genes (n=377, 69.0%) were regulated independently from P4, 29% (n=156) of the genes exhibited agonistic activity and only 2.0% (n=13) of the genes had an opposite

direction after RU486 and P4 treatments. Eighty-six genes were found to be related to diseases of the reproductive system. For example, genes related to adenomyosis, gonadal tumours, ovarian cancer and uterine leiomyoma were identified, including *MALAT1, SYNC, ERBB3, FGFR2, KRT23, MCAM* and *MYC*.

Based on the list of RU486-responsive genes, a gene network covering cell-to-cell interactions and tissue remodelling was identified. The central genes/proteins in this network include cadherin 2 (*CDH2*, down-regulated after RU486 treatment) and a complex between androgen receptor (*AR*, up-regulated after RU486 treatment) and *NFκB*, which mediate the direct and indirect interactions with several RU486-responsive genes. In this network, the genes associated with cell-to-cell contacts and adhesion were down-regulated and included: *CTNND1, JUP, CDH2, IQGAP1* and *COL2A1*. Alternatively, *PDK1* and *ADAM15*, which have the roles in inducing tissue breakdown, were up-regulated (Figure 8). The suppression of cell-adhesion genes and activation of genes responsible for tissue breakdown possibly lead to endometrial shedding after using RU486 for emergency contraception and medical termination of pregnancy.

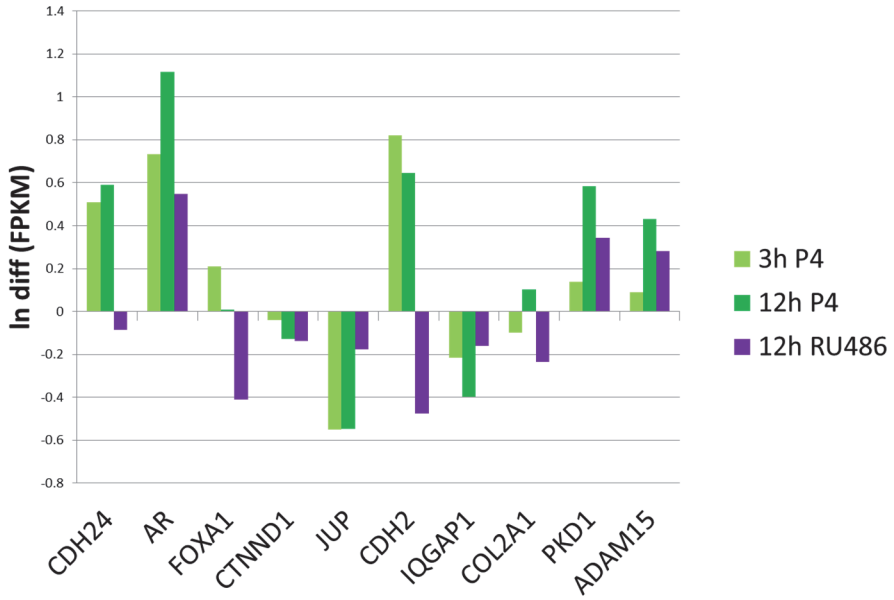

Figure 8. Agonistic and antagonistic activities of RU486 on selected P4-responsive genes in Ishikawa cells. Agonistic activity of RU486 to P4 action was detected for example on *AR, CTNND1, JUP, PKD1* and *ADAM15* expression. RU486 antagonistic activity was seen for instance on *CDH2, FOXA, CDH2, IQGAP1* and *COL2A1* expression. Light green denotes P4 treatment for 3 h; dark green P4 treatment for 12 h; and purple demonstrates 12 h RU486-treatment compared to non-treated Ishikawa cells.

7. Genes regulated by E2 and P4 in endometrium during endometrial preparation for FET in women with recurrent implantation failure (publication III)

Multiple pregnancies are probably the most deleterious outcome of infertility treatment, accompanied by high health risks for both mothers and neonates. In order to avoid multiple pregnancies, single fresh embryo transfers are becoming increasingly popular. At the same time all good-quality spare embryos obtained from the same ovarian stimulation cycle are cryopreserved. Later they are used in frozen embryo transfers (FETs), resulting in improved cumulative pregnancy rate per single IVF.

Furthermore, the previous evidence strongly supports the idea of impaired endometrial receptivity in fresh IVF embryo transfers after ovarian stimulation and supra-physiological steroid-hormone levels compared to FET cycles where the endometrium is matured in more mild conditions (Shapiro et al., 2011). Thus, all this aforementioned information supports the belief that FETs will gain more importance in IVF programs worldwide.

7.1. The endometrial transcriptome of the patients with the recurrent implantation failure (publication III)

FET cycles are actively used in infertility treatment, with almost 130,000 transfers performed in Europe in 2011 (European et al., 2016). Still, there is no clear consensus about the protocols for preparation of the endometrium to receive frozen embryos. Protocols used in FET include natural cycle (NC) and artificial cycle FET (AC-FET) with or without preceding pituitary down-regulation by using GnRH agonists (Hill et al., 2010). In natural cycle FET (NC-FET), the endometrium develops under the endogenous steroid hormonal milieu. The endometrial maturation and the development of a dominant follicle is monitored by ultrasound. FET is timed by triggering ovulation by exogenous LH/hCG or, alternatively by spontaneous LH. In AC-FET exogenous E2 and P4 are used to prepare the endometrium for implantation by starting with daily doses of E2, supplemented with P4 when the endometrium has reached sufficient thickness (Groenewoud et al., 2012).

There is single clear benefit of NC-FET over AC-FET. As in NC medications are not needed, the price for FET is cheaper, albeit, there is a need for more frequent ultrasonographic evaluation to avoid unexpected ovulation. On the other hand, AC-FET allows better timing and planning of FET, which is why it is preferred by clinicians.

We still lack evidence-based information to prefer one endometrial preparation scheme over another. The recent meta-analysis was also unable to bring any clear-cut answer, as there were no differences in the clinical pregnancy

rate, ongoing pregnancy rate, or live birth rate after using different methods for endometrial preparation prior to FET (Groenewoud et al., 2013). Furthermore, we still miss the information - how different FET preparation protocols influence the endometrial transcriptome- as this has been shown to be directly implicated in the 'endometrial receptivity' phenotype.

In order to answer these questions, we conducted the current sub-study to analyse endometrial gene expression profiles in infertile women undergoing FET with two alternative options – NC-FET vs. AC-FET. Our study group was composed of women with recurrent implantation failure – RIF; where all patients had previously had at least three embryo transfers without achieving pregnancy. The inclusion of this difficult group of RIF-patients is particularly intriguing, asthese women have been shown to display altered endometrial receptivity, which may be rescued by using exogenous steroid hormones in AC-FET (Altmae et al., 2010). In order to estimate the effect of steroid hormone supplementation on the endometrial transcriptome, the results of the infertile RIF-patients were compared to those of fertile women by analysing the endometrial biopsy samples from their natural cycle (controls or NC-FC group). The characteristics of the study groups, representing the RIF-patients with endometrial biopsies obtained from the same patients from NC- and AC-FET cycles, and those of fertile control women with natural cycle biopsies are demonstrated in Table 1.

Table 1. Characteristics of the study groups (Table from publication III)**.**

	RIF, NC-FET (*n*=5)	RIF, AC-FET (*n*=5)	NC-FC (*n*=5)
Age (years)	30.2 ± 4.3	32.4 ± 5.0	31.8 ± 3.8
BMI (kg/m^2)	20.7 ± 1.8	20.9 ± 2.0	23.5 ± 2.1
Cycle length (days)	28.2 ± 1.7	28.0 ± 1.4	28.4 ± 0.7
Menses duration (days)	4.4 ± 0.7	4.5 ± 0.7	4.0 ± 0.2
Previous implantation failures	3.4 ± 0.9	3.4 ± 0.6	0
Parity	0	0	1.5 ± 0.2
Endometrial thickness (mm)	9.3 ± 0.9	8.5 ± 1.7	n.a.
Biopsy sample taken	LH+7	Prog+6	LH+7

RIF – recurrent implantation failure; NC-FET – natural cycle-frozen embryo transfer; AC-FET – artificial cycle-frozen embryo transfer; NC-FC – natural cycle-fertile control; BMI – body mass index; LH+ – day since the luteinizing hormone (LH) surge; Prog+ – progesterone administration in days; n.a. – not assessed. Results are mean ± SD.

The two main questions in the third sub-study were formulated as follows: 1) Which is the best endometrial preparation protocol for RIF-patients planning for FET, based on the similarity of the endometrial transcriptome to

that demonstrated for fertile women at NC; and 2) Whether the steroid supplementation of exogenous E2 and P4 used in AC-FET improves the endometrial maturation in the RIF study group. Both of these questions aimed on obtaining better knowledge about the mechanisms of how the steroid hormones are involved in endometrial maturation. In long-term this novel knowledge will help devise better hormonal regimens for FET, even for the patients' group complicated with RIF.

In order to achieve these tasks, we classified the genes according to their endometrial expression in NC-FET, AC-FET, and NC-FC groups, and defined three classes of differentially expressed genes (DEGs). Firstly, we were interested in genes with abnormal expression in natural cycle, but are improved in AC. Secondly, in genes with normal expression in NC, but deteriorated gene-activity in AC due to administered steroid hormones. Thirdly, in genes, characteristic to the RIF-phenotype, with altered expression in both groups of NC- and AC-FET, when compared to the fertile women in NC.

Our comparative microarray transcriptome analysis suggested that NC endometrial gene expression pattern in RIF-patients was more similar to that observed in fertile controls, when compared to AC-prepared endometrium (Figure 9). A more negative impact of AC on the endometrial transcriptome was seen as described in third publication. For this reason, we believe that the NC-FET endometrial transcriptome in RIF is more similar to that of fertile women, while the use of E2- and P4-based drugs in FET drives the unfavourable changes in the gene expression. For instance, numerous genes known to be important in endometrial receptivity were deteriorated with AC, including *CRISP3, HPSE, HAL, C14orf161* (Altmae et al., 2012; Borthwick et al., 2003; Diaz-Gimeno et al., 2011; D. Zhang et al., 2012; Chan et al., 2013; Carson et al., 2002; and Tapia et al., 2008). In addition, genes known to be involved in embryo implantation were also negatively altered with AC, including fibroblast growth factors (*FGF17, FGF8, FGFBP2*)*; INHBC;* interleukins (*IL1F6, IL27, IL29, IL4, IL9R*)*; LEP;* matrix metallopeptidases (*MMP17, MMP27, MMP3*)*; PPARD; PTGER3* and *WNT8A* (Aghajanova et al., 2008; Altmae et al., 2012; Bogacka et al., 2013; Gonzalez et al., 2000; Kao et al., 2002; McGowen et al., 2014; Osteen et al., 1999; van Mourik et al., 2009). Interestingly, genes coding for ERβ (*ESR2*) and FSHR were also down-regulated in the endometrium with AC. These two genes code for hormone receptors that have very recently been shown to participate in endometrial receptivity (Hapangama et al., 2015; Stilley et al., 2014).

In conclusion, these results indicate that NC endometrial gene expression pattern in women with RIF is more comparable to that observed in endometrium of fertile controls, and AC will lead to the substantial deviation of the endometrial transcriptome from the natural state. This means that E2 and P4 supplementation during AC should not be preferred, because AC has a negative impact on endometrial gene expression at least in women with RIF, and thus NC scheme should be preferred for FET.

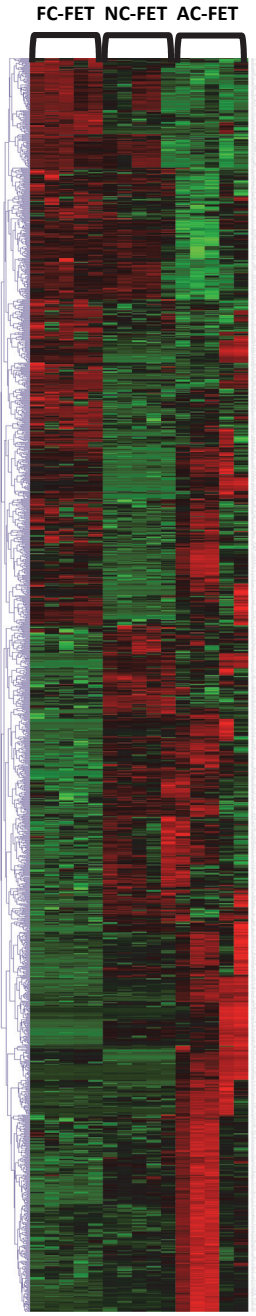

Figure 9. Cluster analysis of gene expression values in the endometrium at WOI in fertile controls (FC-FET), in natural cycle (NC-FET) and artificially prepared cycle (AC-FET) in RIF-patients. Red represents genes with a positive Z-score and green denotes genes with negative Z-score (Figure from publication III).

7.2 E2 and P4 responsive genes present in Endometrial Receptivity Array (ERA) (publication III)

ERA test – Endometrial Receptivity Array is currently the only clinically proven molecular test for evaluating endometrial receptivity. ERA test, a customized microarray, is comprised of 238 genes and it serves as a comprehensive panel of endometrial receptivity biomarkers. ERA test helps to identify patient's timing for WOI which is the basis for scheduling the next embryo transfer. Furthermore, this test has been ascertained to be more accurate and consistent when compared to histological evaluation of human endometrial tissue, It can reveal that some patients have a delayed WOI, others an advanced WOI, or unusually short window for endometrial receptivity. ERA test has also been shown to help achieving a better pregnancy outcome in different infertility patients' groups, including the women with RIF (Blesa et al., 2014; Diaz-Gimeno et al., 2011; Garrido-Gomez et al., 2013; Ruiz-Alonso et al., 2013).

In order to uncover the most critical genes controlled by steroid hormones, the AC-improved/-deteriorated DEGs from third publication were compared with the ERA list of genes. DEGs that improved with AC shared 11 common genes with ERA list: *CALB2*, *COL16A1*, *COMP, EDN3*, *IGFBP1*, *LRRC17*, *OLFM4, POSTN*, *SLC15A1*, *SORD* and *TMEM16A*. Deteriorated-DEGs shared 4 common genes with ERA test: *CRISP3*, *C14orf161*, *HAL* and *HPSE*. In addition, four genes were RIF-specific DEGs and included: *HABP2*, *HLA-DOB*, *SPDEF,* and *TRH* genes (Table 2).

IGFBP1 has been recognized as a marker of decidualization of endometrial stromal cells and its expression is induced in response to cAMP in combination with steroid hormones (Large et al., 2014). E2 and P4 drugs used in AC improved the expression of *IGFBP1* and made it more comparable to fertile controls. Cysteine-rich protein CRISP3 identified in both luminal and glandular epithelium is more abundant during proliferative phase and is secreted into the uterine cavity. Previous data suggests its participation in cell adhesion and proliferation (Evans et al., 2015). In addition, the expression of *CRISP-3* mRNA and protein levels have been described to be higher in endometrium from ectopic pregnancies when compared to intrauterine pregnancies (Horne et al., 2009). In our study, the expression of *CRISP3* deteriorated by the use of AC in preparation for FET. The down-regulation of hyaluronan-binding protein 2 coding gene *HABP2* in women with RIF compared to fertile controls has been described previously (Altmae et al., 2010) and AC supresses its expression even more.

Table 2. Comparison of array data with the Endometrial Receptivity Array (ERA) gene list. Eleven DEGs that improved with AC were also in the ERA list, as were four DEGs that deteriorated with AC and four DEGs specific to RIF (Table from publication III)

Genes whose expression improved with ACs			
Gene	***ERA**	**NC-FET vs, NC-FC**	**AC-FET *vs.* NC-FC**
CALB2	-8.04	5.15	n.d.
COL16A1	-4.89	5.89	n.d.
COMP	30.95	-3.93	n.d.
EDN3	-5.03	3.48	n.d.
IGFBP1	5.35	-4.17	n.d.
LRRC17	-4.64	4.25	n.d.
OLFM4	-9.35	15.35	n.d.
POSTN	-6.04	5.21	n.d.
SLC15A1	5.59	-4.61	n.d.
SORD	-3.21	3.7	n.d.
TMEM16A	-3.13	3.73	n.d.
Genes whose expression deteriorated after AC			
Gene	***ERA**	**NC-FET vs, NC-FC**	**AC-FET *vs.* NC-FC**
CRISP3	5.09	n.d.	-13.46
C14orf161	5.07	n.d.	-3.38
HAL	3.37	n.d.	-3.93
HPSE	5.17	n.d.	-7.48
Genes specific to RIF			
Gene	***ERA**	**NC-FET vs, NC-FC**	**AC-FET *vs.* NC-FC**
HABP2	4.09	-4.48	-5.48
HLA-DOB	-11.06	5.17	12.86
SPDEF	-3.78	9.27	12.20
TRH	-21.69	7.56	9.02

* ERA test values are fold changes obtained from comparisons of receptive *vs.* pre-receptive endometrium (Diaz-Gimeno *et al.*, 2011); RIF – recurrent implantation failure; NC-FET – natural cycle-frozen embryo transfer; AC-FET – artificial cycle-frozen embryo transfer; NC-FC – natural cycle-fertile control; n.d. – non-detectable, meaning that there was no gene expression difference between the RIF patients and fertile controls.

7.3. Analysis of hormone response elements (HREs) in endometrial DEGs (publication III)

With the high prevalence of steroid hormone receptors in different endometrial compartments, it is thought that gene expression of this tissue is greatly controlled by ERs and PRs. The steroid hormones exert their action upon binding to their respective nuclear receptors (NRs), which act thereafter as transcription factors by attaching to the hormone response elements (HREs) in target genes and augment or suppress the transcription of the target genes. HREs

are mostly located in front or inside of a gene, but many binding sites could also be found far away from the transcription start site (TSS), even up to 100 kb (Carroll et al., 2005). Currently, we focused in *in silico* HRE analysis on three promoter regions (-1,000 bp to +150 bp, -10,000 bp to +150 bp, and -50,000 bp to +150 bp from TSS) of differentially expressed genes – DEGs, which were identified in third publication by comparing the endometrial transcriptome in natural and E2-/P4-supplemented artificial cycles. In total 1,273 genes were eligible for TRANSFAC analysis and the upstream sequences of those promoters were searched for estrogen response elements (EREs) and progesterone response elements (PREs/GREs).

Important finding of our analysis was the identification of at least one HRE in the promoter regions of all DEG genes, demonstrating that these genes may be under the control of steroid hormones. If all EREs and PRE/GREs were summarized, the PRE/GRE motifs showed significantly higher prevalence ($p<2.2e-16$) in promoter regions of DEGs compared to other genes in human genome. When all the DEG subgroups were analysed separately, significantly lower frequency ($p=0.0052$) of PRE/GRE sites was determined for deteriorated DEGs compared to genes whose expression improved after AC. In contrast, AC deteriorated genes had significantly higher frequency of EREs compared to AC improved ($p=3.2e-06$) and RIF-specific genes ($p=0.0045$). Thus, in summary, the DEGs whose expression was deteriorated included significantly less PRE/GRE and more ERE sites in their promoter regions, when compared to the genes which expression improved after AC (Figure 10A).

Moreover, a significantly higher number of one particular ERE motif - V$ESR1_01 was identified among the DEGs that deteriorated after AC. For example, genes like *IL9R, MMP17, PTGER3, ESR2* and *GATA3* all included the same ERE motif in their promoter regions. On the other hand, one certain PRE site - V$PR_Q6 was overrepresented in front of the genes that improved with AC including: *ADAD1, CALML5, FAM196A, IFNA5,* and *IL21* genes. In addition, the same PRE motif was also present at a significantly higher rate in front of genes specific to RIF, like *AFM, BRINP3, CNNM1, FAM151A,* and *IL12RB2* (Figure 10B).

Our *in silico* study was the first one where up to 50 kb upstream of gene´s transcription start site was analysed to search for HREs in genes expressed in human endometrium. Our findings support the view that genes, which expression is more vulnerable to exogenously administered steroid hormones, are more responsive to E2. To the contrary, genes, which expression could be improved with steroid hormones are more likely affected by P4. Hence, in the current AC regimens used in clinics for preparing endometrium for FET, E2 could have a stronger negative impact on the endometrial transcriptome, rather than P4, which could restore the ‘natural’ gene expression.

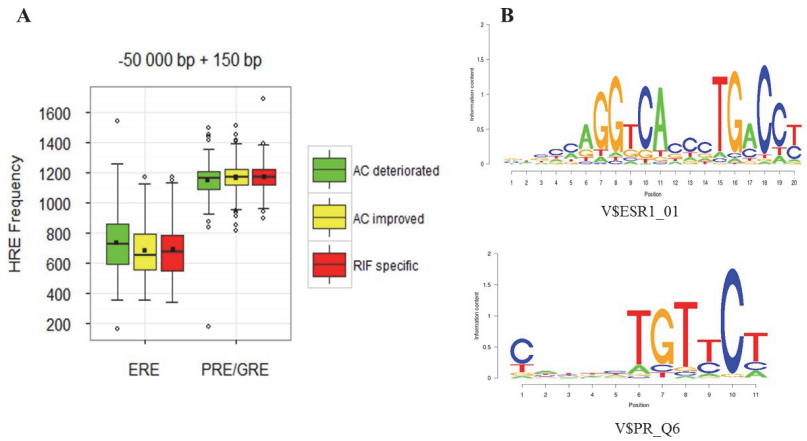

Figure 10. The presence of EREs and PRE/GREs in DEGs of human endometrium. **A.** Tukey test for pairwise comparison of ERE and PRE profiles in promoter regions, located from -50,000 bp to +150 bp from the transcription start site, of AC-deteriorated and -improved, and RIF-specific DEGs. Green denotes DEGs AC-deteriorated; yellow DEGs AC-improved and red are RIF-specific genes. **B.** Two most prevalent HREs found in promoter regions among DEGs identified in third study. Letters abbreviate the nucleotides (A, C, G, and T) and are sized according to their relative occurrence. ESR1 element (V$ESR1_01) (above) was statistically over represented in front of DEGs-deteriorated. PRE (V$PR_Q6) (below) was statistically more frequent in front of genes related to RIF and among DEGs that improved with AC (Figure from publication III).

7.4. Comparison of DEGs in human endometrium with genes that responded to E2 and P4 in studies of endometrial cell lines (publication I, II and III)

The studies of endometrial tissue are unable to reveal the direct effects of individual steroid hormones on 'endometrial receptivity' genes, because *in vivo* conditions of both hormones, E2 and P4, exert their effects simultaneously. Therefore, it is important to compare the results obtained from analysing the endometrial tissue (publication III) with the results arising from testing the endometrial cell lines treated separately either with E2 or P4 (publications I and II). The comparison of endometrial tissue gene expression in natural cycle or after artificial E2-/P4-supplementation with data obtained from endometrial cell lines helps to understand the changes in endometrial genome regulation caused by the endogenous or exogenous steroid hormones (publications I-III).

When we compared the DEGs from endometrial transcriptome obtained from AC vs. NC endometrial tissue with the significantly changed genes after E2 and P4 treatment in Ishikawa cells, 50 hormone responsive genes were found in common as depicted on Figure 11. These genes were identified regardless of the different gene-expression profiling platforms used in analysing the endometrial tissue samples (microarrays) and cell lines (RNA-sequencing).

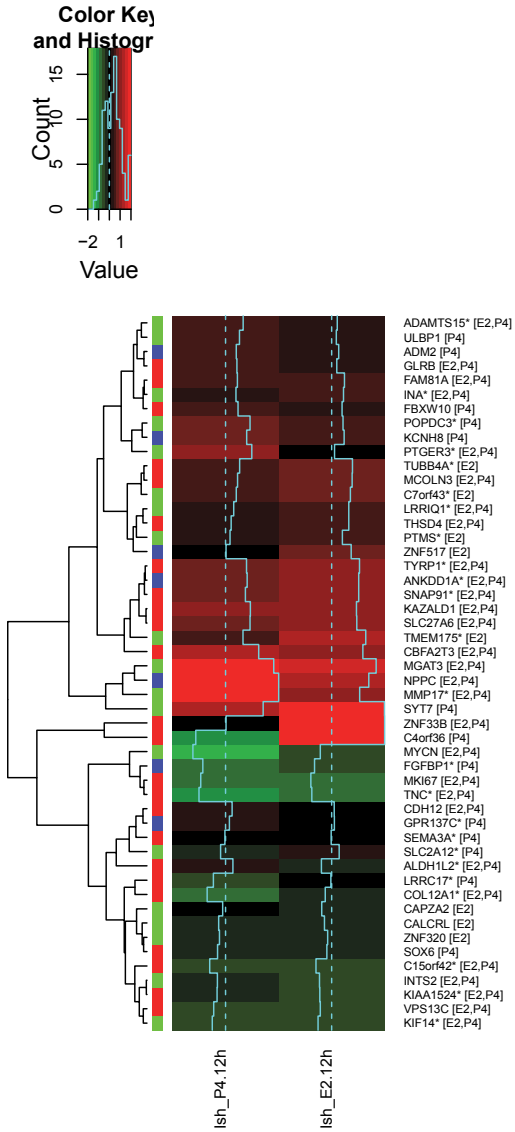

Figure 11. Genes regulated by E2 and P4 in endometrium and Ishikawa cells. Fifty DEGs from the third sub-study (publication III) were also regulated by E2 and P4 in Ishikawa cells (publication II). Red and green denote up- and down-regulation of gene expression in Ishikawa cells, respectively. The dotted line indicates zero and the variable line shows the expression change in response to hormone treatment; [E2] – gene expression regulated by estradiol, [P4] – gene expression regulated by progesterone, and [E2, P4] – gene expression regulated by both estradiol and progesterone. *Designates genes in publication III with an opposite expression direction *vs.* Ishikawa cells (publication II). Red on the y-axis indicates genes whose expression improved with AC, green indicates genes whose expression deteriorated with AC, and blue highlights the RIF-specific genes (Figure from publication III).

We identified 34 and 36 genes in Ishikawa cells with significant regulation in response to E2 and P4 (27 genes were influenced by both hormones), respectively, whose expression also changed in response to AC-prepared endometrium. Among the genes with up-regulated expression in AC, majority were co-regulated by both steroid hormones (75%) or were solely regulated by P4 (21%). To the contrary, the AC-deteriorated genes, which expression become deviated from the natural cycle following AC, were more often regulated by E2 only (32% vs. 4% for AC-improved genes) and the proportion of the E2-/P4-co-regulated genes was lower (47% vs. 75% for AC-improved genes). Our experiments suggest that AC-deteriorated DEGs are more often E2-regulated or are less regulated by both E2 and P4 in Ishikawa cells. In contrast, for AC-improved genes majority of the genes are regulated jointly by E2 and P2. Collectively, this information supports the idea that AC and the use of E2 have negative impact on endometrial gene expression in the subgroup of infertile women with RIF, while the P4 has general favourable effect on endometrial transcriptome (publication III).

Six genes were found in common in all three sub-studies, including: *ITGA10, WFDC2, IFNAR2, PTGER4, SPDEF* and *TNC*. The experimental conditions and the biological relevance of these 'endometrial receptivity' genes are provided in Table 3. Relatively low number of common genes between three publications can be explained with the pre-selection of genes in the first publication, the differences in study material, and methods used. In the first publication endometrial epithelial cell lines HEC1A and RL95-2 were used; in the second study Ishikawa cell line was studied and compared with endometrial biopsies from infertile patients with unexplained infertility; and in the third publication endometrial biopsies obtained in NC and AC from fertile women and infertile patients with RIF were studied. The different analysing platforms included ChIP-qPCR in publication I, RNA-seq in publication II, and microarrays in publication III.

Tenascin C (TNC) is a ECM glycoprotein known to participate in cell differentiation, proliferation and migration (Chiquet-Ehrismann and Chiquet, 2003). In human endometrium TNC expression was initially detected only in proliferative endometrium and its extracellular expression was specified around the stromal cells surrounding the endometrial glands (Tan et al., 2008; Yamanaka et al., 1996). Later, TNC was detected also in secretory phase of the endometrium as a narrow layer around stromal cells immediately adjacent to the glandular epithelium and around the endothelium of tortuous blood vessels (Tan et al., 2008). In addition, *TNC* expression in human endometrium has been shown to increase following luteal support (Zhao et al., 2012). In case of endometriosis abundant expression of TNC has been detected throughout the stroma in ectopic endometrial tissues where E2 has stimulatory effect on its expression (Tan et al., 2008). The proximal promoter region of *TNC* was identified as ERβ target in HEC1A in response to E2 treatment and the promotor was also targeted by PRAB antibody (PRB receptor) in RL95-2 cells following

to P4 treatment. In the second sub-study *TNC* expression was significantly down-regulated by both E2 and P4 in endometrial epithelial Ishikawa cell line. The suppression of *TNC* by steroid hormones in endometrial epithelial cells might be important for its extracellular localization around stromal cells adjacent to the glandular epithelium. In human endometrium from women with RIF the expression of *TNC* was induced in response to AC and became more similar to fertile controls (publication III).

Integrin alpha 10 (*ITGA10*), coding for integrin α chain, belongs to a transmembrane glycoprotein family participating in cell adhesion and cell-surface mediated signalling. Proximal promoter of *ITGA10* was identified as ERα target in HEC1A and PRAB (PRB) target in RL95-2 cells. Integrins are composed of heterodimeric α and β subunits that serve as receptors for the extracellular matrix and are extensively investigated in context of endometrial receptivity (Lessey et al., 1992). The expression of the integrins seems to happen in spatiotemporal manner as glandular epithelium expresses certain integrins, β1 integrin and αVβ3, only during the WOI (Lessey et al., 1994). Most intensively studied integrin in context of implantation is αVβ3 that appears in glandular epithelium at the cycle day 20 and with abundance at the site of embryo attachment (Kang et al 2014). Furthermore, reduced expression and αVβ3 integrin has been related to RIF-patients (Lessey et al., 1995; Lessey et al., 1996b) and the expression of αVβ3 correlates with the down-regulation of epithelial ERs and PRs (Lessey, 1998). In the current thesis numerous integrin coding genes showed hormone responsive expression pattern. For instance, *ITGAV* coding for αV subunit and *ITGB3* coding for β3 subunit were significantly down-regulated by E2 and P4 in Ishikawa cells (publication II). To the contrary, *ITGA7* coding fo*r* α7 and *ITGB8* coding for β8 were significantly up-regulated by both hormones in Ishikawa cells (publication II). In addition, *ITGB6* coding for β6 showed significant up-regulation after P4 treatment in Ishikawa cells in the second sub-study. Finally, *ITGB7* was identified as ER and PR target in HEC1A and RL95-2 cells, and *ITGA2* only in RL95-2 cells.

Prostaglandin E receptor 4 (PTGER4) is known as a G-protein coupled receptor for prostaglandin E2. Prostaglandins increase vascular permeability and endometrial stromal decidualization (Ahmed et al., 1998). The mRNA expression of *PTGER4* is described to be elevated in glandular epithelium and vascular cells in late proliferative phase (Milne et al., 2001). Studies have indicated its induction by E2 and down-regulation by P4; also its significant increase in ectopic endometrium in endometriosis has been found (Moggs et al., 2004; Pabona et al., 2012; Santulli et al., 2014). In our studies both ERα and ERβ showed binding ability to proximal promotor of *PTGER4* in HEC1A cells (publication I). *PTGER4* was expressed in Ishikawa transcriptome in low level and its expression was not changed in response to steroid hormones. The treatment with TAM induced its expression, albeit, not noteworthy (publication II). PTGER4 was significantly down-regulated in women with RIF compared to fertile controls but in AC-prepared endometrium its expression became more similar to fertile controls.

Table 3. The DEGs in endometrial cell lines HEC1A, RL95-2 (publication I), Ishikawa (publication II) and NC vs. AC or RIF-specific human endometrial tissue samples (publication III).

Gene	Experiments	Description of the gene and endometrial context
TNC	ERβ target in HEC1A; PRAB target in RL95-2; E2 and P4 responsive in Ishikawa; AC-improved in endometrium	Extracellular matrix protein with a spatially and temporally restricted tissue distribution. This protein is expressed in various tissues during normal development and diseased conditions. In human endometrium its expression is concentrated in the upper layer of glandular epithelium and in ectopic lesions in endometriosis.
ITGA10	ER target in HEC1A, PR target in RL95-2, RIF-related in human endometrium	Transmembrane glycoproteins composed of non-covalently linked α and β chains. Integrins have been extensively investigated in context of endometrial receptivity.
PTGER4	ER target in HEC1A; PR target in RL95-2 and AC-improved in human endometrium	Member of the G-protein coupled receptor family. This protein is one of four receptors identified for prostaglandin E2 (PGE2). Prostaglandins increase vascular permeability and endometrial stromal decidualization. *PTGER4* is elevated in glandular epithelium and vascular cells in late proliferative phase endometrium.
IFNAR2	PR target in RL95-2 and AC-deteriorated in human endometrium	Type I membrane protein that forms one of the two chains of a receptor for interferons. The protein has been detected on luminal and glandular epithelium, and in stromal cells of ovine uterus.
WFDC2	ER target at HEC1A and AC-improved in human endometrium	Member of the WFDC domain family. Stimulated by E2 in mouse uterus, by E2 analogue bisphenol A in Ishikawa cells and up-regulated in response to E2 in rhesus monkey endometrium during WOI.
SPDEF	PR target in RL95-2 and RIF-associated in human endometrium.	ETS family of transcription factors. Marker gene of receptive endometrium as its expression is confirmed during WOI.

Interferon α and β receptor subunit 2 (IFNAR2) is a type I membrane receptor of interferons. All type I interferons, exert their action through a common receptor, which consists of two subunits, IFNAR1 and IFNAR2 (Roberts et al., 1997). High concentration of IFNAR1 and IFNAR2 have been detected on luminal and glandular epithelium, and also in stromal cells in the ovine uterus (Rosenfeld et al., 2002). This cytokine receptor stimulates Janus protein kinases, which in turn phosphorylate several proteins, including STAT1 and STAT2 (Rosenfeld et al., 2002). However, the previous information about the IFNAR2 expression in human endometrium is rather scarce. In the current thesis we uncovered that *IFNAR2* promoter is targeted by PRAB antibody (PRB receptor); it is induced by P4 in Ishikawa cells although not at significant level (publication I and II); and the gene is down-regulated in AC-prepared endometrium of women with RIF compared to fertile controls (publication III).

WAP four-disulfide core domain 2 secretory protein (WFDC2) acts as a protease inhibitor. Its expression has been stimulated by E2 in mouse uterus and in rhesus monkey endometrium during WOI, and by E2 analogue bisphenol A in Ishikawa cells (Hewitt et al., 2003; Moggs et al., 2004; Naciff et al., 2010). In the current thesis *WFDC2* promoter was targeted by ERβ in HEC1A cells, the gene was constantly expressed at high levels in Ishikawa cells, and it was up-regulated in natural cycle endometrium of RIF-patients when compared to fertile controls. After E2-/P4-supplementation the expression of *WFDC2* was improved and exhibited more similar pattern to fertile controls.

Epithelium-specific transcription factor (*SPDEF*) belongs to ETS family of transcription factors. *SPDEF* is considered as a marker of endometrial receptivity as its expression is confirmed during WOI (Diaz-Gimeno et al., 2011; Hu et al., 2014). Its responsiveness to steroid hormones and bisphenol A have been seen in mouse uterus and Ishikawa cells (Hewitt et al., 2003; Moggs et al., 2004; Naciff et al., 2010). In the current thesis *SPDEF* was identified as PR-target gene in RL95-2 cells and as RIF-associated gene in human endometrium. *SPDEF* was induced by both steroid hormones in Ishikawa cells albeit not in significant level (publication II).

CONCLUSIONS

In the current thesis, the influence of E2 and P4 on gene expression in human endometrium and endometrial cell lines was studied using large-scale genomic methods, like ChIP and qRT-PCR, RNA-sequencing, and microarrays. Altogether more than three thousand E2 and P4 responsive genes were identified in endometrial tissue samples and cell lines within three published studies. These hormone-responsive genes are involved in normal endometrial function and maturation, and participate in embryo implantation processes. However, their expression can be altered in conditions like female infertility or hormone-sensitive malignancies. The obtained results offer a better understanding of steroid hormone action on gene expression in human endometrial tissue, which extends our knowledge about this complex and dynamic tissue and hopefully helps to improve the infertility treatments in the nearest future.

More precise outcomes of the current thesis are as follows:

- ✓ Human endometrial cell lines HEC1A and RL95-2 respond differentially to ovarian steroid hormones, where genomic action of E2 and P4 regulate distinct groups of genes engaged in endometrial receptivity. Significantly higher rate of ER-target genes was found in HEC1A cells, while PR-targets were prevalent in RL95-2 cells. E2 predominates in pre-receptive endometrium (corresponding to the proliferative phase) and P4 exerts its dominant effect on receptive endometrium (corresponding to mid-secretory phase). Thus, our results support the view about HEC1A and RL95-2 cell lines as *in vitro* models of non-receptive and receptive endometrium, respectively.
- ✓ According to the best of our knowledge, our studies were the first to accomplish RNA-sequencing to study the genome-wide action of ovarian steroid hormones (E2 and P4) and their clinically used antagonists: TAM (antagonist for E2) and RU486 (antagonist for P4), on endometrial Ishikawa cells. We observed that nearly 1,700 genes were significantly affected following both the E2 or P4 treatments and overwhelming majority of these transcripts were also present in human endometrial samples, including the well-known endometrial receptivity markers as *APOE, IL6ST,* and *RARB*
- ✓ TAM and RU486 had both agonistic and antagonistic effects on E2- and P4-responsive genes, respectively, but also up-regulated distinct groups of genes independently in Ishikawa cells. The traditional E2-antagonist TAM changed the expression of approximately 1,000 genes from which more than 150 were associated with various diseases of the reproductive system. The most noteworthy was the significant induction of cyclin D1 (*CCND1*) by TAM, which is also remarkably abundant in endometrial carcinomas. The relation between TAM and its up-regulation of *CCND1*

could be one reason why the treatment of breast cancer with TAM is accompanied by a significant rise in endometrial cancer risk.

- ✓ P4 antagonist RU486 clinically goes by the name of mifepristone and is applied for contraceptive purposes to prevent unplanned pregnancies or for early medical abortion. RU486 changed significantly the expression of ca 500 genes, including nearly 100 genes related to diseases of the reproductive system. The genes associated with cell-to-cell adhesions were predominantly down-regulated post-RU486 treatment and included: *CTNND1, JUP, CDH2, IQGAP1,* and *COL2A1*. *This* unequivocally explains the main anti-P4 effects of RU486 as an 'emergency contraceptive' pill, causing endometrial breakdown and tissue shedding.
- ✓ Transcriptome analysis of 15 human endometrial biopsy samples at the time of embryo implantation was used to compare two most actively used endometrial preparation protocols for frozen embryo transfers in patients with previous implantation failure (RIF). The comparisons identified genes, which expression improved or, alternatively, deteriorated after steroid hormone supplemented FET, when compared to the natural reproductive cycle of fertile women. In steroid hormone supplemented artificial cycle FET, E2 and P4 are used to prepare the endometrium for implantation with daily doses of E2 and added P4 when the endometrium has reached sufficient thickness. Based on our transcriptome study, we concluded that the natural cycle endometrial gene expression pattern in RIF group is more similar to that observed in fertile controls. On the otherhand, artificial cycle FET seemed to have a stronger negative impact on expression of genes and pathways crucial to endometrial receptivity, including the altered expression of *ESR2*, *FSHR*, *LEP*, several interleukins, and matrix metalloproteinases. Thereby, the artificial endometrial preparation with supplemented E2 and P4 can lead to more deviated endometrial gene expression profile. For this reason, natural cycle should be favoured for FET, at least for patients with several unsuccessful IVF attempts.
- ✓ We also performed *in silico* analysis of hormone response elements – HREs- for 'endometrial receptivity' genes after E2 or P4 stimulation. In this analysis we were focussed on ER-specific EREs and PR-specific PRE genomic sequences in promoter regions of target genes. We found that all 'endometrial receptivity' genes harbour genomic response elements for ERs and PRs, and are, therefore, directly regulated by E2 and P4. Furthermore, a substantial overrepresentation of EREs was found among the genes with deteriorated expression in artificial cycle, whereas the PREs predominated in genes with amended expression in artificial cycle. Hence, in the artificial cycle protocol used for FET, E2 has a stronger negative impact on the endometrial transcriptome opposed to P4, which is able to restore the 'natural' gene expression.

In conclusion, the current thesis improved our understanding of hormonal regulation of genome activity during the cyclic changes of human endometrium. Our results provide valuable insight into the mechanisms of steroid hormones and their clinically used antagonists in the human endometrium. The limitation of the studies was that endometrial cell lines were mostly used. Future studies are needed to carry out large scale endometrial tissue sample analysis in order to confirm the results of the current study: providing comprehensive models for E2- and P4-mediated gene expression.

ABSTRACT

Achieving a pregnancy in patients with several previous unsuccessful IVF attempts, despite the transfer of good quality embryos to uterus, poses a very challenging task for infertility specialists. Despite growing knowledge about the aetiology of female infertility and constantly improving clinical skills in assisted reproduction, the chances to help these patients are still relatively poor, because of the limited understanding about the mechanisms of endometrial receptivity and embryo implantation. It is likely that one of the risk factors for IVF failure in these patients is hiding in aberrant endometrial maturation, which is unable to attain the receptive state by the time of embryo arrival. Endometrium is receptive for embryo implantation in a very limited time-period called 'window of implantation', usually lasting only for 2-4 days. The development of the endometrium is under the strict control of ovarian steroid hormones – estrogen and progesterone, by enforcing the proliferative and secretory changes in endometrium, respectively. Ovarian steroid hormones regulate endometrial gene expression directly via specific nuclear receptors, which bind onto the promoter areas of target genes and lead to changes in the expression of hormone-responsive genes. In the present thesis genome-wide analyses were applied to study the changes in endometrial gene expression in response to estrogen and progesterone and their clinically proven antagonists: antiestrogen – tamoxifen (TAM), and antiprogestin – mifepristone (RU486). The binding sites for estrogen and progesterone receptors were studied using chromatin immunoprecipitation in purpose to identify potential target genes of steroid hormones in three human endometrial cell lines HEC1A, RL95-2, and Ishikawa. The unique profiles of estrogen and progesterone target genes were found in endometrial cell lines, supporting their use as *in vitro* models for implantation. Transcriptome of Ishikawa cells was used to analyse the genome-wide effects of steroid hormone modulators – TAM and RU486. These studies highlighted the possible adverse effects of TAM on endometrial cells by inducing, for instance, the cyclin D1 gene expression, which has previously been shown to be up-regulated in endometrial carcinomas, and possibly explains why the treatment of breast cancer with TAM is accompanied by a significant rise in endometrial cancer risk. On the other hand, the anti-P4 effects induced the down-regulation of several cell-to-cell adhesion genes, thereby clarifying the action of RU486 as an 'emergency contraceptive pill', causing the endometrial breakdown and tissue shedding. Finally, the studies using the endometrial tissue transcriptomes from infertility patients, with previous recurrent implantation failure, helped us to recommend the most appropriate endometrial preparation protocol for frozen embryo transfers. We concluded that the natural cycle is more favourable than the use of exogenously E2-/P4-supplemented artificial cycle, in terms of similar endometrial transcriptomic signature to fertile controls. To the best of our knowledge, we were also among the first to accomplish the genome-wide analysis for genomic steroid hormone response elements for 'endometrial

receptivity' genes, showing that all of the genes might be directly regulated by steroid hormones and harbour the hormone-response elements at their promoter areas. Taking together, in the current thesis more than 3000 genes were identified which exhibited differential activity in response to estrogen, progesterone, and their clinically applied antagonists. These hormone-responsive genes may be involved in the normal endometrial function and participate in embryo implantation processes, but their expression can be altered in conditions like female infertility or hormone-sensitive malignancies. Collectively, the results of our studies improved the understanding of steroid hormone action on genome activity during the cyclic changes of human endometrial maturation and embryo implantation. We believe that these discoveries may help to improve the results of infertility treatment and counteract their possible side effects.

KOKKUVÕTE

Igas lastetuse ravi kliinikus on patsiente, kellele siiratakse korduvalt hea kvaliteediga embrüoid, aga soovitud rasedust ei teki. Põhjus, miks need patsiendid ei rasestu, peitub suure tõenäosusega selles, et emaka limaskest ehk endomeetrium ei ole vastuvõtlik embrüo kinnitumiseks. Tegelikult on endomeetrium võimeline toetama pesastuvat (implanteeruvat) embrüot ainult väga kitsal ajaperioodil, mida kutsutakse 'implantatsiooni aknaks' ja mille kestus igas reproduktiivses tsüklis on ainult 2-4 päeva. Reproduktiivses eas oleval naisel teeb endomeetrium igal kuul läbi olulised koemuutused. Need tsüklilised muutused on täpselt kontrollitud munasarjade poolt toodetud naissuguhormoonide – östradiooli ja progesterooni poolt. Naissuguhormoonid östradiool ja progesteroon reguleerivad endomeetriumis avalduvate geenide aktiivsust, seostudes esmalt vastavatele tuumaretseptoritele ning kinnitudes seejärel spetsiifilistele geenipiirkondadele. Käesolevas töös kasutati ülegenoomseid uurimismeetodeid, et teha kindlaks, kuidas mõjutavad östradiool ja progesteroon ning nende hormoonide kliiniliselt kasutatavad antagonistid – antiöstrogeen tamoksüfeen ja antiprogestageen mifepristoon geenide avaldumist inimese endomeetriumis. Inimese endomeetriumi HEC1A ja RL95-2 rakuliinides määrati östradiooli ja progesterooni retseptorite genoomsed seondumiskohad ja tuvastati seeläbi potentsiaalsed hormoonide sihtmärkgeenid. Meie uuringud näitasid, et östradioolil ja progesteroonil on spetsiifilised märklaudgeenid ning seetõttu saab endomeetriumi rakuliine kasutada implantatsiooni *in vitro* uuringutes. Hormoontöödeldud Ishikawa endomeetriumi rakuliini analüüs aitas selgitada tamoksüfeeni ja mifepristooni mõju geenide aktiivsusele. Näiteks indutseeris tamoksüfeeni töötlus tsükliinide ekspressiooni endomeetriumis, mida on varasemalt tuvastatud endomeetriumi kartsinoomi rakkudes. Antud leid aitab seetõttu selgitada fakti, miks rinnavähi ravis kasutatav tamoksüfeen suurendab oluliselt emaka limaskesta kasvajate riski. Mifepristooni abil töödeldud endomeetriumi rakuliinides vähenes aga rakuliidestes osalevate valkude ekspressioon, mis selgitab mifepristooni toimet medikamentoosse abordi esilekutsumisel. Steroidhormoonide kasutamine on samuti olulisel kohal emaka limaskesta ettevalmistamisel külmutatud embrüote siirdamiseks viljatuse ravis. Külmutatud embrüoid siiratakse kas loomulikus menstruaaltsüklis või eelneb embrüote siirdamisele östradiooli ja progesterooni ravimite kasutamine. Meie uurisime endomeetriumi limaskesta geenide aktiivsust viljatutel patsientidel, kelle kehavälise viljastamise protseduurid olid varasemalt ebaõnnestunud. Vastavalt meie uuringu tulemustele ei ole nende naiste puhul soovitav eksogeensete steroidhormoonide kasutamine endomeetriumi ettevalmistusel külmutatud embrüote siirdamiseks. Samuti analüüsisime emaka limaskestas avalduvaid ning embrüo implantatsioonis osalevate geenide regulatsiooni ning näitasime, et kõikidel nendel geenidel asetsevad promootorite piirkondades steroidhormoonide seostumissaidid. Kokku tuvastasime inimese endomeetriumi koes ja rakuliinides üle kolme tuhande

geeni, mille aktiivsust reguleerivad steroidhormoonid. Tuvastatud geenid osalevad endomeetriumi küpsemises ja embrüo implantatsiooni protsessides, kuid samuti mängivad suurt rolli naise viljatuse põhjusena ning hormoon-sõltuvate kasvajate tekkes. Käesolevas töös saadud informatsioon steroidhormoonide mõjust inimese endomeetriumi geenieksprессiooni mustrile avardab meie arusaamu emaka limaskesta toimimisest ja tsüklilistest muutustest ning aitab täiendada viljatusravi meetodeid.

REFERENCES

Acosta, A.A., Elberger, L., Borghi, M., Calamera, J.C., Chemes, H., Doncel, G.F., Kliman, H., Lema, B., Lustig, L., and Papier, S. (2000). Endometrial dating and determination of the window of implantation in healthy fertile women. Fertil Steril *73*, 788-798.

Adamson, G.D., and Pasta, D.J. (2010). Endometriosis fertility index: the new, validated endometriosis staging system. Fertil Steril *94*, 1609-1615.

Aghajanova, L., Altmae, S., Bjuresten, K., Hovatta, O., Landgren, B.M., and Stavreus-Evers, A. (2009). Disturbances in the LIF pathway in the endometrium among women with unexplained infertility. Fertil Steril *91*, 2602-2610.

Aghajanova, L., Hamilton, A.E., and Giudice, L.C. (2008). Uterine receptivity to human embryonic implantation: histology, biomarkers, and transcriptomics. Semin Cell Dev Biol *19*, 204-211.

Aghajanova, L., Stavreus-Evers, A., Nikas, Y., Hovatta, O., and Landgren, B.M. (2003). Coexpression of pinopodes and leukemia inhibitory factor, as well as its receptor, in human endometrium. Fertil Steril *79 Suppl 1*, 808-814.

Ahmed, A., Dearn, S., Shams, M., Li, X.F., Sangha, R.K., Rola-Pleszczynski, M., and Jiang, J. (1998). Localization, quantification, and activation of platelet-activating factor receptor in human endometrium during the menstrual cycle: PAF stimulates NO, VEGF, and FAKpp125. FASEB J *12*, 831-843.

Alfarawati, S., Fragouli, E., Colls, P., Stevens, J., Gutierrez-Mateo, C., Schoolcraft, W.B., Katz-Jaffe, M.G., and Wells, D. (2011). The relationship between blastocyst morphology, chromosomal abnormality, and embryo gender. Fertil Steril *95*, 520-524.

Alsalili, M., Yuzpe, A., Tummon, I., Parker, J., Martin, J., Daniel, S., Rebel, M., and Nisker, J. (1995). Cumulative pregnancy rates and pregnancy outcome after in-vitro fertilization: > 5000 cycles at one centre. Hum Reprod *10*, 470-474.

Altmae, S., Martinez-Conejero, J.A., Salumets, A., Simon, C., Horcajadas, J.A., and Stavreus-Evers, A. (2010). Endometrial gene expression analysis at the time of embryo implantation in women with unexplained infertility. Mol Hum Reprod *16*, 178-187.

Altmae, S., Reimand, J., Hovatta, O., Zhang, P., Kere, J., Laisk, T., Saare, M., Peters, M., Vilo, J., Stavreus-Evers, A.*, et al.* (2012). Research resource: interactome of human embryo implantation: identification of gene expression pathways, regulation, and integrated regulatory networks. Mol Endocrinol *26*, 203-217.

Aplin, J.D. (2006). In vitro analysis of trophoblast invasion. Methods Mol Med *122*, 45-57.

Aplin, J.D., Hey, N.A., and Graham, R.A. (1998). Human endometrial MUC1 carries keratan sulfate: characteristic glycoforms in the luminal epithelium at receptivity. Glycobiology *8*, 269-276.

Aplin, J.D., and Kimber, S.J. (2004). Trophoblast-uterine interactions at implantation. Reprod Biol Endocrinol *2*, 48.

Aplin, J.D., Seif, M.W., Graham, R.A., Hey, N.A., Behzad, F., and Campbell, S. (1994). The endometrial cell surface and implantation. Expression of the polymorphic mucin MUC-1 and adhesion molecules during the endometrial cycle. Ann N Y Acad Sci *734*, 103-121.

Ashley, R.L., Clay, C.M., Farmerie, T.A., Niswender, G.D., and Nett, T.M. (2006). Cloning and characterization of an ovine intracellular seven transmembrane receptor for progesterone that mediates calcium mobilization. Endocrinology *147*, 4151-4159.

Attia, G.R., Zeitoun, K., Edwards, D., Johns, A., Carr, B.R., and Bulun, S.E. (2000). Progesterone receptor isoform A but not B is expressed in endometriosis. J Clin Endocrinol Metab *85*, 2897-2902.

Baerwald, A.R., Adams, G.P., and Pierson, R.A. (2012). Ovarian antral folliculogenesis during the human menstrual cycle: a review. Hum Reprod Update *18*, 73-91.

Bagowski, C.P., Myers, J.W., and Ferrell, J.E., Jr. (2001). The classical progesterone receptor associates with p42 MAPK and is involved in phosphatidylinositol 3-kinase signaling in Xenopus oocytes. J Biol Chem *276*, 37708-37714.

Bahce, M., Cohen, J., and Munne, S. (1999). Preimplantation genetic diagnosis of aneuploidy: were we looking at the wrong chromosomes? J Assist Reprod Genet *16*, 176-181.

Baker, T.G. (1963). A Quantitative and Cytological Study of Germ Cells in Human Ovaries. Proceedings of the Royal Society of London Series B, Biological sciences *158*, 417-433.

Balasch, J., and Gratacos, E. (2012). Delayed childbearing: effects on fertility and the outcome of pregnancy. Curr Opin Obstet Gynecol *24*, 187-193.

Barash, A., Dekel, N., Fieldust, S., Segal, I., Schechtman, E., and Granot, I. (2003). Local injury to the endometrium doubles the incidence of successful pregnancies in patients undergoing in vitro fertilization. Fertil Steril *79*, 1317-1322.

Beato, M., and Klug, J. (2000). Steroid hormone receptors: an update. Hum Reprod Update *6*, 225-236.

Bellehumeur, C., Blanchet, J., Fontaine, J.Y., Bourcier, N., and Akoum, A. (2009). Interleukin 1 regulates its own receptors in human endometrial cells via distinct mechanisms. Hum Reprod *24*, 2193-2204.

Bentin-Ley, U., and Lopata, A. (2000). In vitro models of human blastocyst implantation. Baillieres Best Pract Res Clin Obstet Gynaecol *14*, 765-774.

Bergman, L., Beelen, M.L., Gallee, M.P., Hollema, H., Benraadt, J., and van Leeuwen, F.E. (2000). Risk and prognosis of endometrial cancer after

tamoxifen for breast cancer. Comprehensive Cancer Centres' ALERT Group. Assessment of Liver and Endometrial cancer Risk following Tamoxifen. Lancet *356*, 881-887.

Berrodin, T.J., Jelinsky, S.A., Graciani, N., Butera, J.A., Zhang, Z., Nagpal, S., Winneker, R.C., and Yudt, M.R. (2009). Novel progesterone receptor modulators with gene selective and context-dependent partial agonism. Biochem Pharmacol *77*, 204-215.

Berry, J. (2005). Are all aromatase inhibitors the same? A review of controlled clinical trials in breast cancer. Clin Ther *27*, 1671-1684.

Bjornstrom, L., and Sjoberg, M. (2002). Signal transducers and activators of transcription as downstream targets of nongenomic estrogen receptor actions. Mol Endocrinol *16*, 2202-2214.

Blesa, D., Ruiz-Alonso, M., and Simon, C. (2014). Clinical management of endometrial receptivity. Semin Reprod Med *32*, 410-413.

Bogacka, I., Bogacki, M., Kurzynska, A., and Chojnowska, K. (2013). The involvement of peroxisome proliferator activated receptors (PPARs) in prostaglandin F2alpha production by porcine endometrium. Reproductive biology *13*, 309-316.

Bomsel-Helmreich, O., Gougeon, A., Thebault, A., Saltarelli, D., Milgrom, E., Frydman, R., and Papiernik, E. (1979). Healthy and atretic human follicles in the preovulatory phase: differences in evolution of follicular morphology and steroid content of follicular fluid. J Clin Endocrinol Metab *48*, 686-694.

Boomsma, C.M., Kavelaars, A., Eijkemans, M.J., Fauser, B.C., Heijnen, C.J., and Macklon, N.S. (2010). Ovarian stimulation for in vitro fertilization alters the intrauterine cytokine, chemokine, and growth factor milieu encountered by the embryo. Fertil Steril *94*, 1764-1768.

Borthwick, J.M., Charnock-Jones, D.S., Tom, B.D., Hull, M.L., Teirney, R., Phillips, S.C., and Smith, S.K. (2003). Determination of the transcript profile of human endometrium. Mol Hum Reprod *9*, 19-33.

Brinsden, P.R., Alam, V., de Moustier, B., and Engrand, P. (2009). Recombinant human leukemia inhibitory factor does not improve implantation and pregnancy outcomes after assisted reproductive techniques in women with recurrent unexplained implantation failure. Fertil Steril *91*, 1445-1447.

Brogden, R.N., Goa, K.L., and Faulds, D. (1993). Mifepristone. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential. Drugs *45*, 384-409.

Brosens, J.J., and Gellersen, B. (2006). Death or survival--progesterone-dependent cell fate decisions in the human endometrial stroma. J Mol Endocrinol *36*, 389-398.

Buzdar, A.U., and Hortobagyi, G.N. (1998). Tamoxifen and toremifene in breast cancer: comparison of safety and efficacy. J Clin Oncol *16*, 348-353.

Cao, Q.J., Einstein, M.H., Anderson, P.S., Runowicz, C.D., Balan, R., and Jones, J.G. (2002). Expression of COX-2, Ki-67, cyclin D1, and P21 in endometrial endometrioid carcinomas. Int J Gynecol Pathol *21*, 147-154.

Carroll, J.S., Liu, X.S., Brodsky, A.S., Li, W., Meyer, C.A., Szary, A.J., Eeckhoute, J., Shao, W., Hestermann, E.V., Geistlinger, T.R.*, et al.* (2005). Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. Cell *122*, 33-43.

Carson, D.D., Lagow, E., Thathiah, A., Al-Shami, R., Farach-Carson, M.C., Vernon, M., Yuan, L., Fritz, M.A., and Lessey, B. (2002). Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening. Mol Hum Reprod *8*, 871-879.

Chang, K.C., Wang, Y., Bodine, P.V., Nagpal, S., and Komm, B.S. (2010). Gene expression profiling studies of three SERMs and their conjugated estrogen combinations in human breast cancer cells: insights into the unique antagonistic effects of bazedoxifene on conjugated estrogens. J Steroid Biochem Mol Biol *118*, 117-124.

Chauchereau, A., Savouret, J.F., and Milgrom, E. (1992). Control of biosynthesis and post-transcriptional modification of the progesterone receptor. Biol Reprod *46*, 174-177.

Check, J.H., O'Shaughnessy, A., Lurie, D., Fisher, C., and Adelson, H.G. (1995). Evaluation of the mechanism for higher pregnancy rates in donor oocyte recipients by comparison of fresh with frozen embryo transfer pregnancy rates in a shared oocyte programme. Hum Reprod *10*, 3022-3027.

Chiquet-Ehrismann, R., and Chiquet, M. (2003). Tenascins: regulation and putative functions during pathological stress. J Pathol *200*, 488-499.

Cohen, I. (2004). Endometrial pathologies associated with postmenopausal tamoxifen treatment. Gynecol Oncol *94*, 256-266.

Cohen, J., Inge, K.L., Suzman, M., Wiker, S.R., and Wright, G. (1989). Videocinematography of fresh and cryopreserved embryos: a retrospective analysis of embryonic morphology and implantation. Fertil Steril *51*, 820-827.

Cork, B.A., Li, T.C., Warren, M.A., and Laird, S.M. (2001). Interleukin-11 (IL-11) in human endometrium: expression throughout the menstrual cycle and the effects of cytokines on endometrial IL-11 production in vitro. J Reprod Immunol *50*, 3-17.

Coulam, C.B., Jeyendran, R.S., Fishel, L.A., and Roussev, R. (2006). Multiple thrombophilic gene mutations are risk factors for implantation failure. Reprod Biomed Online *12*, 322-327.

Craig, J., Orisaka, M., Wang, H., Orisaka, S., Thompson, W., Zhu, C., Kotsuji, F., and Tsang, B.K. (2007). Gonadotropin and intra-ovarian signals regulating follicle development and atresia: the delicate balance between

life and death. Frontiers in bioscience : a journal and virtual library *12*, 3628-3639.
Critchley, H.O., and Saunders, P.T. (2009). Hormone receptor dynamics in a receptive human endometrium. Reprod Sci *16*, 191-199.
Croxtall, J.D., Elder, M.G., and White, J.O. (1990). Hormonal control of proliferation in the Ishikawa endometrial adenocarcinoma cell line. J Steroid Biochem *35*, 665-669.
Cullinan, E.B., Abbondanzo, S.J., Anderson, P.S., Pollard, J.W., Lessey, B.A., and Stewart, C.L. (1996). Leukemia inhibitory factor (LIF) and LIF receptor expression in human endometrium suggests a potential autocrine/paracrine function in regulating embryo implantation. Proc Natl Acad Sci U S A *93*, 3115-3120.
Dain, L., Ojha, K., Bider, D., Levron, J., Zinchenko, V., Walster, S., and Dirnfeld, M. (2014). Effect of local endometrial injury on pregnancy outcomes in ovum donation cycles. Fertil Steril *102*, 1048-1054.
de Ruijter-Villani, M., Deelen, C., and Stout, T.A. (2015). Expression of leukaemia inhibitory factor at the conceptus?maternal interface during preimplantation development and in the endometrium during the oestrous cycle in the mare. Reprod Fertil Dev.
De Vos, M., Devroey, P., and Fauser, B.C. (2010). Primary ovarian insufficiency. Lancet *376*, 911-921.
DeLoia, J.A., Krasnow, J.S., Brekosky, J., Babaknia, A., Julian, J., and Carson, D.D. (1998). Regional specialization of the cell membrane-associated, polymorphic mucin (MUC1) in human uterine epithelia. Hum Reprod *13*, 2902-2909.
Demirol, A., and Gurgan, T. (2004). Effect of treatment of intrauterine pathologies with office hysteroscopy in patients with recurrent IVF failure. Reprod Biomed Online *8*, 590-594.
Develioglu, O.H., Hsiu, J.G., Nikas, G., Toner, J.P., Oehninger, S., and Jones, H.W., Jr. (1999). Endometrial estrogen and progesterone receptor and pinopode expression in stimulated cycles of oocyte donors. Fertil Steril *71*, 1040-1047.
Devroey, P., Bourgain, C., Macklon, N.S., and Fauser, B.C. (2004). Reproductive biology and IVF: ovarian stimulation and endometrial receptivity. Trends Endocrinol Metab *15*, 84-90.
Dharmaraj, N., Chapela, P.J., Morgado, M., Hawkins, S.M., Lessey, B.A., Young, S.L., and Carson, D.D. (2014). Expression of the transmembrane mucins, MUC1, MUC4 and MUC16, in normal endometrium and in endometriosis. Hum Reprod *29*, 1730-1738.
Diaz-Gimeno, P., Horcajadas, J.A., Martinez-Conejero, J.A., Esteban, F.J., Alama, P., Pellicer, A., and Simon, C. (2011). A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature. Fertil Steril *95*, 50-60, 60 e51-15.

Diaz-Gimeno, P., Ruiz-Alonso, M., Blesa, D., Bosch, N., Martinez-Conejero, J.A., Alama, P., Garrido, N., Pellicer, A., and Simon, C. (2013). The accuracy and reproducibility of the endometrial receptivity array is superior to histology as a diagnostic method for endometrial receptivity. Fertil Steril *99*, 508-517.

Dimitriadis, E., Menkhorst, E., Salamonsen, L.A., and Paiva, P. (2010a). Review: LIF and IL11 in trophoblast-endometrial interactions during the establishment of pregnancy. Placenta *31 Suppl*, S99-104.

Dimitriadis, E., Nie, G., Hannan, N.J., Paiva, P., and Salamonsen, L.A. (2010b). Local regulation of implantation at the human fetal-maternal interface. Int J Dev Biol *54*, 313-322.

Dimitriadis, E., Salamonsen, L.A., and Robb, L. (2000). Expression of interleukin-11 during the human menstrual cycle: coincidence with stromal cell decidualization and relationship to leukaemia inhibitory factor and prolactin. Mol Hum Reprod *6*, 907-914.

Dimitriadis, E., White, C.A., Jones, R.L., and Salamonsen, L.A. (2005). Cytokines, chemokines and growth factors in endometrium related to implantation. Hum Reprod Update *11*, 613-630.

Donnez, J., and Jadoul, P. (2002). What are the implications of myomas on fertility? A need for a debate? Hum Reprod *17*, 1424-1430.

dos Santos, R.L., da Silva, F.B., Ribeiro, R.F., Jr., and Stefanon, I. (2014). Sex hormones in the cardiovascular system. Hormone molecular biology and clinical investigation *18*, 89-103.

Dunn, C.L., Kelly, R.W., and Critchley, H.O. (2003). Decidualization of the human endometrial stromal cell: an enigmatic transformation. Reprod Biomed Online *7*, 151-161.

Edwards, D.P. (2005a). Regulation of signal transduction pathways by estrogen and progesterone. Annu Rev Physiol *67*, 335-376.

Edwards, R.G. (2005b). Historical significance of gonadotrophins in assisted reproduction. Reprod Biomed Online *10 Suppl 3*, 3-10.

Edwards, R.G. (2006). Human implantation: the last barrier in assisted reproduction technologies? Reprod Biomed Online *13*, 887-904.

Ehrmann, D.A. (2005). Polycystic ovary syndrome. N Engl J Med *352*, 1223-1236.

Eisner, J.R., Dumesic, D.A., Kemnitz, J.W., and Abbott, D.H. (2000). Timing of prenatal androgen excess determines differential impairment in insulin secretion and action in adult female rhesus monkeys. J Clin Endocrinol Metab *85*, 1206-1210.

El-Toukhy, T., Coomarasamy, A., Khairy, M., Sunkara, K., Seed, P., Khalaf, Y., and Braude, P. (2008). The relationship between endometrial thickness and outcome of medicated frozen embryo replacement cycles. Fertil Steril *89*, 832-839.

Elram, T., Simon, A., Israel, S., Revel, A., Shveiky, D., and Laufer, N. (2005). Treatment of recurrent IVF failure and human leukocyte antigen similarity by intravenous immunoglobulin. Reprod Biomed Online *11*, 745-749.

Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjold, M., and Gustafsson, J.A. (1997). Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab *82*, 4258-4265.

ESHRE "ART fact sheet 2014": https://www.eshre.eu/guidelines-and-legal/art-fact-sheet.aspx

European, I.V.F.M.C., European Society of Human, R., Embryology, Kupka, M.S., D'Hooghe, T., Ferraretti, A.P., de Mouzon, J., Erb, K., Castilla, J.A., Calhaz-Jorge, C.*, et al.* (2016). Assisted reproductive technology in Europe, 2011: results generated from European registers by ESHREdagger. Hum Reprod *31*, 233-248.

Evans, J., D'Sylva, R., Volpert, M., Jamsai, D., Merriner, D.J., Nie, G., Salamonsen, L.A., and O'Bryan, M.K. (2015). Endometrial CRISP3 is regulated throughout the mouse estrous and human menstrual cycle and facilitates adhesion and proliferation of endometrial epithelial cells. Biol Reprod *92*, 99.

Falkenstein, E., and Wehling, M. (2000). Nongenomically initiated steroid actions. Eur J Clin Invest *30 Suppl 3*, 51-54.

Fauser, B.C., Laven, J.S., Tarlatzis, B.C., Moley, K.H., Critchley, H.O., Taylor, R.N., Berga, S.L., Mermelstein, P.G., Devroey, P., Gianaroli, L.*, et al.* (2011). Sex steroid hormones and reproductive disorders: impact on women's health. Reprod Sci *18*, 702-712.

Ferraretti, A.P., Goossens, V., de Mouzon, J., Bhattacharya, S., Castilla, J.A., Korsak, V., Kupka, M., Nygren, K.G., Nyboe Andersen, A., European, I.V.F.m.*, et al.* (2012). Assisted reproductive technology in Europe, 2008: results generated from European registers by ESHRE. Hum Reprod *27*, 2571-2584.

Ferraretti, A.P., Goossens, V., Kupka, M., Bhattacharya, S., de Mouzon, J., Castilla, J.A., Erb, K., Korsak, V., Nyboe Andersen, A., European, I.V.F.M.C.f.t.E.S.o.H.R.*, et al.* (2013). Assisted reproductive technology in Europe, 2009: results generated from European registers by ESHRE. Hum Reprod *28*, 2318-2331.

Filant, J., DeMayo, F.J., Pru, J.K., Lydon, J.P., and Spencer, T.E. (2014). Fibroblast growth factor receptor two (FGFR2) regulates uterine epithelial integrity and fertility in mice. Biol Reprod *90*, 7.

Filicori, M., Cognigni, G.E., Tabarelli, C., Pocognoli, P., Taraborrelli, S., Spettoli, D., and Ciampaglia, W. (2002). Stimulation and growth of antral ovarian follicles by selective LH activity administration in women. J Clin Endocrinol Metab *87*, 1156-1161.

Fisher, B., Costantino, J.P., Wickerham, D.L., Cecchini, R.S., Cronin, W.M., Robidoux, A., Bevers, T.B., Kavanah, M.T., Atkins, J.N., Margolese,

R.G.*, et al.* (2005). Tamoxifen for the prevention of breast cancer: current status of the National Surgical Adjuvant Breast and Bowel Project P-1 study. J Natl Cancer Inst *97*, 1652-1662.

Fisher, B., Costantino, J.P., Wickerham, D.L., Redmond, C.K., Kavanah, M., Cronin, W.M., Vogel, V., Robidoux, A., Dimitrov, N., Atkins, J.*, et al.* (1998). Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst *90*, 1371-1388.

Foryst-Ludwig, A., and Kintscher, U. (2010). Metabolic impact of estrogen signalling through ERalpha and ERbeta. J Steroid Biochem Mol Biol *122*, 74-81.

Friedler, S., Margalioth, E.J., Kafka, I., and Yaffe, H. (1993). Treatable uterine cause for in-vitro fertilisation failures. Lancet *341*, 1213.

Frye, C.A., and Walf, A.A. (2009). Progesterone reduces depression-like behavior in a murine model of Alzheimer's Disease. Age (Dordr) *31*, 143-153.

Fu, M., Wang, C., Li, Z., Sakamaki, T., and Pestell, R.G. (2004). Minireview: Cyclin D1: normal and abnormal functions. Endocrinology *145*, 5439-5447.

Fugere, P., Scheele, W.H., Shah, A., Strack, T.R., Glant, M.D., and Jolly, E. (2000). Uterine effects of raloxifene in comparison with continuous-combined hormone replacement therapy in postmenopausal women. Am J Obstet Gynecol *182*, 568-574.

Garrido-Gomez, T., Ruiz-Alonso, M., Blesa, D., Diaz-Gimeno, P., Vilella, F., and Simon, C. (2013). Profiling the gene signature of endometrial receptivity: clinical results. Fertil Steril *99*, 1078-1085.

Gellersen, B., Brosens, I.A., and Brosens, J.J. (2007). Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. Semin Reprod Med *25*, 445-453.

Gellersen, B., and Brosens, J. (2003). Cyclic AMP and progesterone receptor cross-talk in human endometrium: a decidualizing affair. J Endocrinol *178*, 357-372.

Germeyer, A., Capp, E., Schlicksupp, F., Jauckus, J., von Rango, U., von Wolff, M., and Strowitzki, T. (2013). Cell-type specific expression and regulation of apolipoprotein D and E in human endometrium. Eur J Obstet Gynecol Reprod Biol *170*, 487-491.

Ghosh, D., De, P., and Sengupta, J. (1994). Luteal phase ovarian oestrogen is not essential for implantation and maintenance of pregnancy from surrogate embryo transfer in the rhesus monkey. Hum Reprod *9*, 629-637.

Giudice, L.C. (1999). Genes associated with embryonic attachment and implantation and the role of progesterone. J Reprod Med *44*, 165-171.

Giudice, L.C., Milkowski, D.A., Lamson, G., Rosenfeld, R.G., and Irwin, J.C. (1991). Insulin-like growth factor binding proteins in human endometrium: steroid-dependent messenger ribonucleic acid expression and protein synthesis. J Clin Endocrinol Metab *72*, 779-787.

Glujovsky, D., Pesce, R., Fiszbajn, G., Sueldo, C., Hart, R.J., and Ciapponi, A. (2010). Endometrial preparation for women undergoing embryo transfer with frozen embryos or embryos derived from donor oocytes. Cochrane Database Syst Rev, CD006359.

Godkin, J.D., and Dore, J.J. (1998). Transforming growth factor beta and the endometrium. Rev Reprod *3*, 1-6.

Gold, L.I., Saxena, B., Mittal, K.R., Marmor, M., Goswami, S., Nactigal, L., Korc, M., and Demopoulos, R.I. (1994). Increased expression of transforming growth factor beta isoforms and basic fibroblast growth factor in complex hyperplasia and adenocarcinoma of the endometrium: evidence for paracrine and autocrine action. Cancer Res *54*, 2347-2358.

Goldberg, J.R., Plescia, M.G., and Anastasio, G.D. (1998). Mifepristone (RU 486): current knowledge and future prospects. Arch Fam Med *7*, 219-222.

Golouh, R., Cufer, T., Sadikov, A., Nussdorfer, P., Usher, P.A., Brunner, N., Schmitt, M., Lesche, R., Maier, S., Timmermans, M.*, et al.* (2008). The prognostic value of Stathmin-1, S100A2, and SYK proteins in ER-positive primary breast cancer patients treated with adjuvant tamoxifen monotherapy: an immunohistochemical study. Breast Cancer Res Treat *110*, 317-326.

Gonzalez, R.R., Caballero-Campo, P., Jasper, M., Mercader, A., Devoto, L., Pellicer, A., and Simon, C. (2000). Leptin and leptin receptor are expressed in the human endometrium and endometrial leptin secretion is regulated by the human blastocyst. J Clin Endocrinol Metab *85*, 4883-4888.

Graham, J.D., and Clarke, C.L. (1997). Physiological action of progesterone in target tissues. Endocr Rev *18*, 502-519.

Graham, J.D., and Clarke, C.L. (2002). Expression and transcriptional activity of progesterone receptor A and progesterone receptor B in mammalian cells. Breast Cancer Res *4*, 187-190.

Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J.M., Argos, P., and Chambon, P. (1986). Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature *320*, 134-139.

Griesinger, G., Diedrich, K., and Edwards, R.G. (2005). Gonadotrophins--past, present and future. Reprod Biomed Online *10 Suppl 3*, 91-92.

Groenewoud, E.R., Cantineau, A.E., Kollen, B.J., Macklon, N.S., and Cohlen, B.J. (2013). What is the optimal means of preparing the endometrium in frozen-thawed embryo transfer cycles? A systematic review and meta-analysis. Hum Reprod Update *19*, 458-470.

Groenewoud, E.R., Macklon, N.S., Cohlen, B.J., and group, A.t.s. (2012). Cryo-thawed embryo transfer: natural versus artificial cycle. A non-inferiority trial. (ANTARCTICA trial). BMC women's health *12*, 27.

Grosse, B., Kachkache, M., Le Mellay, V., and Lieberherr, M. (2000). Membrane signalling and progesterone in female and male osteoblasts. I.

Involvement Of intracellular Ca(2+), inositol trisphosphate, and diacylglycerol, but not cAMP. J Cell Biochem *79*, 334-345.
Guo, F., Li, Y., Liu, Y., Wang, J., Li, Y., and Li, G. (2010). Inhibition of metastasis-associated lung adenocarcinoma transcript 1 in CaSki human cervical cancer cells suppresses cell proliferation and invasion. Acta Biochim Biophys Sin (Shanghai) *42*, 224-229.
Guzeloglu-Kayisli, O., Kayisli, U.A., and Taylor, H.S. (2009). The role of growth factors and cytokines during implantation: endocrine and paracrine interactions. Semin Reprod Med *27*, 62-79.
Hanna, R., Pang, Y., Thomas, P., and Zhu, Y. (2006). Cell-surface expression, progestin binding, and rapid nongenomic signaling of zebrafish membrane progestin receptors alpha and beta in transfected cells. J Endocrinol *190*, 247-260.
Hansen, K.R., Knowlton, N.S., Thyer, A.C., Charleston, J.S., Soules, M.R., and Klein, N.A. (2008). A new model of reproductive aging: the decline in ovarian non-growing follicle number from birth to menopause. Hum Reprod *23*, 699-708.
Haouzi, D., Assou, S., Mahmoud, K., Tondeur, S., Reme, T., Hedon, B., De Vos, J., and Hamamah, S. (2009a). Gene expression profile of human endometrial receptivity: comparison between natural and stimulated cycles for the same patients. Hum Reprod *24*, 1436-1445.
Haouzi, D., Mahmoud, K., Fourar, M., Bendhaou, K., Dechaud, H., De Vos, J., Reme, T., Dewailly, D., and Hamamah, S. (2009b). Identification of new biomarkers of human endometrial receptivity in the natural cycle. Hum Reprod *24*, 198-205.
Hapangama, D.K., Kamal, A.M., and Bulmer, J.N. (2015). Estrogen receptor beta: the guardian of the endometrium. Hum Reprod Update *21*, 174-193.
Harduf, H., Goldman, S., and Shalev, E. (2007). Human uterine epithelial RL95-2 and HEC-1A cell-line adhesiveness: the role of plexin B1. Fertil Steril *87*, 1419-1427.
Harper, M.J. (1992). The implantation window. Baillieres Clin Obstet Gynaecol *6*, 351-371.
Henzl, M.R., and Loomba, P.K. (2003). Transdermal delivery of sex steroids for hormone replacement therapy and contraception. A review of principles and practice. J Reprod Med *48*, 525-540.
Hewitt, S.C., Deroo, B.J., Hansen, K., Collins, J., Grissom, S., Afshari, C.A., and Korach, K.S. (2003). Estrogen receptor-dependent genomic responses in the uterus mirror the biphasic physiological response to estrogen. Mol Endocrinol *17*, 2070-2083.
Hill, M.J., Miller, K.A., and Frattarelli, J.L. (2010). A GnRH agonist and exogenous hormone stimulation protocol has a higher live-birth rate than a natural endogenous hormone protocol for frozen-thawed blastocyst-stage embryo transfer cycles: an analysis of 1391 cycles. Fertil Steril *93*, 416-422.

Hillier, S.G., Reichert, L.E., Jr., and Van Hall, E.V. (1981). Control of preovulatory follicular estrogen biosynthesis in the human ovary. J Clin Endocrinol Metab *52*, 847-856.

Horcajadas, J.A., Riesewijk, A., Martin, J., Cervero, A., Mosselman, S., Pellicer, A., and Simon, C. (2004). Global gene expression profiling of human endometrial receptivity. Journal of reproductive immunology *63*, 41-49.

Horne, A.W., Duncan, W.C., King, A.E., Burgess, S., Lourenco, P.C., Cornes, P., Ghazal, P., Williams, A.R., Udby, L., and Critchley, H.O. (2009). Endometrial cysteine-rich secretory protein 3 is inhibited by human chorionic gonadotrophin, and is increased in the decidua of tubal ectopic pregnancy. Mol Hum Reprod *15*, 287-294.

Horne, A.W., Lalani, E.N., Margara, R.A., Ryder, T.A., Mobberley, M.A., and White, J.O. (2005). The expression pattern of MUC1 glycoforms and other biomarkers of endometrial receptivity in fertile and infertile women. Molecular reproduction and development *72*, 216-229.

Horne, A.W., White, J.O., and Lalani el, N. (2002). Adhesion molecules and the normal endometrium. BJOG *109*, 610-617.

Horne AW, Lalani EN, Margara RA, White JO. The effects of sex steroid hormones and interleukin-1-beta on MUC1 expression in endometrial epithelial cell lines. Reproduction 2006;131:733-742.

Hu, S., Yao, G., Wang, Y., Xu, H., Ji, X., He, Y., Zhu, Q., Chen, Z., and Sun, Y. (2014). Transcriptomic changes during the pre-receptive to receptive transition in human endometrium detected by RNA-Seq. J Clin Endocrinol Metab *99*, E2744-2753.

John, N.J., Linke, M., and Denker, H.W. (1993). Retinoic acid decreases attachment of JAR choriocarcinoma spheroids to a human endometrial cell monolayer in vitro. Placenta *14*, 13-24.

Jones, R.L., Salamonsen, L.A., Zhao, Y.C., Ethier, J.F., Drummond, A.E., and Findlay, J.K. (2002). Expression of activin receptors, follistatin and betaglycan by human endometrial stromal cells; consistent with a role for activins during decidualization. Mol Hum Reprod *8*, 363-374.

Jones, R.L., Stoikos, C., Findlay, J.K., and Salamonsen, L.A. (2006). TGF-beta superfamily expression and actions in the endometrium and placenta. Reproduction *132*, 217-232.

Kabir-Salmani, M., Shiokawa, S., Akimoto, Y., Sakai, K., and Iwashita, M. (2004). The role of alpha(5)beta(1)-integrin in the IGF-I-induced migration of extravillous trophoblast cells during the process of implantation. Mol Hum Reprod *10*, 91-97.

Kang YJ, Forbes K, Carver J, Aplin JD. The role of the osteopontin-integrin alphabeta3 interaction at implantation:functional analysis using three different *in vitro* models. Hum Reprod 2014;29:739-749

Kao, L.C., Tulac, S., Lobo, S., Imani, B., Yang, J.P., Germeyer, A., Osteen, K., Taylor, R.N., Lessey, B.A., and Giudice, L.C. (2002). Global gene profiling in

human endometrium during the window of implantation. Endocrinology *143*, 2119-2138.
Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H., and Chambon, P. (1990). Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J *9*, 1603-1614.
Kelly, M.J., and Ronnekleiv, O.K. (2008). Membrane-initiated estrogen signaling in hypothalamic neurons. Mol Cell Endocrinol *290*, 14-23.
Khorasanizadeh, S., and Rastinejad, F. (2001). Nuclear-receptor interactions on DNA-response elements. Trends Biochem Sci *26*, 384-390.
King, A. (2000). Uterine leukocytes and decidualization. Hum Reprod Update *6*, 28-36.
Klinge, C.M. (2000). Estrogen receptor interaction with co-activators and co-repressors. Steroids *65*, 227-251.
Klinge, C.M., Jernigan, S.C., Mattingly, K.A., Risinger, K.E., and Zhang, J. (2004). Estrogen response element-dependent regulation of transcriptional activation of estrogen receptors alpha and beta by coactivators and corepressors. J Mol Endocrinol *33*, 387-410.
Krikun, G., Schatz, F., Taylor, R., Critchley, H.O., Rogers, P.A., Huang, J., and Lockwood, C.J. (2005). Endometrial endothelial cell steroid receptor expression and steroid effects on gene expression. J Clin Endocrinol Metab *90*, 1812-1818.
Krussel, J.S., Bielfeld, P., Polan, M.L., and Simon, C. (2003). Regulation of embryonic implantation. Eur J Obstet Gynecol Reprod Biol *110 Suppl 1*, S2-9.
Kuiper, G.G., and Gustafsson, J.A. (1997). The novel estrogen receptor-beta subtype: potential role in the cell- and promoter-specific actions of estrogens and anti-estrogens. FEBS Lett *410*, 87-90.
Kuramoto, H., Tamura, S., and Notake, Y. (1972). Establishment of a cell line of human endometrial adenocarcinoma in vitro. Am J Obstet Gynecol *114*, 1012-1019.
Kwan, I., Bhattacharya, S., Kang, A., and Woolner, A. (2014). Monitoring of stimulated cycles in assisted reproduction (IVF and ICSI). Cochrane Database Syst Rev *8*, CD005289.
Kyo, S., Maida, Y., and Inoue, M. (2011). Stem cells in endometrium and endometrial cancer: accumulating evidence and unresolved questions. Cancer Lett *308*, 123-133.
Lange, C.A., Shen, T., and Horwitz, K.B. (2000). Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. Proc Natl Acad Sci U S A *97*, 1032-1037.
Large, M.J., Wetendorf, M., Lanz, R.B., Hartig, S.M., Creighton, C.J., Mancini, M.A., Kovanci, E., Lee, K.F., Threadgill, D.W., Lydon, J.P.*, et al.* (2014).

The epidermal growth factor receptor critically regulates endometrial function during early pregnancy. PLoS Genet *10*, e1004451.
Lass, A., Peat, D., Avery, S., and Brinsden, P. (1998). Histological evaluation of endometrium on the day of oocyte retrieval after gonadotrophin-releasing hormone agonist-follicle stimulating hormone ovulation induction for in-vitro fertilization. Hum Reprod *13*, 3203-3205.
Ledee-Bataille, N., Lapree-Delage, G., Taupin, J.L., Dubanchet, S., Frydman, R., and Chaouat, G. (2002). Concentration of leukaemia inhibitory factor (LIF) in uterine flushing fluid is highly predictive of embryo implantation. Hum Reprod *17*, 213-218.
Ledee, N., Munaut, C., Aubert, J., Serazin, V., Rahmati, M., Chaouat, G., Sandra, O., and Foidart, J.M. (2011). Specific and extensive endometrial deregulation is present before conception in IVF/ICSI repeated implantation failures (IF) or recurrent miscarriages. J Pathol *225*, 554-564.
Lessey, B.A. (1997). Integrins and the endometrium: new markers of uterine receptivity. Ann N Y Acad Sci *828*, 111-122.
Lessey, B.A. (1998). Endometrial integrins and the establishment of uterine receptivity. Hum Reprod *13 Suppl 3*, 247-258; discussion 259-261.
Lessey, B.A., and Castelbaum, A.J. (2002). Integrins and implantation in the human. Reviews in endocrine & metabolic disorders *3*, 107-117.
Lessey, B.A., Castelbaum, A.J., Buck, C.A., Lei, Y., Yowell, C.W., and Sun, J. (1994). Further characterization of endometrial integrins during the menstrual cycle and in pregnancy. Fertil Steril *62*, 497-506.
Lessey, B.A., Castelbaum, A.J., Sawin, S.W., and Sun, J. (1995). Integrins as markers of uterine receptivity in women with primary unexplained infertility. Fertil Steril *63*, 535-542.
Lessey, B.A., Castelbaum, A.J., Wolf, L., Greene, W., Paulson, M., Meyer, W.R., and Fritz, M.A. (2000). Use of integrins to date the endometrium. Fertil Steril *73*, 779-787.
Lessey, B.A., Damjanovich, L., Coutifaris, C., Castelbaum, A., Albelda, S.M., and Buck, C.A. (1992). Integrin adhesion molecules in the human endometrium. Correlation with the normal and abnormal menstrual cycle. J Clin Invest *90*, 188-195.
Lessey, B.A., Gui, Y., Apparao, K.B., Young, S.L., and Mulholland, J. (2002). Regulated expression of heparin-binding EGF-like growth factor (HB-EGF) in the human endometrium: a potential paracrine role during implantation. Molecular reproduction and development *62*, 446-455.
Lessey, B.A., Ilesanmi, A.O., Castelbaum, A.J., Yuan, L., Somkuti, S.G., Chwalisz, K., and Satyaswaroop, P.G. (1996a). Characterization of the functional progesterone receptor in an endometrial adenocarcinoma cell line (Ishikawa): progesterone-induced expression of the alpha1 integrin. J Steroid Biochem Mol Biol *59*, 31-39.
Lessey, B.A., Ilesanmi, A.O., Lessey, M.A., Riben, M., Harris, J.E., and Chwalisz, K. (1996b). Luminal and glandular endometrial epithelium

express integrins differentially throughout the menstrual cycle: implications for implantation, contraception, and infertility. Am J Reprod Immunol *35*, 195-204.

Lessey, B.A., Killam, A.P., Metzger, D.A., Haney, A.F., Greene, G.L., and McCarty, K.S., Jr. (1988). Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle. J Clin Endocrinol Metab *67*, 334-340.

Li, L., Haynes, M.P., and Bender, J.R. (2003). Plasma membrane localization and function of the estrogen receptor alpha variant (ER46) in human endothelial cells. Proc Natl Acad Sci U S A *100*, 4807-4812.

Li, Y., Burns, K.A., Arao, Y., Luh, C.J., and Korach, K.S. (2012). Differential estrogenic actions of endocrine-disrupting chemicals bisphenol A, bisphenol AF, and zearalenone through estrogen receptor alpha and beta in vitro. Environ Health Perspect *120*, 1029-1035.

Lim, P.S., Nazarian, L.N., Wechsler, R.J., Kurtz, A.B., and Parker, L. (2002). The endometrium on routine contrast-enhanced CT in asymptomatic postmenopausal women: avoiding errors in interpretation. Clin Imaging *26*, 325-329.

Lurie, M., Elmalach, I., and Weill, S. (1988). The pattern of epithelial mucin secretion in normal, hyperplastic, and adenocarcinomatous endometrium. Gynecol Oncol *30*, 274-284.

Luu-The, V. (2001). Analysis and characteristics of multiple types of human 17beta-hydroxysteroid dehydrogenase. J Steroid Biochem Mol Biol *76*, 143-151.

Machelon, V., Nome, F., Grosse, B., and Lieberherr, M. (1996). Progesterone triggers rapid transmembrane calcium influx and/or calcium mobilization from endoplasmic reticulum, via a pertussis-insensitive G-protein in granulosa cells in relation to luteinization process. J Cell Biochem *61*, 619-628.

Machin, P., Catasus, L., Pons, C., Munoz, J., Matias-Guiu, X., and Prat, J. (2002). CTNNB1 mutations and beta-catenin expression in endometrial carcinomas. Hum Pathol *33*, 206-212.

Macias, H., and Hinck, L. (2012). Mammary gland development. Wiley Interdiscip Rev Dev Biol *1*, 533-557.

Macklon, N.S., van der Gaast, M.H., Hamilton, A., Fauser, B.C., and Giudice, L.C. (2008). The impact of ovarian stimulation with recombinant FSH in combination with GnRH antagonist on the endometrial transcriptome in the window of implantation. Reprod Sci *15*, 357-365.

Margalioth, E.J., Ben-Chetrit, A., Gal, M., and Eldar-Geva, T. (2006). Investigation and treatment of repeated implantation failure following IVF-ET. Hum Reprod *21*, 3036-3043.

Martin, J., Dominguez, F., Avila, S., Castrillo, J.L., Remohi, J., Pellicer, A., and Simon, C. (2002). Human endometrial receptivity: gene regulation. J Reprod Immunol *55*, 131-139.

Maruyama, T., Masuda, H., Ono, M., Kajitani, T., and Yoshimura, Y. (2010). Human uterine stem/progenitor cells: their possible role in uterine physiology and pathology. Reproduction *140*, 11-22.

Mascarenhas, M.N., Flaxman, S.R., Boerma, T., Vanderpoel, S., and Stevens, G.A. (2012). National, regional, and global trends in infertility prevalence since 1990: a systematic analysis of 277 health surveys. PLoS medicine *9*, e1001356.

McGowen, M.R., Erez, O., Romero, R., and Wildman, D.E. (2014). The evolution of embryo implantation. Int J Dev Biol *58*, 155-161.

Mikhailik, A., Mazella, J., Liang, S., and Tseng, L. (2009). Notch ligand-dependent gene expression in human endometrial stromal cells. Biochem Biophys Res Commun *388*, 479-482.

Milne, S.A., Perchick, G.B., Boddy, S.C., and Jabbour, H.N. (2001). Expression, localization, and signaling of PGE(2) and EP2/EP4 receptors in human nonpregnant endometrium across the menstrual cycle. J Clin Endocrinol Metab *86*, 4453-4459.

Mindnich, R., Moller, G., and Adamski, J. (2004). The role of 17 beta-hydroxysteroid dehydrogenases. Mol Cell Endocrinol *218*, 7-20.

Mirkin, S., Arslan, M., Churikov, D., Corica, A., Diaz, J.I., Williams, S., Bocca, S., and Oehninger, S. (2005). In search of candidate genes critically expressed in the human endometrium during the window of implantation. Hum Reprod *20*, 2104-2117.

Mirkin, S., Nikas, G., Hsiu, J.G., Diaz, J., and Oehninger, S. (2004). Gene expression profiles and structural/functional features of the peri-implantation endometrium in natural and gonadotropin-stimulated cycles. J Clin Endocrinol Metab *89*, 5742-5752.

Moggs, J.G., Tinwell, H., Spurway, T., Chang, H.S., Pate, I., Lim, F.L., Moore, D.J., Soames, A., Stuckey, R., Currie, R.*, et al.* (2004). Phenotypic anchoring of gene expression changes during estrogen-induced uterine growth. Environ Health Perspect *112*, 1589-1606.

Mohan, S., and Baylink, D.J. (2002). IGF-binding proteins are multifunctional and act via IGF-dependent and -independent mechanisms. J Endocrinol *175*, 19-31.

Moore, J.T., McKee, D.D., Slentz-Kesler, K., Moore, L.B., Jones, S.A., Horne, E.L., Su, J.L., Kliewer, S.A., Lehmann, J.M., and Willson, T.M. (1998). Cloning and characterization of human estrogen receptor beta isoforms. Biochem Biophys Res Commun *247*, 75-78.

Morriss-Kay, G. (1992). Retinoic acid receptors in normal growth and development. Cancer surveys *14*, 181-193.

Munsell, M.F., Sprague, B.L., Berry, D.A., Chisholm, G., and Trentham-Dietz, A. (2014). Body mass index and breast cancer risk according to postmenopausal estrogen-progestin use and hormone receptor status. Epidemiologic reviews *36*, 114-136.

Mylonas, I., Jeschke, U., Shabani, N., Kuhn, C., Kunze, S., Dian, D., Friedl, C., Kupka, M.S., and Friese, K. (2007). Steroid receptors ERalpha, ERbeta, PR-A and PR-B are differentially expressed in normal and atrophic human endometrium. Histol Histopathol *22*, 169-176.

Naciff, J.M., Khambatta, Z.S., Reichling, T.D., Carr, G.J., Tiesman, J.P., Singleton, D.W., Khan, S.A., and Daston, G.P. (2010). The genomic response of Ishikawa cells to bisphenol A exposure is dose- and time-dependent. Toxicology *270*, 137-149.

Nagashima, T., Li, Q., Clementi, C., Lydon, J.P., DeMayo, F.J., and Matzuk, M.M. (2013). BMPR2 is required for postimplantation uterine function and pregnancy maintenance. J Clin Invest *123*, 2539-2550.

Nargund, G., and Frydman, R. (2007). Towards a more physiological approach to IVF. Reprod Biomed Online *14*, 550-552.

Nawroth, F., and Ludwig, M. (2005). What is the 'ideal' duration of progesterone supplementation before the transfer of cryopreserved-thawed embryos in estrogen/progesterone replacement protocols? Hum Reprod *20*, 1127-1134.

Nelson, L.R., and Bulun, S.E. (2001). Estrogen production and action. J Am Acad Dermatol *45*, S116-124.

Niakan, K.K., Han, J., Pedersen, R.A., Simon, C., and Pera, R.A. (2012). Human pre-implantation embryo development. Development *139*, 829-841.

Nikas, G., Drakakis, P., Loutradis, D., Mara-Skoufari, C., Koumantakis, E., Michalas, S., and Psychoyos, A. (1995). Uterine pinopodes as markers of the 'nidation window' in cycling women receiving exogenous oestradiol and progesterone. Hum Reprod *10*, 1208-1213.

Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M., and Gustafsson, J.A. (2001). Mechanisms of estrogen action. Physiol Rev *81*, 1535-1565.

Nishida, M., Kasahara, K., Kaneko, M., Iwasaki, H., and Hayashi, K. (1985). [Establishment of a new human endometrial adenocarcinoma cell line, Ishikawa cells, containing estrogen and progesterone receptors]. Nihon Sanka Fujinka Gakkai Zasshi *37*, 1103-1111.

Noyes, R.W. (1956). Uniformity of secretory endometrium; study of multiple sections from 100 uteri removed at operation. Fertil Steril *7*, 103-109.

O'Malley, B.W., and Means, A.R. (1974). Female steroid hormones and target cell nuclei. Science *183*, 610-620.

Ombelet, W., Cooke, I., Dyer, S., Serour, G., and Devroey, P. (2008). Infertility and the provision of infertility medical services in developing countries. Hum Reprod Update *14*, 605-621.

Osteen, K.G., Keller, N.R., Feltus, F.A., and Melner, M.H. (1999). Paracrine regulation of matrix metalloproteinase expression in the normal human endometrium. Gynecol Obstet Invest *48 Suppl 1*, 2-13.

Pabona, J.M., Simmen, F.A., Nikiforov, M.A., Zhuang, D., Shankar, K., Velarde, M.C., Zelenko, Z., Giudice, L.C., and Simmen, R.C. (2012). Kruppel-like

factor 9 and progesterone receptor coregulation of decidualizing endometrial stromal cells: implications for the pathogenesis of endometriosis. J Clin Endocrinol Metab *97*, E376-392.

Palermo, G., Joris, H., Devroey, P., and Van Steirteghem, A.C. (1992). Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet *340*, 17-18.

Pallottini, V., Bulzomi, P., Galluzzo, P., Martini, C., and Marino, M. (2008). Estrogen regulation of adipose tissue functions: involvement of estrogen receptor isoforms. Infect Disord Drug Targets *8*, 52-60.

Papanikolaou, E.G., Bourgain, C., Kolibianakis, E., Tournaye, H., and Devroey, P. (2005). Steroid receptor expression in late follicular phase endometrium in GnRH antagonist IVF cycles is already altered, indicating initiation of early luteal phase transformation in the absence of secretory changes. Hum Reprod *20*, 1541-1547.

Paulson, R.J. (2011). Hormonal induction of endometrial receptivity. Fertil Steril *96*, 530-535.

Pennings, G., and Ombelet, W. (2007). Coming soon to your clinic: patient-friendly ART. Hum Reprod *22*, 2075-2079.

Penot, G., Le Peron, C., Merot, Y., Grimaud-Fanouillere, E., Ferriere, F., Boujrad, N., Kah, O., Saligaut, C., Ducouret, B., Metivier, R.*, et al.* (2005). The human estrogen receptor-alpha isoform hERalpha46 antagonizes the proliferative influence of hERalpha66 in MCF7 breast cancer cells. Endocrinology *146*, 5474-5484.

Polanski, L.T., Baumgarten, M.N., Quenby, S., Brosens, J., Campbell, B.K., and Raine-Fenning, N.J. (2014). What exactly do we mean by 'recurrent implantation failure'? A systematic review and opinion. Reprod Biomed Online *28*, 409-423.

Pomp, E.R., Rosendaal, F.R., and Doggen, C.J. (2008). Smoking increases the risk of venous thrombosis and acts synergistically with oral contraceptive use. American journal of hematology *83*, 97-102.

Punyadeera, C., Dassen, H., Klomp, J., Dunselman, G., Kamps, R., Dijcks, F., Ederveen, A., de Goeij, A., and Groothuis, P. (2005). Oestrogen-modulated gene expression in the human endometrium. Cell Mol Life Sci *62*, 239-250.

Quinn, C.E., and Casper, R.F. (2009). Pinopodes: a questionable role in endometrial receptivity. Hum Reprod Update *15*, 229-236.

Ragusa, A., de Carolis, C., dal Lago, A., Miriello, D., Ruggiero, G., Brucato, A., Pisoni, M.P., Muscara, M., Merati, R., Maccario, L.*, et al.* (2004). Progesterone supplement in pregnancy: an immunologic therapy? Lupus *13*, 639-642.

Raziel, A., Friedler, S., Schachter, M., Kasterstein, E., Strassburger, D., and Ron-El, R. (2002). Increased frequency of female partner chromosomal abnormalities in patients with high-order implantation failure after in vitro fertilization. Fertil Steril *78*, 515-519.

Revankar, C.M., Cimino, D.F., Sklar, L.A., Arterburn, J.B., and Prossnitz, E.R. (2005). A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science *307*, 1625-1630.

Report of National Institute for Health Development 2015: http://www.tai.ee/et/terviseandmed/tervisestatistika-ja-uuringute-andmebaas/uuendused/3608-kehavaline-viljastamine-2013-2014

Riesewijk, A., Martin, J., van Os, R., Horcajadas, J.A., Polman, J., Pellicer, A., Mosselman, S., and Simon, C. (2003). Gene expression profiling of human endometrial receptivity on days LH+2 versus LH+7 by microarray technology. Molecular human reproduction *9*, 253-264.

Roberts, R.M., Liu, L., and Alexenko, A. (1997). New and atypical families of type I interferons in mammals: comparative functions, structures, and evolutionary relationships. Progress in nucleic acid research and molecular biology *56*, 287-325.

Rohde, L.H., and Carson, D.D. (1993). Heparin-like glycosaminoglycans participate in binding of a human trophoblastic cell line (JAR) to a human uterine epithelial cell line (RL95). J Cell Physiol *155*, 185-196.

Rosenfeld, C.S., Han, C.S., Alexenko, A.P., Spencer, T.E., and Roberts, R.M. (2002). Expression of interferon receptor subunits, IFNAR1 and IFNAR2, in the ovine uterus. Biol Reprod *67*, 847-853.

Roy, D., Cai, Q., Felty, Q., and Narayan, S. (2007). Estrogen-induced generation of reactive oxygen and nitrogen species, gene damage, and estrogen-dependent cancers. J Toxicol Environ Health B Crit Rev *10*, 235-257.

Ruiz-Alonso, M., Blesa, D., Diaz-Gimeno, P., Gomez, E., Fernandez-Sanchez, M., Carranza, F., Carrera, J., Vilella, F., Pellicer, A., and Simon, C. (2013). The endometrial receptivity array for diagnosis and personalized embryo transfer as a treatment for patients with repeated implantation failure. Fertil Steril *100*, 818-824.

Russell, R., Gori, I., Pellegrini, C., Kumar, R., Achtari, C., and Canny, G.O. (2011). Lipoxin A4 is a novel estrogen receptor modulator. FASEB J *25*, 4326-4337.

Ryan, I.P., Schriock, E.D., and Taylor, R.N. (1994). Isolation, characterization, and comparison of human endometrial and endometriosis cells in vitro. J Clin Endocrinol Metab *78*, 642-649.

Samalecos, A., and Gellersen, B. (2008). Systematic expression analysis and antibody screening do not support the existence of naturally occurring progesterone receptor (PR)-C, PR-M, or other truncated PR isoforms. Endocrinology *149*, 5872-5887.

Sandalinas, M., Sadowy, S., Alikani, M., Calderon, G., Cohen, J., and Munne, S. (2001). Developmental ability of chromosomally abnormal human embryos to develop to the blastocyst stage. Hum Reprod *16*, 1954-1958.

Saner, K.J., Welter, B.H., Zhang, F., Hansen, E., Dupont, B., Wei, Y., and Price, T.M. (2003). Cloning and expression of a novel, truncated, progesterone receptor. Mol Cell Endocrinol *200*, 155-163.

Santulli, P., Borghese, B., Noel, J.C., Fayt, I., Anaf, V., de Ziegler, D., Batteux, F., Vaiman, D., and Chapron, C. (2014). Hormonal therapy deregulates prostaglandin-endoperoxidase synthase 2 (PTGS2) expression in endometriotic tissues. J Clin Endocrinol Metab *99*, 881-890.

Scarpin, K.M., Graham, J.D., Mote, P.A., and Clarke, C.L. (2009). Progesterone action in human tissues: regulation by progesterone receptor (PR) isoform expression, nuclear positioning and coregulator expression. Nucl Recept Signal *7*, e009.

Schauer, M., Chalepakis, G., Willmann, T., and Beato, M. (1989). Binding of hormone accelerates the kinetics of glucocorticoid and progesterone receptor binding to DNA. Proc Natl Acad Sci U S A *86*, 1123-1127.

Schilling, B., and Yeh, J. (2000). Transforming growth factor-beta(1), -beta(2), -beta(3) and their type I and II receptors in human term placenta. Gynecol Obstet Invest *50*, 19-23.

Scholz, A., Truss, M., and Beato, M. (1998). Hormone-induced recruitment of Sp1 mediates estrogen activation of the rabbit uteroglobin gene in endometrial epithelium. J Biol Chem *273*, 4360-4366.

Schumacher, M., Guennoun, R., Stein, D.G., and De Nicola, A.F. (2007). Progesterone: therapeutic opportunities for neuroprotection and myelin repair. Pharmacol Ther *116*, 77-106.

Seifert-Klauss, V., and Prior, J.C. (2010). Progesterone and bone: actions promoting bone health in women. J Osteoporos *2010*, 845180.

Seifert-Klauss, V., Schmidmayr, M., Hobmaier, E., and Wimmer, T. (2012). Progesterone and bone: a closer link than previously realized. Climacteric *15 Suppl 1*, 26-31.

Shapiro, B.S., Daneshmand, S.T., Garner, F.C., Aguirre, M., Hudson, C., and Thomas, S. (2011). Evidence of impaired endometrial receptivity after ovarian stimulation for in vitro fertilization: a prospective randomized trial comparing fresh and frozen-thawed embryo transfer in normal responders. Fertil Steril *96*, 344-348.

Sher, G., and Fisch, J.D. (2002). Effect of vaginal sildenafil on the outcome of in vitro fertilization (IVF) after multiple IVF failures attributed to poor endometrial development. Fertil Steril *78*, 1073-1076.

Simon, C. (2008). New perspectives in medically assisted procreation. Gynecol Endocrinol *24*, 485.

Smith, C.L., Htun, H., Wolford, R.G., and Hager, G.L. (1997). Differential activity of progesterone and glucocorticoid receptors on mouse mammary tumor virus templates differing in chromatin structure. J Biol Chem *272*, 14227-14235.

Smitz, J., Bourgain, C., Van Waesberghe, L., Camus, M., Devroey, P., and Van Steirteghem, A.C. (1993). A prospective randomized study on oestradiol valerate supplementation in addition to intravaginal micronized progesterone in buserelin and HMG induced superovulation. Hum Reprod *8*, 40-45.

Smitz, J., Devroey, P., Faguer, B., Bourgain, C., Camus, M., and Van Steirteghem, A.C. (1992). A prospective randomized comparison of intramuscular or intravaginal natural progesterone as a luteal phase and early pregnancy supplement. Hum Reprod *7*, 168-175.

Spencer, T.E., Graf, G.H., and Bazer, F.W. (1995). Sulfated glycoprotein-1 (SGP-1) expression in ovine endometrium during the oestrous cycle and early pregnancy. Reprod Fertil Dev *7*, 1053-1060.

Stavreus-Evers, A., Aghajanova, L., Brismar, H., Eriksson, H., Landgren, B.M., and Hovatta, O. (2002). Co-existence of heparin-binding epidermal growth factor-like growth factor and pinopodes in human endometrium at the time of implantation. Mol Hum Reprod *8*, 765-769.

Stavreus-Evers, A., Nikas, G., Sahlin, L., Eriksson, H., and Landgren, B.M. (2001). Formation of pinopodes in human endometrium is associated with the concentrations of progesterone and progesterone receptors. Fertil Steril *76*, 782-791.

Steck, T., Giess, R., Suetterlin, M.W., Bolland, M., Wiest, S., Poehls, U.G., and Dietl, J. (2004). Leukaemia inhibitory factor (LIF) gene mutations in women with unexplained infertility and recurrent failure of implantation after IVF and embryo transfer. Eur J Obstet Gynecol Reprod Biol *112*, 69-73.

Steptoe, P.C., and Edwards, R.G. (1978). Birth after the reimplantation of a human embryo. Lancet *2*, 366.

Stewart, C.L. (1994). The role of leukemia inhibitory factor (LIF) and other cytokines in regulating implantation in mammals. Ann N Y Acad Sci *734*, 157-165.

Stilley, J.A., Christensen, D.E., Dahlem, K.B., Guan, R., Santillan, D.A., England, S.K., Al-Hendy, A., Kirby, P.A., and Segaloff, D.L. (2014). FSH receptor (FSHR) expression in human extragonadal reproductive tissues and the developing placenta, and the impact of its deletion on pregnancy in mice. Biol Reprod *91*, 74.

Zegers-Hochschild, F., Adamson, G.D., de Mouzon, J., Ishihara, O., Mansour, R., Nygren, K., Sullivan, E., Vanderpoel, S., International Committee for Monitoring Assisted Reproductive, T., and World Health, O. (2009). International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of ART terminology, 2009. Fertil Steril *92*, 1520-1524.

Zhang, X., Bocca, S., Franchi, A., Anderson, S., Kaur, M., Bajic, V.B., and Oehninger, S. (2010). Do GnRH analogues directly affect human endometrial epithelial cell gene expression? Mol Hum Reprod *16*, 347-360.

Zhao, C., Matthews, J., Tujague, M., Wan, J., Strom, A., Toresson, G., Lam, E.W., Cheng, G., Gustafsson, J.A., and Dahlman-Wright, K. (2007). Estrogen receptor beta2 negatively regulates the transactivation of

estrogen receptor alpha in human breast cancer cells. Cancer Res *67*, 3955-3962.
Zhao, Y., Zacur, H., Cheadle, C., Ning, N., Fan, J., and Vlahos, N.F. (2012). Effect of luteal-phase support on endometrial microRNA expression following controlled ovarian stimulation. Reprod Biol Endocrinol *10*, 72.
Zhou, J., Dsupin, B.A., Giudice, L.C., and Bondy, C.A. (1994). Insulin-like growth factor system gene expression in human endometrium during the menstrual cycle. J Clin Endocrinol Metab *79*, 1723-1734.
Zhu, Y., Rice, C.D., Pang, Y., Pace, M., and Thomas, P. (2003). Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc Natl Acad Sci U S A *100*, 2231-2236.
Tabibzadeh, S. (1998). Molecular control of the implantation window. Hum Reprod Update *4*, 465-471.
Takimoto, G.S., Tung, L., Abdel-Hafiz, H., Abel, M.G., Sartorius, C.A., Richer, J.K., Jacobsen, B.M., Bain, D.L., and Horwitz, K.B. (2003). Functional properties of the N-terminal region of progesterone receptors and their mechanistic relationship to structure. J Steroid Biochem Mol Biol *85*, 209-219.
Talbi, S., Hamilton, A.E., Vo, K.C., Tulac, S., Overgaard, M.T., Dosiou, C., Le Shay, N., Nezhat, C.N., Kempson, R., Lessey, B.A.*, et al.* (2006). Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. Endocrinology *147*, 1097-1121.
Tamada, H., Das, S.K., Andrews, G.K., and Dey, S.K. (1991). Cell-type-specific expression of transforming growth factor-alpha in the mouse uterus during the peri-implantation period. Biol Reprod *45*, 365-372.
Tamm-Rosenstein, K., Simm, J., Suhorutshenko, M., Salumets, A., and Metsis, M. (2013). Changes in the transcriptome of the human endometrial Ishikawa cancer cell line induced by estrogen, progesterone, tamoxifen, and mifepristone (RU486) as detected by RNA-sequencing. PLoS One *8*, e68907.
Tamm, K., Room, M., Salumets, A., and Metsis, M. (2009). Genes targeted by the estrogen and progesterone receptors in the human endometrial cell lines HEC1A and RL95-2. Reprod Biol Endocrinol *7*, 150.
Tan, B.K., Vandekerckhove, P., Kennedy, R., and Keay, S.D. (2005). Investigation and current management of recurrent IVF treatment failure in the UK. BJOG *112*, 773-780.
Tan, O., Ornek, T., Seval, Y., Sati, L., and Arici, A. (2008). Tenascin is highly expressed in endometriosis and its expression is upregulated by estrogen. Fertil Steril *89*, 1082-1089.
Tang, Y.T., Hu, T., Arterburn, M., Boyle, B., Bright, J.M., Emtage, P.C., and Funk, W.D. (2005). PAQR proteins: a novel membrane receptor family

defined by an ancient 7-transmembrane pass motif. J Mol Evol *61*, 372-380.
Tarlatzis, B.C., Toncheva, D.I., and Vatev, I.T. (2000). Significance of chromosomal aberrations for the unsuccessful procedures of assisted reproduction. Eur J Obstet Gynecol Reprod Biol *88*, 181-187.
Tesarik, J., and Mendoza, C. (1993). Insights into the function of a sperm-surface progesterone receptor: evidence of ligand-induced receptor aggregation and the implication of proteolysis. Exp Cell Res *205*, 111-117.
Thie, M., Harrach-Ruprecht, B., Sauer, H., Fuchs, P., Albers, A., and Denker, H.W. (1995). Cell adhesion to the apical pole of epithelium: a function of cell polarity. Eur J Cell Biol *66*, 180-191.
Thomas, P., Pang, Y., Dong, J., Groenen, P., Kelder, J., de Vlieg, J., Zhu, Y., and Tubbs, C. (2007). Steroid and G protein binding characteristics of the seatrout and human progestin membrane receptor alpha subtypes and their evolutionary origins. Endocrinology *148*, 705-718.
Thomas, P., Pang, Y., Filardo, E.J., and Dong, J. (2005). Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology *146*, 624-632.
Tierney, E.P., Tulac, S., Huang, S.T., and Giudice, L.C. (2003). Activation of the protein kinase A pathway in human endometrial stromal cells reveals sequential categorical gene regulation. Physiol Genomics *16*, 47-66.
Tokumoto, M., Nagahama, Y., Thomas, P., and Tokumoto, T. (2006). Cloning and identification of a membrane progestin receptor in goldfish ovaries and evidence it is an intermediary in oocyte meiotic maturation. Gen Comp Endocrinol *145*, 101-108.
Toran-Allerand, C.D., Guan, X., MacLusky, N.J., Horvath, T.L., Diano, S., Singh, M., Connolly, E.S., Jr., Nethrapalli, I.S., and Tinnikov, A.A. (2002). ER-X: a novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury. J Neurosci *22*, 8391-8401.
Tourgeman, D.E., Slater, C.C., Stanczyk, F.Z., and Paulson, R.J. (2001). Endocrine and clinical effects of micronized estradiol administered vaginally or orally. Fertil Steril *75*, 200-202.
Tung, L., Mohamed, M.K., Hoeffler, J.P., Takimoto, G.S., and Horwitz, K.B. (1993). Antagonist-occupied human progesterone B-receptors activate transcription without binding to progesterone response elements and are dominantly inhibited by A-receptors. Mol Endocrinol *7*, 1256-1265.
Ubaldi, F., Rienzi, L., Baroni, E., Ferrero, S., Iacobelli, M., Minasi, M.G., Sapienza, F., Romano, S., Colasante, A., Litwicka, K.*, et al.* (2007). Hopes and facts about mild ovarian stimulation. Reprod Biomed Online *14*, 675-681.
Wakeling, A.E., and Bowler, J. (1991). Development of novel oestrogen-receptor antagonists. Biochem Soc Trans *19*, 899-901.

Walf, A.A., and Frye, C.A. (2010). Estradiol reduces anxiety- and depression-like behavior of aged female mice. Physiology & behavior *99*, 169-174.
World Cancer Research Fund International: www.wcrf.org/
van der Linden, M., Buckingham, K., Farquhar, C., Kremer, J.A., and Metwally, M. (2015). Luteal phase support for assisted reproduction cycles. Cochrane Database Syst Rev *7*, CD009154.
van Mourik, M.S., Macklon, N.S., and Heijnen, C.J. (2009). Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. J Leukoc Biol *85*, 4-19.
Wang, H., Critchley, H.O., Kelly, R.W., Shen, D., and Baird, D.T. (1998). Progesterone receptor subtype B is differentially regulated in human endometrial stroma. Mol Hum Reprod *4*, 407-412.
Wang, Z., Zhang, X., Shen, P., Loggie, B.W., Chang, Y., and Deuel, T.F. (2005). Identification, cloning, and expression of human estrogen receptor-alpha36, a novel variant of human estrogen receptor-alpha66. Biochem Biophys Res Commun *336*, 1023-1027.
Vanneste, E., Voet, T., Le Caignec, C., Ampe, M., Konings, P., Melotte, C., Debrock, S., Amyere, M., Vikkula, M., Schuit, F.*, et al.* (2009). Chromosome instability is common in human cleavage-stage embryos. Nat Med *15*, 577-583.
Warner, M., and Gustafsson, J.A. (2006). Nongenomic effects of estrogen: why all the uncertainty? Steroids *71*, 91-95.
Way, D.L., Grosso, D.S., Davis, J.R., Surwit, E.A., and Christian, C.D. (1983). Characterization of a new human endometrial carcinoma (RL95-2) established in tissue culture. In Vitro *19*, 147-158.
Weckstein, L.N., Jacobson, A., Galen, D., Hampton, K., and Hammel, J. (1997). Low-dose aspirin for oocyte donation recipients with a thin endometrium: prospective, randomized study. Fertil Steril *68*, 927-930.
Vegeto, E., Shahbaz, M.M., Wen, D.X., Goldman, M.E., O'Malley, B.W., and McDonnell, D.P. (1993). Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function. Mol Endocrinol *7*, 1244-1255.
Wei, L.L., and Miner, R. (1994). Evidence for the existence of a third progesterone receptor protein in human breast cancer cell line T47D. Cancer Res *54*, 340-343.
Weiss, B.D. (1993). RU 486. The progesterone antagonist. Arch Fam Med *2*, 63-69; discussion 70.
Wen, D.X., Xu, Y.F., Mais, D.E., Goldman, M.E., and McDonnell, D.P. (1994). The A and B isoforms of the human progesterone receptor operate through distinct signaling pathways within target cells. Mol Cell Biol *14*, 8356-8364.
Venkatesh, T., Suresh, P.S., and Tsutsumi, R. (2014). New insights into the genetic basis of infertility. Appl Clin Genet *7*, 235-243.

Venken, K., Callewaert, F., Boonen, S., and Vanderschueren, D. (2008). Sex hormones, their receptors and bone health. Osteoporos Int *19*, 1517-1525.
White, C.A., Dimitriadis, E., Sharkey, A.M., and Salamonsen, L.A. (2005). Interleukin-11 inhibits expression of insulin-like growth factor binding protein-5 mRNA in decidualizing human endometrial stromal cells. Mol Hum Reprod *11*, 649-658.
White, P.C. (2009). Neonatal screening for congenital adrenal hyperplasia. Nat Rev Endocrinol *5*, 490-498.
Whitman, M., and Melton, D.A. (1989). Growth factors in early embryogenesis. Annual review of cell biology *5*, 93-117.
Wiegratz, I., and Thaler, C.J. (2011). Hormonal contraception--what kind, when, and for whom? Dtsch Arztebl Int *108*, 495-505; quiz 506.
Wolf, I.M., Heitzer, M.D., Grubisha, M., and DeFranco, D.B. (2008). Coactivators and nuclear receptor transactivation. J Cell Biochem *104*, 1580-1586.
Wong, Y.F., Cheung, T.H., Lo, K.W., Yim, S.F., Siu, N.S., Chan, S.C., Ho, T.W., Wong, K.W., Yu, M.Y., Wang, V.W.*, et al.* (2007). Identification of molecular markers and signaling pathway in endometrial cancer in Hong Kong Chinese women by genome-wide gene expression profiling. Oncogene *26*, 1971-1982.
Voullaire, L., Wilton, L., McBain, J., Callaghan, T., and Williamson, R. (2002). Chromosome abnormalities identified by comparative genomic hybridization in embryos from women with repeated implantation failure. Mol Hum Reprod *8*, 1035-1041.
Wu, J., Liang, Y., Nawaz, Z., and Hyder, S.M. (2005). Complex agonist-like properties of ICI 182,780 (Faslodex) in human breast cancer cells that predominantly express progesterone receptor-B: implications for treatment resistance. Int J Oncol *27*, 1647-1659.
Yamanaka, M., Taga, M., and Minaguchi, H. (1996). Immunohistological localization of tenascin in the human endometrium. Gynecol Obstet Invest *41*, 247-252.
Yeung, T.W., Chai, J., Li, R.H., Lee, V.C., Ho, P.C., and Ng, E.H. (2014). The effect of endometrial injury on ongoing pregnancy rate in unselected subfertile women undergoing in vitro fertilization: a randomized controlled trial. Hum Reprod *29*, 2474-2481.

ACKNOWLEDGEMENTS

During my PhD studies, I have met many inspiring people who have taught me the most interesting language – language of science. I am grateful to all my supervisors who have helped me to carry out this research. I appreciate Professor Andres Salumets for introducing me the world of ART and his guidance and support during the entire study. Professor Madis Metsis for his flying ideas at the time when new advanced sequencing technologies became available. I wish to thank Jaak Simm for the interesting discussions on the borderline of the biology and informatics and his contribution on bioinformatics analysis. Beside my advisors, I would like to thank Anu Aaspõllu for the support and encouragement.

This study has been part of the other research projects in Competence Centre on Health Technologies. In addition, I sincerely thank Institute of Gene Technology for the chance to finalize my studies.

I also wish to express my gratitude to all co-authors and colleagues for their participation and help on the publications included to this thesis. Especially Miia - my steroid sister, for her contribution and long lab hours during the first study; Marina for her technical support and positive attitude at the time of the second study; and Signe, for coordinating the third study from long distance, which resulted to be a very (re)productive. My big gratitude goes to Velve Kõiv for her punctuality and administrative help.

In addition, I would like to thank my fellow lab-mates Agne, Anne, Minna, Merilin, Ranno, Esta, Tiina, Peeter and others for the interesting discussions and positive lab atmosphere.

As an embryologist I have been very lucky to see the beginning of the life under the microscope. I would like to thank all my colleagues from Nova Vita Clinic, CRM London and Create Health. Especially my IVF supervisors Kaie, Fatima and Rebecca for teaching me “baby techniques”. My sincere thanks go to doctors I have been proud to work with: Dr Elle Talving, Dr Peeter Karits, Dr Robert Forman and Dr Piotr Pierzynski.

Finally, my biggest gratitude goes to my family and husband Margus for their constant support and belief in me. Undoubtedly, the most important mentors in my life are my children, Marii and Markus, who teach me every day how not to lose the curiosity and help me to see miracles in little things.

PUBLICATION I

I **Tamm K***, Rõõm M*, Salumets A, Metsis M. (2009). Genes targeted by the estrogen and progesterone receptors in the human endometrial cell lines HEC1A and RL95-2. Reproductive biology and endocrinology, 7 (170), 1-12.

Reproductive Biology and Endocrinology

BioMed Central

Research

Open Access

Genes targeted by the estrogen and progesterone receptors in the human endometrial cell lines HEC1A and RL95-2

Karin Tamm*†[1,2], Miia Rõõm†[1], Andres Salumets[2,3,4,5] and Madis Metsis[1,5]

Address: [1]Centre for Biology of Integrated Systems, Tallinn University of Technology, Tallinn, Estonia, [2]Nova Vita Clinic, Centre for infertility treatment and medical genetics, Tallinn, Estonia, [3]Department of Obstetrics and Gynecology, University of Tartu, Tartu, Estonia, [4]Department of Biotechnology, Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia and [5]Competence Centre on Reproductive Medicine and Biology, Tallinn, Estonia

Email: Karin Tamm* - karin.tamm@mail.ee; Miia Rõõm - miia.room@ttu.ee; Andres Salumets - asalumets@novavita.ee; Madis Metsis - madis.metsis@ttu.ee

* Corresponding author †Equal contributors

Published: 24 December 2009

Reproductive Biology and Endocrinology 2009, **7**:150 doi:10.1186/1477-7827-7-150

Received: 9 October 2009
Accepted: 24 December 2009

This article is available from: http://www.rbej.com/content/7/1/150

Abstract

Background: When the steroid hormones estrogen and progesterone bind to nuclear receptors, they have transcriptional impact on target genes in the human endometrium. These transcriptional changes have a critical function in preparing the endometrium for embryo implantation.

Methods: 382 genes were selected, differentially expressed in the receptive endometrium, to study their responsiveness of estrogen and progesterone. The endometrial cell lines HEC1A and RL95-2 were used as experimental models for the non-receptive and receptive endometrium, respectively. Putative targets for activated steroid hormone receptors were investigated by chromatin immunoprecipitation (ChIP) using receptor-specific antibodies. Promoter occupancy of the selected genes by steroid receptors was detected in ChIP-purified DNA by quantitative PCR (qPCR). Expression analysis by reverse transcriptase (RT)-PCR was used to further investigate hormone dependent mRNA expression regulation of a subset of genes.

Results: ChIP-qPCR analysis demonstrated that each steroid hormone receptor had distinct group of target genes in the endometrial cell lines. After estradiol treatment, expression of estrogen receptor target genes predominated in HEC1A cells (n = 137) compared to RL95-2 cells (n = 35). In contrast, expression of progesterone receptor target genes was higher in RL95-2 cells (n = 83) than in HEC1A cells (n = 7) after progesterone treatment. RT-PCR analysis of 20 genes demonstrated transcriptional changes after estradiol or progesterone treatment of the cell lines.

Conclusions: Combined results from ChIP-qPCR and RT-PCR analysis showed different patterns of steroid hormone receptor occupancy at target genes, corresponding to activation or suppression of gene expression after hormone treatment of HEC1A and RL95-2 cell lines.

Background

The human endometrium is a dynamic tissue that undergoes cyclic changes in preparation for endometrial receptivity and embryo implantation. Endometrial development consists of proliferative and secretory phases, and the two major regulators of this process are the ovarian steroid hormones 17β-estradiol (E2) and progesterone (P4). In the proliferative phase, estrogens stim-

ulate the proliferation of the epithelial and stromal components of the endometrium, while in the secretory phase P4 is involved in glandular differentiation and inhibition of E2-mediated cell proliferation [1]. In the absence of implantation, declining levels of P4 and E2 signal the degeneration of the endometrial tissue, which is followed by regeneration during the next cycle.

The biological activities of E2 and P4 are mediated mainly by nuclear receptors (NRs). Binding of a steroid hormone to its cognate receptor results in a conformational change in the NR that allows the ligand-NR complex to bind with high affinity to response elements in DNA and regulate transcription of target genes. Two types of E2 receptors, ERα and ERβ, encoded by separate genes, are found in humans [2,3]. Although ERα and ERβ are present in all endometrial cell types over the entire menstrual cycle, they are expressed at higher levels during the proliferative phase and show lower activity during the secretory phase because of the suppressive effect of P4 [4].

P4 signalling is also mediated by two receptors, PRA and PRB [5], which are encoded by the same gene but transcribed from different promoters, resulting in a PRB that has an additional 164 amino acids at the N-terminus [6]. PRB is a stronger transcriptional activator in most cell types, while PRA acts often as a dominant negative repressor for PRB activity [7,8]. PRA and PRB levels are similar during the proliferative phase. In the early secretory phase, PRA is dominant, while higher PRB levels during the mid-secretory phase have been described [9]. The expression of the PR gene in endometrial glands is controlled by E2 and P4, where E2 induces PR synthesis and P4 down-regulates the expression of its own receptor [1].

Implantation of the developing embryo involves a molecular dialogue between the endometrium and blastocyst that involves a number of specific mediators including membrane receptors, components of the extra-cellular matrix, growth factors, cytokines and lipid components of the cell membranes [10]. The endometrium is receptive for embryo attachment only during a restricted period called the "implantation window" (IW). In humans, the IW is limited to days 20-24 of the menstrual cycle and is achieved through the coordinated action of P4 and E2. Thus, an imbalance of steroid hormone levels and their ratios could influence the regulation of target genes, leading to female infertility by disturbing endometrial receptivity during the IW.

Microarray technology has led to genome-wide identification of gene expression pathways involved in implantation events. Based on five transcriptome studies [11-15], we selected 382 genes with different expression levels during the IW in human endometrium. The aim of this study was to investigate whether these pre-selected genes could be directly regulated by E2 and P4 through their specific receptors. To achieve the purpose, to study hormone dependent receptivity of the endometrium, we used two human uterine epithelial cell lines as *in vitro* models.

HEC1A was used as a model of non-receptive endometrium, and RL95-2 was used as a model of receptive endometrium [16-19]. The cell lines were chosen based on earlier studies which have demonstrated that RL95-2 cells have stronger adhesiveness for human JAR choriocarcinoma multicellular spheroids compared to HEC1A cells [20-22] and are thus considered as a model of the receptive endometrium. Following hormone treatment of the cells, binding of steroid hormone receptors to the promoters of selected genes was investigated by chromatin immunoprecipitation (ChIP) with specific antibodies, and detection of the isolated genomic sequences with quantitative PCR (qPCR). E2- and P4-dependent gene regulation was confirmed for 20 genes by reverse transcriptase (RT)-PCR.

Methods

Endometrial cell culture

HEC1A (#HTB-112, American Type Culture Collection, ATCC, Teddington, UK) cells were grown in McCoy 5A medium (#A1324, AppliChem GmbH, Darmstadt, Germany), while RL95-2 (#CRL-1671, ATCC) cells were maintained in DMEM/F12 (AppliChem GmbH) medium with 10 mM Hepes and 5 μl/ml insulin. Both media were supplemented with 2 g/L sodium bicarbonate (AppliChem GmbH), 10% fetal bovine serum (FBS) (AppliChem GmbH) and 1% penicillin/streptomycin (AppliChem GmbH). Both studied cell lines express E2 and P4 specific nuclear receptors (ERα, ERβ, PRA, PRB) as indicated by the ATCC. For hormonal treatment, E2 (Sigma-Aldrich, Helsinki, Finland) or P4 (4-Pregnene-3,20-dione, Sigma-Aldrich) was added to the culture media to a final concentration of 10^{-8}M as described before [23,24]. For cultures with hormone supplements, dextran-coated charcoal-treated FBS and media without phenol red were used for 48 h prior to experiments to adapt similar conditions and avoid possible hormone-like (estrogenic) activity of Phenol red. Hormone treatment was 45 min for ChIP experiments and 3 h, 6 h or 12 h for mRNA experiments.

Chromatin immunoprecipitation

Chromatin was immunoprecipitated as previously described [25], using following antibodies: monoclonal mouse anti-human ERα antibody (D-12, sc-8005); polyclonal rabbit anti-human ERβ antibody (H-150, sc-8974); monoclonal mouse anti-human PRAB antibody (AB-52, sc-810) that recognizes both human PRA and PRB receptors (AB-52, sc-810); and monoclonal mouse anti-human

PRB antibody (B-30, sc-811) that recognizes only the additional NH2-terminal stretch of PRB receptor (Santa Cruz Biotech, CA, USA). Preliminary experiments determined an optimal formaldehyde cross-linking time of 15 min for both cell lines. Chromatin was fragmented to an average size of 0.5 - 2 kb using a Vibra-Cell ultrasonic processor (Sonics, Newtown, CT USA). Antibody-antigen complexes were precipitated with GammaBind™ plus Sepharose™ (GE Healthcare Life Sciences, Uppsala, Sweden), eluted with hot 0.1% SDS and uncrosslinked overnight at 65°C in the presence of Protein K (Sigma-Aldrich). DNA was purified using a Gel Extraction Kit (Qiagen, Helsinki, Finland). Control experiments used preimmune total IgG (Cell Signalling Technology, Inc, Danvers, MA, USA) to estimate the non-specific binding by unspecific antibodies.

ChIP-qPCR for detection of genomic sequences

Specific genomic regions in the ChIP DNA samples were detected by qPCR. The 382 genomic targets for PCR were selected from previously published endometrial tissue microarray data (See additional file 1) [11-15] using two criteria: (i) ≥ 2-fold up- or down-regulated mRNA expression during IW and (ii) evolutionary conservation of human and murine orthologous sequences. Primers were designed to detect and amplify a region from +1000 to -5000 bp from the transcription start site using the Primer3 program [26] detecting NR involvement in formation of the basal transcription complex. All primers were designed to have a melting temperature (Tm) of 60°C. Primer specificity was controlled using the alignment algorithms BLAT/iPCR [27] and BLAST [28] to search the whole human genome. Primers were obtained from MWG Biotech (Edsberg, Germany). PCR was performed using HotStarTaq Master Mix (Qiagen) with 0.05 ng of template DNA and 1.25 pmol of primers for each reaction, according to the manufacturer's instructions.

PCR products were detected by qPCR using 384-well plates and a SYBR green detection method with an ABI HT7900 RT-PCR machine (Applied Biosystems, Foster City, CA, USA). PCR conditions were: HotStarTaq DNA Polymerase activation step for 14.5 min at 95°C, 40 cycles of denaturation for 30 s at 95°C, annealing for 45 s at 58°C and extension for 45 s at 72°C. A dissociation step was added to confirm the purity of PCR products by melting-curve analysis with 5 min ramping from 60°C to 95°C.

RT-PCR

RNA was extracted using an RNeasy Mini Kit (Qiagen). Cells were washed once with phosphate-buffered saline prior to extraction, lysed directly in the tissue culture dish and homogenized by 10 strokes with an insulin syringe. RNA was prepared according to the manufacturer's protocol. Genes (n = 20, *ADAMDEC1*, *CD86*, *ETV1*, *FLT1*, *FOXA2*, *GRIP1*, *HES1*, *HOXA1*, *IHH*, *KLK3*, *MEF2D*, *MMP7*, *NCOA1*, *OTOF*, *PLXNA2*, *RELB*, *SMARCA2*, *TBX19*, *TNC*, and *ZNF54*) were chosen for RT-PCR analysis based on positive ChIP experiment results, focusing mostly on transcription factors (See additional file 2). For mRNA analysis, 5 μg of total RNA was subjected to reverse transcription, using SuperScript® III First-Strand Synthesis SuperMix (#18080-400, Invitrogen Life Technologies, Carlsbad, CA, USA), as described by the manufacturer. *GAPDH* specific primers were used to normalize the cDNA synthesis with forward and reverse primers CTCTCTGCTCCTCCTGTTCGAC and TGAGCGATGTGGCTCGGCT, respectively. cDNA specific primer pairs were designed using Primer3 software and tested for unspecific priming against human genomic and mRNA sequences using iPCR software (MWG Biotech). Primers were designed to generate amplicons of 75-150 bp with a Tm of 60°C. PCR conditions for reverse transcriptase products were as described above with the exception of a 15 s annealing step at 58°C, and a 30 s extension time to account for the shorter PCR products.

RT-PCR data analysis

Quantitative qPCR results were analysed using the publicly available software Miner [29]. Outliers were excluded manually when the mean coefficient of variation (CV) for CT (cycle threshold) for triplicates was >1%. Expression values for all transcripts were normalized to the endogenous control of *GAPDH*, and ratios relative to non-treated samples were generated. Relative gene expression ratios were calculated according to GED (Gene Expression Difference) by CT protocol [30], using the average efficiency of each gene and the CT of each triplicate. To compare the effects of hormone treatments on HEC1A and RL95-2 cell lines, statistical analysis was performed on log2-transformed values of gene expression ratios using an unpaired Student's t-test at a 95% confidence level.

Annotation of genes

Gene classifications were carried out using the PANTHER (Protein Analysis THrough Evolutionary Relationships), [31] classification system that specifies genes by functions based on published evidence and evolutionary relationships that predict gene function in the absence of direct experimental evidence. Clustering analysis was performed with the Cluster 3.0 program [32], using uncentered correlation and complete centroid linkage. Inverted CT (CTinv) values were used for clustering qPCR data. The baseline was set to 40 and CTinv was calculated as CTinv = 40-Ct. Visualisation of clustering data was done using Java TreeView software.

Gene annotation, sequence information, gene descriptions and accession numbers (IDs) were downloaded

from BioMart [33] NCBI [34] and UCSC genome browser [35] databases. All datasets were imported and kept in a database supported by MySQL database management software [36].

Results

E2- and P4-receptor target genes in HEC1A and RL95-2 cells

Two endometrial cell lines, HEC1A and RL95-2, were used to investigate the steroid hormone-dependent binding of NRs to the promoters of 382 IW-specific genes (See additional file 1). Although the native receptive endometrium is under the combined influence of both E2 and P4, we performed hormonal treatments separately to reveal E2 or P4 dependent action through their specific nuclear receptors. Cells were treated with either E2 or P4 followed by ChIP using antibodies against the steroid hormone receptors: ERα, ERβ, PRB, or PRAB (which recognizes both PRA and PRB receptors). Quantitative qPCR with specific primers was used to detect NR-binding to the promoter region of the selected genes. HEC1A cells were treated with E2 to simulate the non-receptive-like endometrium and with P4 for comparing P4 effects on the two endometrial cell lines. Out of 382 investigated genes, 137 were ER targets: 101 were bound by ERα, 96 were bound by ERβ, and 60 were targets of both receptors (Figure 1A). When the targets common to both ERs (n = 56) and the targets shared by ERs and PRs (n = 7) were excluded, 40 genes were found to be exclusively regulated by ERα and 34 genes by ERβ (Table 1).

The amino acid sequence of the progesterone receptor PRA is contained within the sequence of PRB, so producing antibodies specific to PRA is not feasible. Therefore, PRA target genes were found by subtracting anti-PRB targets from the common pool of all PRAB-targets as PRB and PRAB overlapping target genes were considered to be PRB-specific. Seven target genes for P4-mediated action in HEC1A cells were identified, 5 for PRA and 2 for PRB (Figure 1A). All PR targets overlapped with ER targets and thus they were not considered to be unique targets for P4 action in HEC1A cells (Table 2).

The RL95-2 cell line was used as a model for the receptive endometrium with E2 and P4 treatments performed separately. ChIP-qPCR analysis revealed four distinct groups of target genes for each steroid hormone receptor (ERα, ERβ, PRA, and PRB) in RL95-2 cells (Figure 1B). After P4 treatment, anti-PR antibodies recognized chromatin from 83 out of the 382 potential target genes; anti PRB for 40 and anti-PRAB for 52, including overlapping targets for two antibodies. After removing common targets between PRs and ERs and subtracting anti-PRB targets from the common pool of all PRAB-targets, 37 genes were considered to be unique targets for PRA and 8 for PRB (Table 3). Although PRAB antibody should detect both PRA and PRB receptor subtypes, 31 genes were targeted solely by PRB and not identified by PRAB antibody. Twenty-five of them were found to be PRB specific, while six were additionally co-regulated by at least one of the ERs. These 25 unique PRB targets were also regarded as PRB-specific, thus resulting in 33 unique PRB target genes (Table 3). E2 treatment of RL95-2 cells resulted in 35 genes bound by ERs, where in total 17 loci were immunoprecipitated by ERα and 24 by ERβ antibody (Figure 1B), including overlapping targets for two antibodies. After deducted common targets, we discovered nine unique target genes for ERα, eight for ERβ (Table 4), and five common targets for both ERs (data not shown).

The total number of genes targeted after E2 treatment of HEC1A and RL95-2 cells is shown in Figure 1. Approximately four times as many genes were bound by ERs in HEC1A cells than in RL95-2 cells, which had 137 and 35 target genes, respectively. These results confirmed that HEC1A is a better model of the non-receptive endometrium with E2 exerting its primary role in tissue proliferation. In contrast, P4 treatment resulted in 10 times more PR targets in RL95-2 cells (n = 83) than in HEC1A cells (n = 7), supporting the view of RL95-2 cell line as a model for the receptive endometrium with P4 preparing the endometrium for embryo nidation.

E2- and P4-mediated mRNA expression in HEC1A and RL95-2 cell lines

To elucidate the biological significance of the molecular interactions seen by ChIP, we complemented the initial results with mRNA expression studies for 20 genes (See additional file 2). All selected genes were detected as NR targets by ChIP-qPCR and half (n = 10) of them were classified as transcription factors. The other criteria after transcription factors was to find genes which has not been described as an ER or PR targets before.

HEC1A and RL95-2 cells were treated with E2 or P4 for 3, 6, or 12 h. At least 2-fold up- or down-regulation of mRNA expression levels were detected for 12 genes (*CD86*, *FOXA2*, *IHH*, *MEF2D*, *MMP7*, *NCOA1*, *OTOF*, *PLXNA2*, *RELB*, *SMARCA2*, *TNC*, and *ZNF549*) out of 20 genes studied (Table 5). Although, the activities of transcription factor coding genes *GRIP1* and *TBX19* changed significantly during the hormonal treatments, these alterations remained below the 2-fold threshold. cDNA amplification was not observed for 6 genes *ADAMDEC1*, *ETV1*, *FLT1*, *HES1*, *HOXA1* and *KLK3* in either cell line regardless of hormonal treatment (data not shown). Surprisingly, the observed changes in E2- or P4-dependent mRNA expression occasionally conflicted with the ChIP-qPCR results. For example, although the secreted growth factor Indian hedgehog (*IHH*) and otoferlin (*OTOF*) were

Table 1: List of unique target genes for ERα and ERβ in HEC1A cell line after E2 treatment

Gene symbol*	Gene name
Unique targets for ERα	
ABCB10	ATP-binding cassette, subfamily B, member 10
ADCYAP1	Adenylate cyclase-activating polypeptide 1
AGL	Amylo-1,6-glucosidase, 4-alpha- glucanotransferase
APRT	Adenine phosphoribosyltransferase
ARID5B	AT-rich interaction domain-containing protein 5B
C3orf1	Chromosome 3 open reading frame 1
CCL11	Chemokine, CC motif, ligand 11
CCL7	Chemokine, CC motif, ligand 7
CCT6B	Chaperonin-containing T-complex polypeptide 1, subunit 6B
CD86	Cd86 antigen
CEACAM3	Carcinoembryonic antigen-related cell adhesion molecule 3
CLDN4	Claudin 4
ECM1	Extracellular matrix protein 1
EDN3	Endothelin 3
FBLN2	Fibulin 2
FOSL1	FOS-like antigen 1
GRIK1	Glutamate receptor, ionotropic, kainate 1
GSN	Gelsolin
HOXA1	Homeobox A1
ITGB7	Integrin, beta-7
ITPA	Inosine triphosphatase
LGALS8	Lectin, galactoside-binding, soluble, 8
MB	Myoglobin
NEK6	Never in mitosis gene a-related kinase 6
NID2	Nidogen 2
ODF2	Outer dense fiber of sperm tails 2
POFUT1	Protein o-fucosyltransferase 1
PWP1	PWP1 homolog (S. Cerevisiae)
RELB	v-rel avian reticuloendotheliosis viral oncogene homolog b
SEC61B	SEC61 complex, beta subunit
SFRS16	Splicing factor, arginine/serine- rich 16
SGCB	Sarcoglycan, beta
SPARC	Secreted protein, acidic, cysteine-rich
SPATA2	Spermatogenesis-associated protein 2
SSFA2	Sperm-specific antigen 2
TFAP2C	Transcription factor AP2-gamma
TRH	Thyrotropin-releasing hormone deficiency
TRIM16	Tripartite motif-containing protein 16
WNT5A	Wingless-type MMTV integration site family, member 5A
XCL2	Chemokine, C motif, ligand 2
Unique targets for ERβ	
AQP3	Aquaporin 3
B3GAT3	Beta-1,3-glucuronyltransferase 3
BMP10	Bone morphogenetic protein 10
CLU	Clusterin
COG5	Component of oligomeric golgi complex 5
CRSP9	Mediator complex subunit 7
DMBT1	Deleted in malignant brain tumors 1
DNAH9	Dynein, axonemal, heavy chain 9
FECH	Protoporphyria, erythropoietic
FEN1	FLAP structure-specific endonuclease 1
FYB	FYN-binding protein
GBAS	Glioblastoma amplified sequence
GPX3	Glutathione peroxidase 3
GRIP1	Glutamate receptor-interacting protein 1
HBB	Hemoglobin--beta locus
KIAA0427	KIAA0427
LGTN	Ligatin
MEF2D	Myocyte enhancer factor 2D
NINJ1	Nerve injury-induced protein 1
PDGFA	Platelet-derived growth factor, alpha polypeptide
PENK	Proenkephalin
PLXNB3	Plexin B3
PRODH	Proline dehydrogenase
SC65	Synaptonemal complex protein SC65
SERPIND1	Heparin cofactor II
SLC16A4	Solute carrier family 16 (monocarboxylic acid transporter), member 4
TDG	Thymine-DNA glycosylase
TFDP1	Transcription factor DP1
TGM2	Transglutaminase 2
TNC	Tenascin C
TNFRSF10C	Tumor necrosis factor receptor superfamily, member 10c
TP63	Tumor protein p63
TRIP10	Thyroid hormone receptor interactor 10
WFDC2	WAP four-disulfide core domain 2

(*) Official gene symbol by HUGO Gene Nomenclature Committee (HGNC).

detected as E2-ER targets in HEC1A cells, E2-induced mRNA expression was observed in RL95-2 cells but not in HEC1A cells.

From the selected 20 genes five genes showed significant cell line-specific mRNA expression either in HEC1A or RL95-2 cell line (Table 5, in bold). *IHH* expression was detected in RL95-2 cell line-specific manner until 6 h after E2 treatment or 3 h after P4 treatment, but was down-regulated after longer hormonal exposure. In addition to *IHH*, the expression of three other genes: plexin A2 (*PLXNA2*), *OTOF* and a SWI/SNF related family member (*SMARCA2*) were induced by E2 or P4 in the RL95-2 cell line. In HEC1A cells, on the contrary, the expression of matrix metalloprotease 7 (*MMP7*) was detected in non-treated HEC1A cells and was gradually down-regulated after treatments by both hormones.

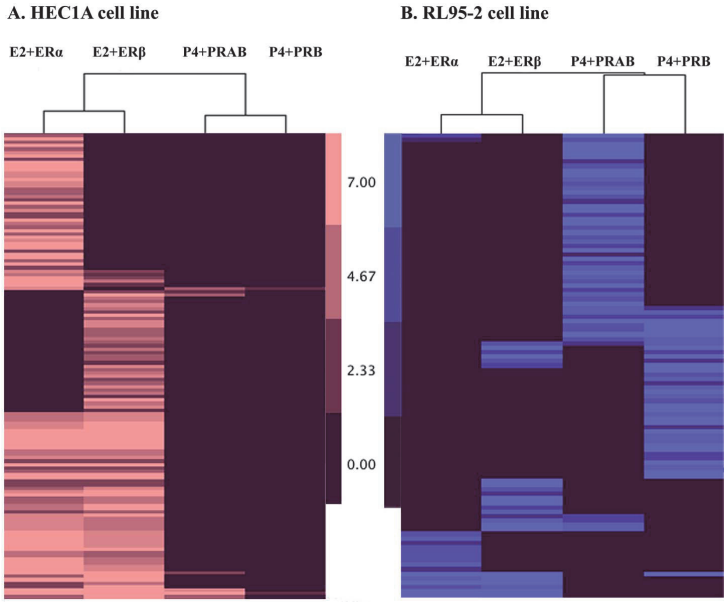

Figure 1
Cluster analysis of ERα, ERβ, PRAB, PRB target genes in HEC1A and RL95-2 cells after E2 or P4 treatment. Target genes revealed from ChIP-qPCR experiment were clustered with Cluster 3.0 program and visualized using Java TreeView software. A. HEC1A cell line. E2+ERα: 101 ERα target genes after E2 treatment; E2+ERβ: 96 ERβ target genes after E2 treatment; P4+PRAB: 7 PRAB target genes after P4 treatment; and P4+PRB: 2 PRB target genes after P4 treatment. B. RL95-2 cell line. E2+ERα: 17 ERα target genes after E2 treatment; E2+ERβ: 24. ERβ target genes after E2 treatment; P4+PRAB: 52 PRAB target genes after P4 treatment; and P4+PRB: 40 PRB target genes after P4 treatment.

Analysis of gene expression in HEC1A and RL95-2 cell lines showed almost opposite responses to E2 and P4. A significant difference ($p < 0.05$) in transcript levels was observed between the two cell lines in the expression of nine genes after E2 treatment (Figure 2). Furthermore, *CD86*, *FOXA2*, *GRIP1*, *NCOA1*, *RELB*, *TBX19*, *TNC*, and *ZNF549* genes showed opposite regulation in the two cell lines, with mRNA levels up-regulated in RL95-2 cells but down-regulated in HEC1A cells after E2 treatment when compared to non-treated samples (Figure 2). The expression of *MEF2D* was also significantly different between the cell lines, but unlike the other genes, it was up-regulated by E2 in both cell lines. P4 similarly exerted opposite effects on gene expression in the two endometrial cell lines as mRNA levels of the three transcription factor genes *FOXA2*, *NCOA1*, *TBX19*, and extracellular matrix tenascin-C (*TNC*) gene were significantly ($p < 0.05$, Figure 3) up-regulated in RL95-2 cells and down-regulated in HEC1A cells post P4 treatment.

Table 2: List of PRA and PRB target genes in HEC1A cell line after P4 treatment

Gene symbol*	Gene name
Targets for PRA	
CD28	CD28 molecule
COL5A2	Collagen, type V, alpha-2
ETS2	V-Ets avian erythroblastosis virus e26 oncogene homolog 2
MYST4	Histone acetyltransferase MYST4
OTOF	Otoferlin
Targets for PRB	
TCF4	Transcription factor 4
ZNF167	Zinc finger protein 167

(*) Official gene symbol by HUGO Gene Nomenclature Committee (HGNC).

Discussion

The exact molecular characteristics of the embryo impantation are still not completely characterised because of the complexity of using human embryos and endometrial tissue in reseach, therefore other means must be elucidated for research of receptivity of the endometrium. Used RL95-2 and HEC1A cell lines are both described as endometrial epithelial cell lines derived from adenocarcinoma cells. Our interest to investigate the suitability of

Table 3: List of unique target genes for PRA and PRB in RL95-2 cell line after P4 treatment

Gene symbol*	Gene name
Unique targets for PRA	
APRT	Adenine phosphoribosyltransferase
BNIP1	Bcl2/adenovirus E1B 19-kD protein-interacting protein 1
CCT6B	Chaperonin-containing T-complex polypeptide 1, subunit 6B
CLDN4	Claudin 4
COL4A6	Collagen, type IV, alpha-6
DNAJB1	DNAJ/HSP40 homolog, subfamily b, member 1
ECM1	Extracellular matrix protein 1
EXT1	Exostosin 1
EXTL2	Exostosin-like 2
FGF9	Fibroblast growth factor 9
FLT1	Fms-related tyrosine kinase 1
GPLD1	Phospholipase D1, glycosylphosphatidylinositol-specific
IFNAR2	Interferon, alpha, beta, and omega, receptor 2
ITGA10	Integrin, alpha-10
ITGA2	Integrin, alpha-2
ITGB7	Integrin, beta-7
KIAA0427	KIAA0427
LAMB1	Laminin, beta-1
MAOA	Monoamine oxidase A
MB	Myoglobin
MYST4	Histone acetyltransferase MYST4
NCOA1	Nuclear receptor coactivator 1
NCOR2	Nuclear receptor corepressor 2
NUP155	Nucleoporin, 155-kD
PDGFA	Platelet-derived growth factor, alpha polypeptide
SLC29A2	Solute carrier family 29 (nucleoside transporter), member 2
SOX4	SRY-box 4
TGFA	Transforming growth factor, alpha
TIAL1	Tia1 cytotoxic granule-associated rna-binding protein-like 1
TNC	Tenascin C
TRIP10	Thyroid hormone receptor interactor 10
TRMT11	tRNA methyltransferase 11 homolog (S. Cerevisiae)
UBE3C	Ubiquitin protein ligase E3C
UBTF	Upstream binding transcription factor (RNA polymerase I)
VEGFA	Vascular endothelial growth factor A
XCL2	Chemokine, C motif, ligand 2
ZNF549	Zinc finger protein 549
Unique targets for PRB	
ARID5B	AT rich interactive domain 5B (MRF1-like)
ARL1	ADP-ribosylation factor-like 1
BRCA2	Breast cancer 2
C2orf3	Chromosome 2 open reading frame 3
CORT	Cortistatin
CROT	Carnitine O-octanoyltransferase
EIF3I	Eukaryotic translation initiation factor 3, subunit I
ETV1	ETS variant gene 1
FGF12	Fibroblast growth factor 12
GADD45A	Growth arrest- and DNA damage-inducible gene GADD45, alpha
GATA2	GATA-binding protein 2
GTF2F2	General transcription factor IIf, polypeptide 2, 30-kD
HES1	Hairy and enhancer of split 1 homolog
IHH	Indian hedgehog
KLK3	Kallikrein-related peptidase 3
MEF2D	Myocyte enhancer factor 2D
NCR3	Natural cytotoxicity triggering receptor 3
NF1	Neurofibromatosis, type 1
NFIX	Nuclear factor I/X (CCAAT-binding transcription factor)
NUP98	Nucleoporin, 98-kD
POSTN	Periostin
PWP1	PWP1 homolog (S. Cerevisiae)
RAD54L	Rad54, s. Cerevisiae, homolog-like
RNF126	Ring finger protein 126
SEMA3F	Semaphorin 3F
SNTG1	Syntrophin, gamma-1
SPATA2	Spermatogenesis-associated protein 2
SPDEF	SAM pointed domain containing ets transcription factor
STC1	Stanniocalcin 1
STIM1	Stromal interaction molecule 1
TDRKH	Tudor and KH domains-containing protein
TLX2	T-cell leukemia, homeobox 2
UMOD	Uromodulin

(*) Official gene symbol by HUGO Gene Nomenclature Committee (HGNC).

selected cell lines as an *in vitro* models of non-receptive and receptive endometrium was based on several studies published recently [21,22,37,38]. RL95-2 cell line has been characterized as a model of receptive endometrium by its ability to mimic relevant properties of the adhesion competent endometrial lining compared to HEC1A [21,22].

The steroid hormones E2 and P4 bind to NRs to play a key role in preparing the endometrium for implantation of an embryo. NRs regulate transcription by binding to the proximal or distal regulatory regions of specific genes. Transcriptional changes regulated by NRs have been suggested to involve chromosomal looping or PolII tracking that allows enhancers to interact with the transcription complex at the promoter [39]. In this study, we used ChIP-qPCR method to investigate whether genes previously identified as IW-specific become direct targets of NRs after hormonal treatment of endometrial cells.

Combining new technologies based on microarrays and high-throughput sequencing with the today's knowledge of entire human genome allows defining of all *in vivo* targets for transcription machinery in a single experiment

Table 4: List of unique target genes for ERα and ERβ in RL95-2 cell line after E2 treatment

Gene symbol*	Gene name	Unique targets for ERβ	
Unique targets for ERα			
ANXA2	Annexin A2	ETS2	v-ets avian erythroblastosis virus e26 oncogene homolog 2
CHRNB2	Cholinergic receptor, neuronal nicotinic, beta polypeptide 2	*FOXA2*	Forkhead box A2
COL3A1	Collagen, type III, alpha-1	*MMP26*	Matrix metalloproteinase 26
GSN	Gelsolin	*NINJ1*	Nerve injury-induced protein 1
RPS6KB2	Ribosomal protein S6 kinase, 70-kD, 2	*SERPIND1*	Heparin cofactor II
SECTM1	Secreted and transmembrane 1	*SFRS16*	Splicing factor, arginine/serine-rich 16
SMARCA2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 2	*TBX19*	T-box 19
SC65	Synaptonemal complex protein SC65	*VDAC1*	Voltage-dependent anion channel 1
VLDLR	Very low density lipoprotein receptor		

(*) Official gene symbol by HUGO Gene Nomenclature Committee (HGNC).

Table 5: Genes > 2 fold up- or down-regulated after hormone treatment in HEC1A and/or RL95-2 cell line

Up-regulation > 2 fold			ChIP target post E2 treatment		ChIP target post P4 treatment
HEC1A	Molecular function*	E2		P4	
MEF2D	Other transcription factor; Nucleic acid binding	6 h	HEC1A ERβ	3 h	RL95-2 PRB
RL95-2		E2		P4	
CD86	Immunoglobulin receptor family member; Membrane-bound signaling molecule; Defense/immunity protein	3 h	HEC1A ERα	NS	ND
MEF2D	Other transcription factor; Nucleic acid binding	3 h	HEC1A ERβ	3 h	RL95-2 PRB
OTOF	Otoferlin	12 h	HEC1A ERα and ERβ	3 h	HEC1A PRA
PLXNA2	Tyrosine protein kinase receptor;Protein kinase	3 h	RL95-2 ERβ	3 h	RL95-2 PRA
RELB	Other transcription factor	3 h	HEC1A ERα	NS	ND
SMARCA2	DNA helicase	3 h	RL95-2 ERα	3 h	ND
TNC	Cell adhesion molecule; Extracellular matrix glycoprotein	3 h	HEC1A ERβ	3 h	RL95-2 PRA
Down-regulation > 2 fold					
HEC1A	Molecular function*	E2		P4	
CD86	Immunoglobulin receptor family member; Membrane-bound signaling molecule; Defense/immunity protein	6 h	HEC1A ERα	NS	ND
FOXA2	Other transcription factor; Nucleic acid binding	3 h	RL95-2 ERβ	12 h	ND
MMP7	Metalloprotease; Other extracellular matrix	12 h	HEC1A ERα and ERβ	12 h	ND
NCOA1	Transcription factor; Acetyltransferα se	3 h	ND	6 h	RL95-2 PRA
RELB	Other transcription factor	3 h	HEC1A ERα	6 h	ND
TNC	Cell adhesion molecule; Extracellular matrix glycoprotein	3 h	HEC1A ERβ	6 h	RL95-2 PRA
ZNF549	KRAB box transcription factor		HEC1A ERα and ERβ	6 h	RL95-2 PRA
RL95-2		E2		P4	
CD86	Immunoglobulin receptor family member; Membrane-bound signaling molecule; Defense/immunity protein	12 h	HEC1A ERα	NS	ND
IHH	Other signaling molecule; Protease	12 h	HEC1A ERα/ERβ	6 h	RL95-2 PRB

(*) Molecular functions were obtained from PANTHER. In bold are genes with cell line-specific expression. NS: not significant gene expression, ND: not detected. Official gene symbols according to HUGO Gene Nomenclature Committee (HGNC).

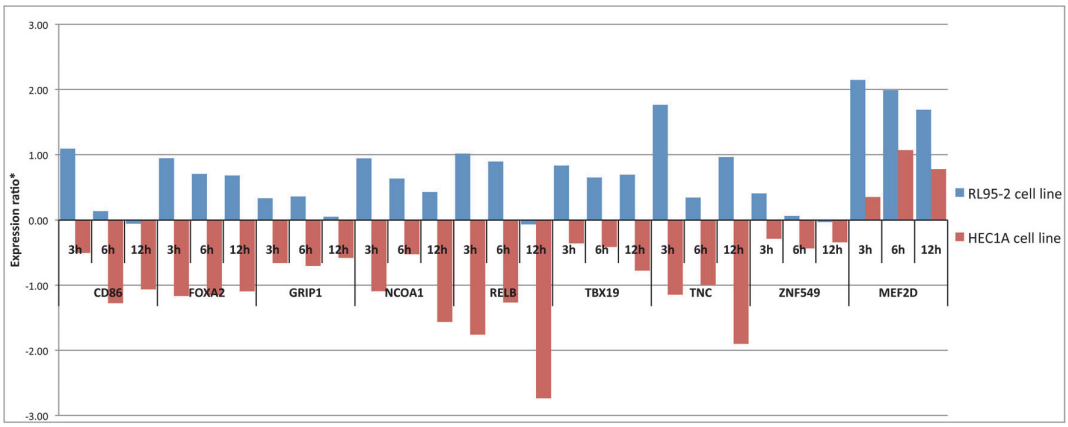

Figure 2
E2 mediated time-dependant gene expression in RL95-2 (blue) and HEC1A (red) cell lines compared to non-treated samples. Expression profiles of *CD86*, *FOXA2*, *GRIP1*, *NCOA1*, *RELB*, *TBX19*, *TNC*, *ZNF549*, and *MEF2D* genes had significant difference ($p < 0.05$, two-tailed, unpaired Student's t-test with 95% confidence of log2-transformed expression ratios relative to baseline expression) in mRNA expression ratios (*) between RL95-2 and HEC1A cells after E2 treatment. E2 exposure predominantly induced mRNA level of gene expression in RL95-2 cells but down-regulated in HEC1A cells (except for *MEF2D* gene).

[23,24]. Since NR activity is often tissue specific, we used primers specifically designed to detect the promoter regions of 382 genes known to exhibit differential expression in the human receptive endometrium. Our specific aim was to investigate whether the steroid hormone actions in endometrial cell lines HEC1A and RL95-2 support their use as non-receptive and receptive endometrial models, respectively.

In non-receptive endometrium, the primary role of E2 is in tissue regeneration and proliferation. Analysis of the number of ER target genes identified by ChIP-qPCR after E2 treatment under our experimental conditions, revealed

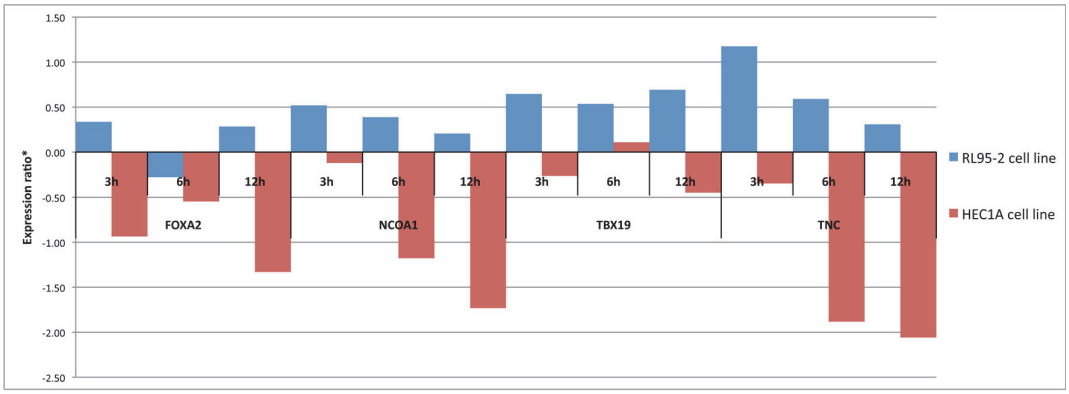

Figure 3
P4 mediated time-dependant gene expression in RL95-2 (blue) and HEC1A (red) cell lines compared to non-treated samples. Expression profiles of *FOXA2*, *NCOA1*, *TBX19* and *TNC* genes had significant difference ($p < 0.05$, two-tailed, unpaired Student's t-test with 95% confidence of log2-transformed expression ratios relative to baseline expression) in mRNA expression ratios (*) between RL95-2 and HEC1A cells after P4 treatment. P4 mostly induced mRNA level in RL95-2 cells but down-regulated in HEC1A cells.

almost 4-fold difference between HEC1A (n = 137) and RL95-2 (n = 35) cells. This finding supports the idea of HEC1A as a good model for non-receptive endometrium being predominantly governed by E2 control. In addition to unique targets of ERα and ERβ, we identified a subset of 60 genes in HEC1A cells and 5 genes in RL95-2 cells that were common for both ERs.

Previous *in vitro* studies have demonstrated that both homodimers ERα/ERα or ERβ/ERβ and heterodimers ERα/ERβ can be formed when both ER subtypes are expressed in the same cell [40]. Moreover, it has been shown that in the absence of ERα, ERβ can either inhibit ERα-mediated gene transcription or partly replace ERα as a transcription factor [41]. Thus, the common ERα and ERβ targets identified in this study could represent genes either activated by heterodimeric complexes or could reflect competitive binding of the two separate E2 receptors to the same regulatory element.

In receptive endometrium, the primary role of P4 is the preparation of the endometrium for embryo implantation. From our set of 382 genes, 83 were bound by PRs in RL95-2 cells, which was ten times more than in the HEC1A cell line (n = 7). Harduf and colleagues have demonstrated that there is a higher PRA/PRB ratio in RL95-2 cells compared to HEC1A cells indicating the possible important role of PRA during implantation window [22]. Even the both cell lines express E2 and P4 specific receptors (ATCC) the lack of specific antibody against PRA has made it difficult to compare the exact ratio levels of two PRs.

To investigate transcriptional changes that occur after NR binds to its promoter region, we assessed the expression of 20 selected genes. RT-PCR analysis showed that 12 out of selected 20 IW-specific genes had at least 2-fold up- or down-regulation in mRNA expression level after hormonal treatment. Five genes were expressed in cell line-specific manner as the expression of *IHH*, *PLXNA2*, *OTOF*, and *SMARCA2* was only observed in RL95-2 cells, while *MMP7* activity was detected exclusively in HEC1A cells.

We included *IHH* as a classical example of steroid hormone-dependent gene regulation during IW. Previous *in vivo* and *in vitro* studies revealed that the expression of *IHH* in the murine uterus is solely P4 dependent and essential for embryo implantation [42-44]. Our results showed that *IHH* was the target gene for PRB after P4 treatment in RL95-2 cells. Indeed, RT-PCR analysis confirmed *IHH* expression only in RL95-2 cells. Interestingly, the mRNA levels of *IHH* in RL95-2 cells were suppressed after the use of either E2 or P4 hormones (Table 5), differentially from murine uterus where *IHH* expression depends only on P4 [42-44]. Yet, the recent work has indicated that the stromal not epithelial PR is critical for P4-mediated induction of *IHH* expression in the mouse uterus [45]. Since the HEC1A and RL95-2 cell lines represent the epithelial cells of the endometrium, *IHH* expression in this endometrial compartment may be regulated by other hormonal mechanisms from internal tissue layers. Species difference between the estrous cycle of mice and the menstrual cycle in women is an additional factor to account for this discrepancy.

Our gene expression analysis focused mainly on transcription factors. Since the development and regeneration of endometrial tissue is largely governed by E2 and P4, a transcriptional regulatory feedback system is needed to mediate these dynamic and complex changes. The majority of E2-regulated genes are thought to be up-regulated after short (1-8 h) hormonal treatment, while most of the hormone-responsive genes are down-regulated after longer (12-48 h) treatment [39]. We examined the changes in mRNA expression following 3-12 h of hormonal exposure in order to investigate the rapid transcriptional changes caused by steroid hormones. We found that the increase of mRNA level in selected genes mainly occurred after short-term (3-6 h) treatment, while the majority of suppressive effects become visible after 6-12 h of treatment (Table 5, Figures 2 and 3).

Interestingly, when hormone responsiveness in two endometrial cell lines was compared, we found that hormonal treatments had almost opposite effects on gene expression. Treatments with E2 or P4 resulted in significant up- and down-regulation of genes in RL95-2 and HEC1A cells, respectively. Genes for the transcription factors *FOXA2*, *NCOA1*, *TBX19*, and the extracellular matrix glycoprotein *TNC* had markedly different and opposite mRNA expression levels after E2 or P4 treatment in both of the cell lines investigated. All four genes were either down-regulated by both hormones in HEC1A or up-regulated in RL95-2 cells (Figures 2 and 3). In addition to already mentioned genes, E2 had also an inverse effect on *CD86*, *GRIP1*, *RELB*, and *ZNF549* mRNA expression in the two endometrial cell lines, up-regulated in RL95-2 and down-regulated in HEC1A cells.

Some of the genes (*ADAMDEC1*, *ETV1*, *FLT1*, *HES1*, *HOXA1* and *KLK3*) did not show any evidence of gene activity in both cell lines in spite of hormonal treatment. As all genes were selected from previous expression studies with human endometrium tissue samples the revealed effect could be cell line specific. In addition, we have to take into account that NR binding alone is not always sufficient to induce gene expression changes. The various combinations of transcription co-activators or -repressors are also required for proper gene activity, depending on the specific cell and tissue background [46-48]. In study-

ing steroid hormone signalling, the non-genomic actions of steroid hormones in cross-talk between the growth factor receptors and cytoplasmic response must also be considered along with the direct transcriptional effects mediated by NRs [49,50].

In present study we gained new information about the specific action of E2 and P4 during different stages of human endometrial development using a combination of ChIP-qPCR and RT-PCR. HEC1A and RL95-2 cell lines showed different sets of ER and PR target genes in response to hormonal stimuli as more target genes were detected for ERs in HEC1A cells than for PRs in RL95-2 cells. We also observed that hormone treatment had different impacts on gene expression levels in the two cell lines. The ChIP and RT-PCR results show that E2 and P4 actions in the human endometrium are more complex than the classic steroid hormone effects mediated through NRs.

Conclusions

The presented data demonstrates that the endometrial cell lines HEC1A and RL95-2 are suitable *in vitro* models for evaluating the effects of steroid hormones in non-receptive and receptive endometrium, respectively. This study deepens our understanding on the hormone responsive gene regulation during the cyclic changes in the human endometrium. However, further studies are needed to elucidate the complex mechanism by which endometrium acquires its receptivity.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KT carried out experimental studies and prepared the draft version of the manuscript. MR participated in experimental work and performed data analysis of ChIP and mRNA expression studies. MM conceived the study, mainly designed and coordinated the work and performed the bioinformatic analysis. AS contributed to the design of the study and helped with the manuscript preparation. All authors read and approved the final version of the manuscript.

Additional material

Additional file 1

***Supplementary Table 1**. Genes used in ChIP-qPCR for ER and PR binding site analysis.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1477-7827-7-150-S1.XLS]

Additional file 2

***Suplementary Table 2**. Genes used in mRNA analysis.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1477-7827-7-150-S2.XLS]

Acknowledgements

This work has been supported by the Tallinn University of Technology (Targeted project no. B611), Estonian Ministry of Education and Science (Targeted project no. SF0180044s09) and Enterprise Estonia (Grant no. EU30200).

References

1. Graham RA, Seif MW, Aplin JD, Li TC, Cooke ID, Rogers AW, Dockery P: **An endometrial factor in unexplained infertility.** *Bmj* 1990, **300(6737):**1428-1431.
2. Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjold M, Gustafsson JA: **Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern.** *J Clin Endocrinol Metab* 1997, **82(12):**4258-4265.
3. Kuiper GG, Gustafsson JA: **The novel estrogen receptor-beta subtype: potential role in the cell- and promoter-specific actions of estrogens and anti-estrogens.** *FEBS Lett* 1997, **410(1):**87-90.
4. Matsuzaki S, Fukaya T, Suzuki T, Murakami T, Sasano H, Yajima A: **Oestrogen receptor alpha and beta mRNA expression in human endometrium throughout the menstrual cycle.** *Mol Hum Reprod* 1999, **5(6):**559-564.
5. Wen DX, Xu YF, Mais DE, Goldman ME, McDonnell DP: **The A and B isoforms of the human progesterone receptor operate through distinct signaling pathways within target cells.** *Mol Cell Biol* 1994, **14(12):**8356-8364.
6. Kastner P, Bocquel MT, Turcotte B, Garnier JM, Horwitz KB, Chambon P, Gronemeyer H: **Transient expression of human and chicken progesterone receptors does not support alternative translational initiation from a single mRNA as the mechanism generating two receptor isoforms.** *J Biol Chem* 1990, **265(21):**12163-12167.
7. Tung L, Mohamed MK, Hoeffler JP, Takimoto GS, Horwitz KB: **Antagonist-occupied human progesterone B-receptors activate transcription without binding to progesterone response elements and are dominantly inhibited by A-receptors.** *Mol Endocrinol* 1993, **7(10):**1256-1265.
8. Vegeto E, Shahbaz MM, Wen DX, Goldman ME, O'Malley BW, McDonnell DP: **Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function.** *Mol Endocrinol* 1993, **7(10):**1244-1255.
9. Arnett-Mansfield RL, DeFazio A, Mote PA, Clarke CL: **Subnuclear distribution of progesterone receptors A and B in normal and malignant endometrium.** *J Clin Endocrinol Metab* 2004, **89(3):**1429-1442.
10. Dimitriadis E, White CA, Jones RL, Salamonsen LA: **Cytokines, chemokines and growth factors in endometrium related to implantation.** *Hum Reprod Update* 2005, **11(6):**613-630.
11. Kao LC, Tulac S, Lobo S, Imani B, Yang JP, Germeyer A, Osteen K, Taylor RN, Lessey BA, Giudice LC: **Global gene profiling in human endometrium during the window of implantation.** *Endocrinology* 2002, **143(6):**2119-2138.
12. Krikun G, Schatz F, Taylor R, Critchley HO, Rogers PA, Huang J, Lockwood CJ: **Endometrial endothelial cell steroid receptor expression and steroid effects on gene expression.** *J Clin Endocrinol Metab* 2005, **90(3):**1812-1818.
13. Mirkin S, Arslan M, Churikov D, Corica A, Diaz JI, Williams S, Bocca S, Oehninger S: **In search of candidate genes critically expressed in the human endometrium during the window of implantation.** *Hum Reprod* 2005, **20(8):**2104-2117.
14. Punyadeera C, Dassen H, Klomp J, Dunselman G, Kamps R, Dijcks F, Ederveen A, de Goeij A, Groothuis P: **Oestrogen-modulated**

gene expression in the human endometrium. *Cell Mol Life Sci* 2005, **62(2):**239-250.
15. Carson : **Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening.** *Molecular Human Reproduction* 2002, **8(No 9):**871-879.
16. Kuramoto H, Tamura S, Notake Y: **Establishment of a cell line of human endometrial adenocarcinoma in vitro.** *Am J Obstet Gynecol* 1972, **114(8):**1012-1019.
17. Way DL, Grosso DS, Davis JR, Surwit EA, Christian CD: **Characterization of a new human endometrial carcinoma (RL95-2) established in tissue culture.** *In Vitro* 1983, **19(3 Pt 1):**147-158.
18. John NJ, Linke M, Denker HW: **Retinoic acid decreases attachment of JAR choriocarcinoma spheroids to a human endometrial cell monolayer in vitro.** *Placenta* 1993, **14(1):**13-24.
19. Rohde LH, Carson DD: **Heparin-like glycosaminoglycans participate in binding of a human trophoblastic cell line (JAR) to a human uterine epithelial cell line (RL95).** *J Cell Physiol* 1993, **155(1):**185-196.
20. Thie M, Fuchs P, Butz S, Sieckmann F, Hoschutzky H, Kemler R, Denker HW: **Adhesiveness of the apical surface of uterine epithelial cells: the role of junctional complex integrity.** *Eur J Cell Biol* 1996, **70(3):**221-232.
21. Harduf H, Goldman S, Shalev E: **Human uterine epithelial RL95-2 and HEC-1A cell-line adhesiveness: the role of plexin B1.** *Fertil Steril* 2007, **87(6):**1419-1427.
22. Harduf H, Goldman S, Shalev E: **Progesterone receptor A and c-Met mediates spheroids-endometrium attachment.** *Reprod Biol Endocrinol* 2009, **7:**14.
23. Carroll JS, Brown M: **Estrogen receptor target gene: an evolving concept.** *Mol Endocrinol* 2006, **20(8):**1707-1714.
24. Lin CY, Vega VB, Thomsen JS, Zhang T, Kong SL, Xie M, Chiu KP, Lipovich L, Barnett DH, Stossi F, *et al.*: **Whole-genome cartography of estrogen receptor alpha binding sites.** *PLoS Genet* 2007, **3(6):**e87.
25. Weinmann AS: **Novel ChIP-based strategies to uncover transcription factor target genes in the immune system.** *Nat Rev Immunol* 2004, **4(5):**381-386.
26. **Primer3 programm** [http://frodo.wi.mit.edu/primer3/]
27. Kent WJ: **BLAT--the BLAST-like alignment tool.** *Genome Res* 2002, **12(4):**656-664.
28. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: **Gapped BLAST and PSI-BLAST: a new generation of protein database search programs.** *Nucleic Acids Res* 1997, **25(17):**3389-3402.
29. **Miner software** [http://www.miner.ewindup.info]
30. Schefe JH, Lehmann KE, Buschmann IR, Unger T, Funke-Kaiser H: **Quantitative real-time RT-PCR data analysis: current concepts and the novel "gene expression's CT difference" formula.** *J Mol Med* 2006, **84(11):**901-910.
31. **Protein Analysis THrough Evolutionary Relationships** [http://www.pantherdb.org]
32. **Cluster 3.0 program** [http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/software.htm]
33. **BioMart** [http://www.biomart.org]
34. **National Center for Biotechnology Information** [http://www.ncbi.nlm.nih.gov]
35. **UCSC genome browser** [http://genome.ucsc.edu]
36. **MySQL database management software** [http://www.mysql.com]
37. Heneweer C, Kruse LH, Kindhauser F, Schmidt M, Jakobs KH, Denker HW, Thie M: **Adhesiveness of human uterine epithelial RL95-2 cells to trophoblast: rho protein regulation.** *Mol Hum Reprod* 2002, **8(11):**1014-1022.
38. Rahnama F, Thompson B, Steiner M, Shafiei F, Lobie PE, Mitchell MD: **Epigenetic regulation of E-cadherin controls endometrial receptivity.** *Endocrinology* 2009, **150(3):**1466-1472.
39. Kininis M, Kraus WL: **A global view of transcriptional regulation by nuclear receptors: gene expression, factor localization, and DNA sequence analysis.** *Nucl Recept Signal* 2008, **6:**e005.
40. Cowley SM, Hoare S, Mosselman S, Parker MG: **Estrogen receptors alpha and beta form heterodimers on DNA.** *J Biol Chem* 1997, **272(32):**19858-19862.
41. Lindberg MK, Moverare S, Skrtic S, Gao H, Dahlman-Wright K, Gustafsson JA, Ohlsson C: **Estrogen receptor (ER)-beta reduces ERalpha-regulated gene transcription, supporting a "ying yang" relationship between ERalpha and ERbeta in mice.** *Mol Endocrinol* 2003, **17(2):**203-208.
42. Takamoto N, Zhao B, Tsai SY, DeMayo FJ: **Identification of Indian hedgehog as a progesterone-responsive gene in the murine uterus.** *Mol Endocrinol* 2002, **16(10):**2338-2348.
43. Khatua A, Wang X, Ding T, Zhang Q, Reese J, DeMayo FJ, Paria BC: **Indian hedgehog, but not histidine decarboxylase or amphiregulin, is a progesterone-regulated uterine gene in hamsters.** *Endocrinology* 2006, **147(9):**4079-4092.
44. Lee K, Jeong J, Kwak I, Yu CT, Lanske B, Soegiarto DW, Toftgard R, Tsai MJ, Tsai S, Lydon JP, *et al.*: **Indian hedgehog is a major mediator of progesterone signaling in the mouse uterus.** *Nat Genet* 2006, **38(10):**1204-1209.
45. Simon L, Spiewak KA, Ekman GC, Kim J, Lydon JP, Bagchi MK, Bagchi IC, DeMayo FJ, Cooke PS: **Stromal progesterone receptors mediate induction of Indian Hedgehog (IHH) in uterine epithelium and its downstream targets in uterine stroma.** *Endocrinology* 2009, **150(8):**3871-3876.
46. Torchia J, Glass C, Rosenfeld MG: **Co-activators and co-repressors in the integration of transcriptional responses.** *Curr Opin Cell Biol* 1998, **10(3):**373-383.
47. Khan OY, Nawaz Z: **Nuclear hormone receptor co-regulators.** *Curr Opin Drug Discov Devel* 2003, **6(5):**692-701.
48. Ellmann S, Sticht H, Thiel F, Beckmann MW, Strick R, Strissel PL: **Estrogen and progesterone receptors: from molecular structures to clinical targets.** *Cell Mol Life Sci* 2009, **66(15):**2405-2426.
49. Losel R, Wehling M: **Nongenomic actions of steroid hormones.** *Nat Rev Mol Cell Biol* 2003, **4(1):**46-56.
50. Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, *et al.*: **Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity.** *Cell* 2001, **104(5):**719-730.

PUBLICATION II

Tamm-Rosenstein K, Simm J, Suhorutšenko M, Salumets A, Metsis M, (2013). Changes in the Transcriptome of the Human Endometrial Ishikawa Cancer Cell Line Induced by Estrogen, Progesterone, Tamoxifen and Mifepristone (RU486) as Detected by RNA-Sequencing. PloS ONE, 8, e68907.

OPEN ACCESS Freely available online

PLOS ONE

Changes in the Transcriptome of the Human Endometrial Ishikawa Cancer Cell Line Induced by Estrogen, Progesterone, Tamoxifen, and Mifepristone (RU486) as Detected by RNA-Sequencing

Karin Tamm-Rosenstein[1,2,3]*, Jaak Simm[1], Marina Suhorutshenko[1], Andres Salumets[2,4,5], Madis Metsis[2,6]

1 Centre for Biology of Integrated Systems, Tallinn University of Technology, Tallinn, Estonia, **2** Competence Centre on Reproductive Medicine and Biology, Tartu, Estonia, **3** Nova Vita Clinic, Tallinn, Estonia, **4** Department of Obstetrics and Gynaecology, University of Tartu, Tartu, Estonia, **5** Institute of Biomedicine, University of Tartu, Tartu, Estonia, **6** Institute of Mathematics and Natural Sciences, Tallinn University, Tallinn, Estonia

Abstract

Background: Estrogen (E2) and progesterone (P4) are key players in the maturation of the human endometrium. The corresponding steroid hormone modulators, tamoxifen (TAM) and mifepristone (RU486) are widely used in breast cancer therapy and for contraception purposes, respectively.

Methodology/Principal findings: Gene expression profiling of the human endometrial Ishikawa cancer cell line treated with E2 and P4 for 3 h and 12 h, and TAM and RU486 for 12 h, was performed using RNA-sequencing. High levels of mRNA were detected for genes, including *PSAP, ATP5G2, ATP5H*, and *GNB2L1* following E2 or P4 treatment. A total of 82 biomarkers for endometrial biology were identified among E2 induced genes, and 93 among P4 responsive genes. Identified biomarkers included: *EZH2, MDK, MUC1, SLIT2,* and *IL6ST*, which are genes previously associated with endometrial receptivity. Moreover, 98.8% and 98.6% of E2 and P4 responsive genes in Ishikawa cells, respectively, were also detected in two human mid-secretory endometrial biopsy samples. TAM treatment exhibited both antagonistic and agonistic effects of E2, and also regulated a subset of genes independently. The cell cycle regulator cyclin D1 (*CCND1*) showed significant up-regulation following treatment with TAM. RU486 did not appear to act as a pure antagonist of P4 and a functional analysis of RU486 response identified genes related to adhesion and apoptosis, including down-regulated genes associated with cell-cell contacts and adhesion as *CTNND1, JUP, CDH2, IQGAP1,* and *COL2A1*.

Conclusions: Significant changes in gene expression by the Ishikawa cell line were detected after treatments with E2, P4, TAM, and RU486. These transcriptome data provide valuable insight into potential biomarkers related to endometrial receptivity, and also facilitate an understanding of the molecular changes that take place in the endometrium in the early stages of breast cancer treatment and contraception usage.

Citation: Tamm-Rosenstein K, Simm J, Suhorutshenko M, Salumets A, Metsis M (2013) Changes in the Transcriptome of the Human Endometrial Ishikawa Cancer Cell Line Induced by Estrogen, Progesterone, Tamoxifen, and Mifepristone (RU486) as Detected by RNA-Sequencing. PLoS ONE 8(7): e68907. doi:10.1371/journal.pone.0068907

Editor: John P. Lydon, Baylor college of Medicine, United States of America

Received May 3, 2013; **Accepted** June 7, 2013; **Published** July 16, 2013

Funding: This work has been supported by the Tallinn University of Technology (Targeted project no. B611), Estonian Ministry of Education and Science (Targeted project no. SF0180044s09) and Enterprise Estonia (Grant no. EU30020).

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: karin.tamm@ttu.ee

Introduction

The ovarian steroid hormones, estrogen (E2) and progesterone (P4), play crucial roles in regulating normal functions of the human endometrium. For example, during a normal menstrual cycle, proliferation, differentiation, and degeneration of the endometrium occur in response to varying E2 and P4 levels. In the proliferative phase, E2 stimulates the proliferation of epithelial cells and stromal components of the endometrium. In the secretory phase, P4 modulates glandular differentiation and an inhibition of estrogen-mediated proliferation [1]. It is during the mid-secretory phase that the endometrium achieves a phenotype compatible with successful embryo implantation.

A better understanding of the biology and functioning of the human endometrium is vital to improving our knowledge about female infertility, and for the design of treatments for these conditions. Correspondingly, a search for markers of endometrial receptivity and novel approaches to improve implantation rates during infertility treatments have been conducted. In recent years, numerous studies involving microarray expression analysis have identified a wide range of genes up- or down-regulated in the human endometrium during the time of embryo implantation [2–10]. Each study identified many candidate genes believed to be critical to the embryo implantation process. However, few genes were consistently reported.

The genomic activities of E2 and P4 are mainly mediated by nuclear receptors. When E2 or P4 are bound to their receptors,

they can bind response elements in DNA with high affinity and regulate the transcription of target genes. In humans, there are two types of estrogen receptors, ERα and ERβ, and these are encoded by separate genes [11,12]. ERα and ERβ are expressed in all endometrial cell types throughout the entire menstrual cycle, and undergo changes in expression and activity. For example, ERα and ERβ are expressed at higher levels during the proliferative phase, yet exhibit lower activity during the secretory phase when they are subject to the suppressive effects of P4. It is after the proliferative phase that P4 mediates E2-based priming of the endometrium towards a state of receptivity.

In contrast with ERα and ERβ, the progesterone receptors (PRs), PRA and PRB, are encoded by the same gene (*PGR*), yet are transcribed from different promoters. As a result, PRB includes an additional 164 amino acids at its N-terminus [13,14]. Both isoforms are expressed in the stroma and epithelium of the endometrium during the proliferative phase. However, the expression of both receptors decreases sharply in the epithelium during the early to mid-secretory phase [15]. Endometrial receptivity appears to be tightly associated with the down-regulation of epithelial PRs, while the stroma only maintains expression of PRA during the secretory phase [16]. Expression of PR genes in the endometrial glandular epithelium is controlled by E2 and P4, with E2 inducing PR synthesis and P4 down-regulating expression of its own receptor [1].

Selective ER modulators (SERMs) have the ability to interact with ERs as agonists or antagonists depending on the target tissue and to modulate signal transduction pathways of E2-responsive genes [17]. For example, tamoxifen (TAM) binds with high affinity to ERs, thereby blocking the action of native E2. Due to its antagonistic activity of E2, TAM has been widely used in breast cancer therapy. However, one of the most troublesome side effects of breast cancer treatments with TAM appears to be its proliferative effect on the endometrium [18,19]. For example, endometrial pathologies associated with TAM treatments include hyperplasia, polyps, carcinomas, and sarcomas [20]. Similar to SERMs, selective progesterone receptor modulators (SPRMs) have been developed to antagonize processes activated by P4. Mifepristone (RU486) is a P4 antagonist that competes with endogenous P4 for receptor binding [21] and is used to end an early pregnancy. RU486 also exhibits a 2-to-10-fold higher affinity towards PRs compared to P4 [22].

The exact basis for the differential, tissue-specific signalling of E2 and P4 is still not fully understood, and a better understanding of E2 and P4 actions is needed to evaluate their roles in regulating endometrial gene expression. In this study, the human uterine-derived epithelial cancer cell line, Ishikawa, was used. It is one of the most well-characterized human endometrial cell lines currently available. Ishikawa cells were derived from a well-differentiated adenocarcinoma of the human endometrial epithelium that expressed functional steroid receptors for E2 and P4 [23–25]. As a result, this cell lines represents an ideal model for studying the response of the endometrial epithelium to E2 and P4. In this study, high-throughput RNA-sequencing (RNA-Seq) was applied to studies of E2- and P4-dependent transcriptomes in an endometrial context. The steroid hormone receptor modulators, TAM and RU486, were also used to study receptor-dependent signal transduction, and to evaluate agonistic or antagonistic activity in the Ishikawa cell line. Finally, the significant E2- and P4-dependent genes identified in the Ishikawa cell line were assayed in endometrial biopsy samples collected at the receptive mid-secretory phase.

Materials and Methods

Cell Culture

The Ishikawa cell line was provided by Prof. Anneli Stavreus-Evers (Uppsala University, Sweden). Cells were grown in DMEM medium (PAA, Pasching, Austria), supplemented with 5% fetal bovine serum (FBS; PAA) and 1% penicillin/streptomycin (PAA), at 37°C and 5% CO_2. For hormonal treatments, E2 (β-Estradiol) or P4 (4-Pregnene-3,20-dione) were added to the culture media to a final concentration of 10^{-8} M. The steroid hormone modulators, tamoxifen (TAM, 4-hydroxytamoxifen) and mifepristone (RU486), were added to culture media to a final concentration of 1 μM. All hormones and modulators were ordered from Sigma-Aldrich (Schnelldorf, Germany), with E2 and P4 dissolved in dimethylsulfoxide (DMSO) and TAM and RU486 in ethanol (EtOH). Control samples were treated with vehicle only. For cultures with hormone supplements, dextran-coated, charcoal-treated FBS and media without phenol red were used 48 h prior to experiments to avoid possible hormone-like activity of phenol red.

RNA Extraction

Total RNA was extracted from untreated cells and cells after 3 h and 12 h of hormone treatment using RNeasy Mini Kits (Qiagen, Valencia, USA). Endometrial biopsies were collected from two patients (ages, 34 and 38 years) with unexplained infertility treated at the Nova Vita Clinic. Biopsies were collected on days LH+7 to LH+9 according to urine ovulation tests. Tissue samples were homogenized with Tissue lyzer (Qiagen) and total RNA was extracted from previously formalin-fixed (3.7%) endometrial biopsies using an RNeasy FFPE kit (Qiagen) according to the manufacturer's instructions. The study was carried out on accordance with the local ethical standards and was approved by the Ethics Review Committee on Human Research of the University of Tartu, with written consent obtained from both study participants.

RNA Library Preparation

RNA libraries for cell line and endometrial samples were prepared using an Illumina TruSeq RNA Sample Prep Kit (FC-122-1001, Illumina, San Diego, USA) according to the manufacturer's instructions. For cell line samples, mRNA was purified using polyA selection, and was subsequently fragmented chemically. For tissue samples, total RNA was collected without polyA selection and the fragmentation step was shortened based on the previous formalin treatment of the samples. In all samples, RNAs were converted into single-stranded cDNAs using random hexamer priming. Multiplexing with different adapter indexes was performed and the quality of the resulting library was checked using a Bioanalyzer (Agilent, Waldbronn, Germany).

RNA-Seq and Data Analysis

Single-end (SE) sequencing of 75 bp was performed using an Illumina Genome Analyzer II (Illumina, San Diego, USA). Bowtie programming was used to provide an initial alignment of sequences to human genome 19 (Hg19), with default settings used to find only perfect matches. Sequenced fragments were aligned to the *H. Sapiens* reference genome (Hg19) provided by University of California Santa Cruz (UCSC) Genome browser using a TopHat v1.2.0 algorithm with default settings [26]. The aligned reads were subsequently processed into transcripts using Cufflinks v1.1.0 [27], with abundances estimated and analysed to examine differential expression patterns between cell line samples. Cufflinks constructed a minimum set of transcripts to best describe the reads in the dataset. The Benjamin-Hochberg correction for multiple testing

was applied to the P values of significant genes with a false discovery rate (FDR) value of 0.05. Normalized RNA-Seq fragment counts indicating the relative abundances of the transcripts were used. Abundances were reported in units of FPKM (e.g., Fragments Per Kilobase of transcript per Million of fragments mapped). The output files of Cufflinks were analysed with Cuffcompare along with the reference from the UCSC Table Browser (Homo sapiens GRCh37/Hg19) [28]. Cuffcompare classifies each transcript as known or novel. Cuffdiff re-estimates the abundance of transcripts listed by Cuffcompare and tests for differential expression between the selected experiments. If one of the experiments (either control or treatment) had 0 FPKM, the log change became infinite. We expressed the log change in these cases as +14 for up-regulation and -14 for down-regulation.

Functional Analysis

For the functional classification of genes that exhibited significant differential expression profiles in response to different steroid hormone and their analogue treatments, Ingenuity Pathway Analysis (IPA) 9.0 software (Ingenuity Systems) was used. The IPA transcription factor module was used to predict the gene expression changes detected regarding to potential bindings of ERs and PRs. In addition, IPA biomarker analysis filters identified potential biomarkers in selected tissues.

Data Visualization

R statistics software (version 2.14.0) (http://www.R-project.org/) was used to process and visualize the results from Cufflinks analyses. Calculation of general statistics, including common and unique counts of significantly affected genes, were performed in R using a custom script. For heatmap visualizations, the R package gplots (version 2.10.1) (http://CRAN.R-project.org/package = gplots) was used. In addition, differences in the FPKM values of the treated samples versus the non-treated samples were calculated in the heatmaps. The largest absolute FPKM difference for each gene was identified, and was used to normalize FPKM data for each gene. Thus, the resulting values lie between −1 and 1, and a value of 0 corresponds to an absence of change compared to the non-treated sample. Based on these normalized expression values, genes were positioned in the heatmap by hierarchical clustering.

Results

The Transcriptome of the Ishikawa Cell Line Before and After Treatment with E2, P4, and Respective Modulators

PolyA-selected RNA from the human endometrial cell line, Ishikawa, was subjected to SE-sequencing with 75 basepair long reads. Reference measurements for each sample were then made based on the $8–11\times10^6$ reads that were obtained. The goal of this sequencing effort was to provide an overall gene expression profile of the Ishikawa cell line in order to identify changes in gene expression that occur during the early response of this cell line to steroid hormones and their modulators. Altogether, seven samples were analysed, and these included non-treated cells, cells treated with E2 or P4 for 3 and 12 h, and cells treated with TAM or RU486 modulators for 12 h. The majority of reads from each sample (e.g., >70%) were successfully aligned to the human genome version 19 (Hg19). Statistical values of these alignments and the number of genes identified, including both known and unknown genes, are listed in Table 1. The relative abundances of fragments were calculated using Cufflinks, and were reported in units of FPKMs in order to describe expressed genes (e.g., fragments) observed from RNA-Seq experiments. In Table 1, the number of genes with different FPKM abundances, as well as the numbers of genes which exhibited significant changes in expression following hormone/modulator treatment, were compared with non-treated cells. In addition, the most responsive genes identified from the Ishikawa cell line were compared with human endometrium biopsy samples (n = 2) collected during the time of embryo implantation. A complete list of the expressed genes identified and their FPKM values are available in Table S1.

One of the advantages of a RNA-Seq analysis is the ability to detect relatively high expression levels of genes. A subset of genes from the Ishikawa cell line had FPKM values that were greater than 1000 after hormone/modulator treatments (Table 2). These included genes encoding prosaposin (*PSAP*), ATP synthases, *ATP5G2* and *ATP5H*, and guanine nucleotide binding protein (*GNB2L1*). These genes were very highly expressed in response to E2 or P4 treatment. The expressions of *ATP5G2*, *ATP5H* and *GNB2L1* have not been shown to be related to endometrium before. Alternatively, genes encoding the S100 calcium binding proteins A2 (*S100A2*) and A6 (*S100A6*), heat shock protein 90 kDa alpha (*HSP90AA1*), and HSPA8, as well as pyruvate kinase in muscle (*PKM2*), exhibited high levels of expression 12 h after treatment with TAM. Among these genes only the expression of *S100A2* has been related to TAM treatment in breast cancer tissue but not in endometrium [29]. In addition, the gene for ferritin light polypeptide (*FTL*), also not detected in endometrium in former studies, was found to be highly expressed 12 h after treatment with E2, P4, and TAM (Table 2).

Significant Gene Expression Changes in the Ishikawa Cell Line After E2 and P4 Treatments

Relative mRNA expression levels (in units of FPKM) for E2- and P4-treated cells were compared with non-treated cells using Cuffdiff software (version 1.1.0) and a 5% FDR. The number of genes that exhibited significant changes in expression after respective treatments are listed in Table 1. In addition, only known genes were included in subsequent analyses of gene expression data.

A total of 1691 known genes (Table S2) were found to be significantly affected in Ishikawa cells following treatments with E2 for 3 h (n = 1084) and 12 h (n = 1121) compared to non-treated cells. Of those genes, 614 were significantly up-regulated, and 470 were significantly down-regulated after 3 h of E2 treatment. When treatment with E2 was extended to 12 h, induction of 715 genes, and suppression of 406 genes, was detected.

In majority of genes 12 h TAM treatment showed antagonistic activity of E2. Twelve hours of treatment with TAM resulted in low or undetectable levels of mRNA for 654 (91.5%) genes of the 715 genes that exhibited higher mRNA levels following treatment with E2 for 12 h. An additional 406 genes were found to be down-regulated following treatment with E2 for 12 h, and 75.1% (n = 305) of these genes exhibited only minor changes in gene activity, or were associated with an absence of regulation, 12 h after treatment with TAM.

Based on the data obtained, TAM did not act as a pure antagonist of E2 in the Ishikawa cell line. For example, of the 715 genes up-regulated after treatment with E2 for 12 h, 61 (8.5%) were also significantly up-regulated following treatment with TAM. In addition, among the 406 genes that were found to be down-regulated following treatment with E2 for 12 h, 101 (24.9%) were similarly down-regulated following treatment with TAM. In combination, these data demonstrate that TAM is both antagonistic and agonistic for E2 in the Ishikawa cell line (Figure S1).

Following treatment with P4, a total of 1692 known genes exhibited significant differences in expression (Table S3). Of these

Table 1. RNA-Seq statistics of E2, P4, TAM, and RU486 treated and non-treated Ishikawa cells.

		Ishikawa E2 & TAM			Ishikawa P4 & RU486		
	Non-treated	**3 h E2**	**12 h E2**	**12 h TAM**	**3 h P4**	**12 h P4**	**12 h RU486**
Reads aligned to Tophat	10 465 431	9 967 453	8 093 476	10 912 784	10 628 213	11 058 071	10 983 639
Total genes	16813	16874	16784	16834	16951	16934	16840
% unknown genes	29.07%	29.09%	28.89%	28.86%	29.43%	29.24%	29.06%
% known genes	70.93%	70.91%	71.11%	71.14%	70.57%	70.76%	70.94%
FPKM 0–10	4577	4361	4226	4611	4437	4406	4607
FPKM 10–100	6487	6701	6894	6486	6653	6722	6495
FPKM 100–1000	820	859	767	831	831	807	804
FPKM >1000	41	44	48	47	41	47	40
Significant genes*		1084	1121	1013	1082	1097	546
% endometrium (n = 2)**		98.6%	99.2%	98.6%	98.2%	99.0%	98.5%

*Significant genes (5% FDR) are counted from known genes and compared to non-treated cells.
**E2 and P4 significant genes present in human endometrium during the time of embryo implantation.
doi:10.1371/journal.pone.0068907.t001

genes, 1082 exhibited changes after 3 h of treatment, and 1097 were detected following 12 h of treatment, both compared to non-treated Ishikawa cells. Of these genes, 592 (54.7%) were significantly up-regulated, and 490 (45.3%) were down-regulated 3 h after treatment with P4, while 631 (57.5%) were up-regulated and 466 (42.5%) were down-regulated 12 h after treatment with P4.

When Ishikawa cells were treated with RU486 for 12 h, low or undetectable levels of mRNA were detected for 84.0% (n = 530) of genes which were significantly up-regulated following treatment with P4 for 12 h (n = 631). Another 466 genes were found to be down-regulated following treatment with P4 for 12 h, and of these, 88.2% (n = 411) exhibited minor down-regulation, or showed no regulation, following treatment with RU486 for 12 h.

Of the 631 genes that were up-regulated in response to 12 h P4 treatment, 101 (16.0%) of these genes were also significantly up-regulated following treatment with RU486. Alternatively, of the 466 genes down-regulated in response to treatment with P4 for 12 h, 55 (11.8%) exhibited a similar down-regulation following treatment with RU486. Moreover, similar to TAM, RU486 exhibited both agonistic and antagonistic activity of P4 in Ishikawa cells (Figure S2).

Of the 1691 genes significantly responsive to E2, and the 1692 genes significantly responsive to P4, 1051 were common to both groups, suggesting that they are regulated by both hormones. Relative majority of the genes identified with significant changes after E2 and P4 treatment, have not been mentioned in endometrial context before.

Potential ER and PR Targets Among the E2 and P4 Significant Genes Identified

An IPA analysis of genes found to be responsive to E2 revealed 20 potential target genes for the E2 receptor, ERα (*ESR1*), based on a database of experimentally observed receptor interactions. For example, *ABCA3*, *CELSR2*, *CYP1A1*, *DDX17*, *EFEMP1*, *ENO1*, *FOSL2*, *GREB1*, *KCNK6*, *MAPK12*, *MYC*, *PDCD4*, *PGR*, *SHANK3*, *TGFA*, and *TPM1* were significantly up-regulated following treatment with E2 for 12 h. Conversely, *CCNG2*, *KCTD6*, *LDLR*, and *PRLR* were down-regulated. Of these gene products, PGR and MAPK12 participate in glucocorticoid

Table 2. Selection of genes with FPKM >1000 after hormone/modulator treatments.

Gene name	Description	Non-treated	3 h E2	12 h E2	12 h TAM	3 h P4	12 h P4	12 h RU486
S100A2	S100 calcium binding protein A2	178.9	145.2	75.3	1469.4*	182.2	137.6	105.8
S100A6	S100 calcium binding protein A6	451.9	421.9	351	1141.6*	414.4	330.9	492
PSAP	prosaposin	875.8	988.4	1021.6*	710.2	933.2	962.8	771.1
HSPA8	heat shock 70 kDa protein 8	840.8	802.4	880.6	1496.2*	702.5	925.8	982.1
ATP5G2	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit C2	961.8	1055.3*	1100.2*	858.3	1041.1*	1232.6*	779.1
HSP90AA1	heat shock protein 90 kDa alpha	733.3	742.7	815	1277.4*	760.9	891	884.7
PKM2	pyruvate kinase, muscle	642.5	565.3	641.8	1019.0*	538.2	597.6	653.3
ATP5H	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit d	974.9	1054.7*	914.1	910.7	1178.3*	907.9	890
FTL	ferritin, light polypeptide	761	687	1027.5*	1130.9*	726.7	1056.5*	989.7
GNB2L1	guanine nucleotide binding protein	851.3	948.5	1027.8*	778.9	891.9	1006.5*	748.8

*FPKM abundance >1000.
doi:10.1371/journal.pone.0068907.t002

receptor signalling, whereas MYC and CYP1A1 are involved in aryl hydrocarbon receptor (AHR) signalling (Figure S3).

After 12 h of treatment with P4, expression of *CDKN1C, F3, FKBP5, GAS6, IL1R1, NQO2, PFKP, TSC22D3* and *PGR* were found to be up-regulated, while *EZR, ACSL1, AKAP13, CCNB2, GLUL, MYCN, PPIF,* and *SNTB2* were found to be down-regulated. Of these, CDKN1C, FKBP5, and PGR contribute to glucocorticoid receptor signalling, while ACSL1 and PFKP have roles in gluconeogenesis (Figure S4).

Biomarker Analysis of E2 and P4 Significant Genes from the Ishikawa Cell Line

Next, potential biomarkers among the E2 (n = 1691) and P4 (n = 1692) responsive genes were examined. Only molecules previously detected in the human endometrium were considered for the IPA biomarker analysis performed, and molecules were further filtered to include those related to reproductive system diseases or endocrine system disorders.

IPA biomarker analysis identified 82 potential biomarkers among E2 significant genes and 93 potential biomarkers among P4 significant genes. There were 62 potential biomarker molecules common to both groups. A complete list of biomarkers expressed in the human uterus is compared with biomarkers identified in Ishikawa cells 3 h and 12 h after E2, P4, and respective modulator treatments (Table S4). Selection of unique E2 and P4 dependent biomarkers that have been related to endometrial receptivity and embryo implantation are listed in Table 3. Regarding the former, these included the following genes related to the endometrium and embryo implantation: *MUC1, EZH2, HMGCR, MDK, PRDM2, PXN,* and *SLIT2* (Table 3). For genes common to both E2 and P4 responsive genes, potential biomarkers were identified that were related to endometrial receptivity or endometriosis, and these included: *ARG2, ANXA1, AR, BMPR2, CDKN1C, CXCL16, EGFR, FGFR1, HMGA1, IGFR2, IL1R1, JAG1, let-7, MCAM, NCOA3, NOTCH1, PDCD4, PGR, RACGAP1, TMSB10,* and *TNC* (Table S4).

Similarly, among P4 responsive genes, potential biomarkers were identified that were related to the development of the endometrium or early pregnancy: *CTNNA1, ERBB3, FGFR2, IGFBP5, IKBKB, IL6ST, KCNMA1, NOTCH3, S100A4, STAT3, TCF7L2, TGFB1,* and *TGFBR3* (Table 3).

A Comparison of the Significant E2 and P4 Responsive Genes Identified in the Ishikawa Cell Line with a Human Endometrial Transcriptome

To predict whether the E2 and P4 responsive genes identified in Ishikawa cells were also expressed in a human endometrium, and/or have roles in the implantation process for embryos, expression of these genes were assayed in two human endometrial tissue samples collected from mid-secretory endometrium. According to RNA-Seq data, 1671 (98.8%) of E2 responsive genes, and 1668 (98.6%) of P4 responsive genes, identified in the Ishikawa cell line were also found to be expressed in human endometrium samples obtained from two patients that underwent endometrial biopsy. These human endometrium samples were only used for comparative purposes. In Table 1, the expressional abundances (e.g., FPKMs) of E2 and P4 biomarkers detected in human endometrial biopsy samples are compared with non-treated Ishikawa cells. Among the IPA-identified E2 and P4 biomarkers present in Ishikawa cells, *EZH2, MDK, MUC1, SLIT2,* and *IL6ST* were also found to be present in human endometrial transcriptomes (Figure 1).

TAM Responsive Genes in the Ishikawa Cell Line that are Related to Reproductive System Diseases

Following the treatment of Ishikawa cells with TAM for 12 h, the expression of 1013 genes were found to be significantly changed compared to non-treated Ishikawa cells. Of these genes, 432 were up-regulated and 581 were down-regulated (Table S5). These results demonstrate that TAM has both antagonistic and agonistic activity towards E2 in Ishikawa cells. Moreover, in addition to influencing E2 regulated genes, TAM significantly altered the expression of 789 genes independently from E2 (Figure 2A).

Using an IPA core analysis, the predicted function of TAM responsive genes was obtained. A total of 168 genes were found to be related to different reproductive system diseases, including uterine, ovarian, and cervical cancers, as well as genital tumors, amenorrhea, metrorrhagia, and polycystic ovary syndrome (Table 4).

One of the main signalling pathways identified from TAM responsive genes was associated with regulation of DNA replication, recombination, and repair, as well as cell cycle progression, and cellular assembly and organization. Moreover, TAM responsive genes encoded molecules that directly, or indirectly, were associated with the cell cycle regulator, cyclin D1 (*CCND1*). Correspondingly, *CCND1* was found to be significantly up-regulated 12 h after treatment with TAM (Figure 3).

RU486 Significant Genes in the Ishikawa Cell Line are Related to Reproductive System Diseases

Treatment of Ishikawa cells with the P4 antagonist, RU486, for 12 h resulted in significant changes in the expression of 546 genes compared with untreated cells, with 255 genes up-regulated and 291 genes down-regulated (Table S6). Similar to TAM, RU486 exhibits both agonistic and antagonistic activities for P4 in Ishikawa cells. For example, 377 genes responsive to RU486 after 12 h did not show significant changes in expression after treatment with P4 for 12 h. Therefore, these genes are regulated independently by RU486 and in the absence of P4 in Ishikawa cells (Figure 2B).

Of the 546 genes found to be responsive to treatment with RU486, 86 encoded molecules related to diseases of the reproductive system. For example, molecules related to adenomyosis, gonadal tumours, metrorrhagia, and uterine leiomyoma were identified (Table 4). Based on the genes that were responsive to treatment with RU486, a signalling pathway related to gene expression, cell-to-cell signalling and interactions, and tissue development was identified. The central molecules in this network, including cadherin 2 (CDH2) and a complex between AR and NFκB, mediate direct and indirect interactions with RU486 responsive genes (Figure 4). For example, the transcriptional co-repressor gene, *NCOR2,* was up-regulated as was the androgen receptor (*AR*) gene. However, transcription factor, FOXA1, was down-regulated. Genes associated with the cell to cell contact and adhesion were also down-regulated, and included: *CTNND1, JUP, CDH2, IQGAP1,* and *COL2A1.* Alternatively, *PDK1* and *ADAM15* were up-regulated, and have roles in the induction of tissue breakdown. Most of the molecules in this network were also related to cell death and apoptosis.

Discussion

The aim of this study was to define the transcriptional response of an endometrial model to treatment with E2, P4, TAM, and RU486. To our knowledge, this is the first report of the application of RNA-Seq to the study of early genome-wide effects in a human

Table 3. Selection of biomarkers related to reproductive system diseases among E2 and P4 significant genes in Ishikawa cell line.

E2 Unique Symbol	Entrez Gene Name	E2 3 h Log Ratio	E2 12 h Log Ratio	TAM 12 h Log Ratio
EZH2	enhancer of zeste homolog 2 (Drosophila)	−0.184	−14	−0.089
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	−0.164	−0.484	−0.042
MDK	midkine (neurite growth-promoting factor 2)	0.296	0.513	−0.211
MUC1	mucin 1, cell surface associated	−0.117	0.557	−0.264
PRDM2	PR domain containing 2, with ZNF domain	−0.333	−0.012	−0.189
PXN	paxillin	0.175	0.417	0.042
SLIT2	slit homolog 2 (Drosophila)	−0.261	−0.447	0.39
P4 Unique Symbol	**Entrez Gene Name**	**P4 3 h Log Ratio**	**P4 12 h Log Ratio**	**RU486 12 h Log Ratio**
CTNNA1	catenin (cadherin-associated protein), alpha 1, 102 kDa	−0.175	−0.265	−0.042
ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	−0.069	−0.264	−0.273
FGFR2	fibroblast growth factor receptor 2	0.125	−0.545	−0.49
IGFBP5	insulin-like growth factor binding protein 5	−0.265	−0.562	0.069
IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	0.228	0.448	0.298
IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	0.176	−0.014	−0.114
KCNMA1	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	0.966	0.465	−0.42
NOTCH3	notch 3	−0.384	−0.559	−0.065
S100A4	S100 calcium binding protein A4	0.09	−0.785	0.031
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	−0.223	−0.003	0.065
TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box)	−0.207	−0.473	−0.072
TGFB1	transforming growth factor, beta 1	0.47	1.189	0.548
TGFBR3	transforming growth factor, beta receptor III	0.504	0.336	0.308

Expression changes are provided in logarithmic scale calculated as following: log (Expression treated/Expression non-treated).
doi:10.1371/journal.pone.0068907.t003

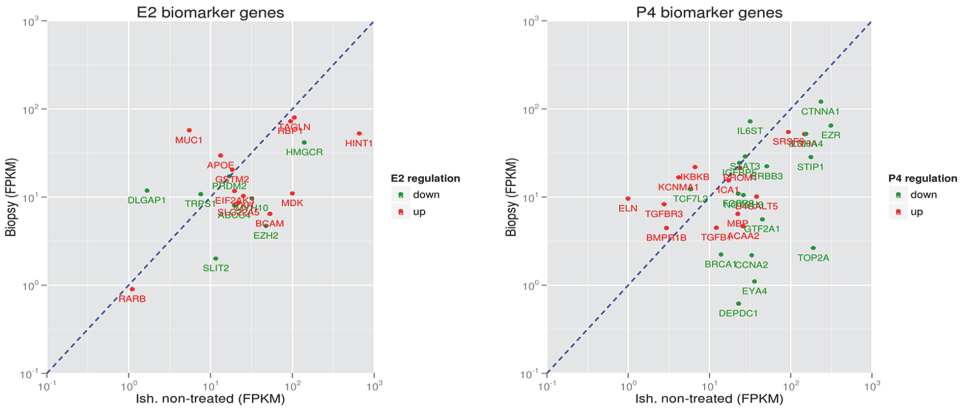

Figure 1. Selection of endometrial specific biomarkers found in 12 h E2 (left) and P4 (right) treated Ishikawa cells and their relative abundance in human endometrial biopsy samples at the time of embryo implantation (n = 2). Red genes up-regulated in E2 and P4 treated Ishikawa cells compared to non-treated cells; green genes down-regulated. Genes situated on the left side of the diagonal line show higher relative abundance (FPKM) in human endometrial biopsy sample compared to non-treated Ishikawa cells. Genes situated on the right side of the diagonal line show lower relative abundance (FPKM) in human endometrial biopsy sample compared to non-treated Ishikawa cells.
doi:10.1371/journal.pone.0068907.g001

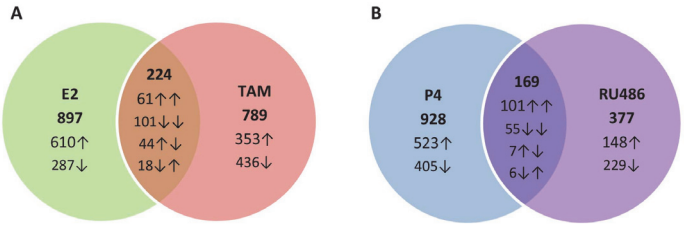

Figure 2. Venn diagram showing significant gene expression changes 12 h E2, TAM, P4 or RU486 treatment, relative to non-treated Ishikawa cells. A Unique and common genes after 12 h E2 and TAM treatment. B Unique and common genes after 12 h P4 and RU486 treatment. The numbers given within each of the circles represent the number of significantly changed genes unique to treatment, and arrows show the manner they are regulated (up- or down-regulation compared to non-treated Ishikawa cells). Overlaps indicate the number of commonly changed genes.
doi:10.1371/journal.pone.0068907.g002

endometrial cell line. Moreover, the majority of genes that were found to be significantly responsive to E2 and P4 in the Ishikawa cell line were also detected in human endometrial biopsies collected at embryo implantation.

During the last decade, microarrays have been the most commonly used method for performing endometrial transcriptome analyses in order to identify genes differentially expressed in proliferative and secretory phases. However, the genes identified in these various studies have not been consistent. These differences could be due to variations in the study design used, and/or other limiting factors of microarray analysis. The prominent limitations associated with microarray analyses include hybridization and cross-hybridization artefacts, differences in data analysis, and low and variable coverage of all genes present in commercially available array platforms [30]. RNA-Seq is a method that has very low, if any, background signal since DNA sequences can be mapped to unique regions of the genome [31]. In addition, unlike DNA microarrays, RNA-Seq does not have any upper limit for quantification, thereby facilitating the detection of genes expressed at very low or very high levels.

Various genes were found to have very high mRNA expression levels following treatment of cells with E2 or P4. These included *PSAP*, *ATP5G2*, *ATP5H*, and *GNB2L1*, which all showed highly abundant transcripts in response to E2 or P4. Housekeeping genes were also highly expressed in all experiments. In previous work, glycoprotein coding gene *PSAP* has been shown to be up-regulated in the endometrium during GnRH antagonist-treated cycles [7]. This protein has been shown to participate in lysosomal hydrolysis of sphingolipids [32]. *PSAP* also has a predicted estrogen response element (ERE) site in its promoter region [33]. The data from the present study are consistent with these previous results, with high levels of *PSAP* detected 12 h after E2 treatment. The ATP synthase subunits, *ATP5G2* and *ATP5H*, as well as the cell proliferation-inducing gene, *GNB2L1*, were found to be highly expressed following treatments with E2 and P4. Although these three genes have not been described in relation to the endometrium in earlier studies, their high levels of expression in response to steroid hormones could be crucial for achieving a receptive state in the endometrium, a tissue which undergoes rapid developmental changes on a monthly basis.

Using Cufflinks and an IPA biomarker filter, genes found to be significantly affected by E2 and P4 were also genes that have been previously characterized as important to the functioning of the endometrium. For example, the expression of histone methyltransferase (*EZH2*) was found to be down-regulated 12 h after E2 treatment compared to non-treated cells, yet the expression was remained 12 h after TAM treatment. Moreover, loss of EZH2 activity in the endometrium has been shown to contribute to the epigenetic programming of decidualizing endometrial stromal cells [34]. An increase in level of *MUC1* mRNA was detected 12 h after treatment with E2 compared to non-treated Ishikawa cells. *MUC1* mRNA was also detected in P4- and RU486-treated Ishikawa cells, although the increases observed were not statistically significant. In the latter case, this may be due to the short duration of the P4 treatment, and different results may be obtained if longer hormonal treatments were used. However, data regarding the role of MUC1 continues to be conflicting as some investigators report an increase in MUC1 in the endometrium during the receptive phase of the endometrium [35,36], while others have reported the opposite result, including the disappearance of MUC1 from pinopodes [37,38]. *MUC1* expression was also found to be not solely dependent on P4 receptors but most likely mediated via non-genomic pathways [39].

Paxillin plays an important role in cellular cytoskeletal formation, and the gene for paxillin (*PXN*) was found to undergo significant up-regulation 12 h after treatment with E2. While treatment with P4 also slightly increased the abundance of *PXN*, the increase was not significant. During the endometrial decidualization process, PXN has been shown to participate in integrin-mediated signal transduction pathways [40]. In these pathways, fibroblast growth factor receptor 2 (*FGFR2*) also has a role, and is expressed in the endometrium at the beginning of the secretory phase. This expression profile coincides with the development of endometrial oedema and the formation of a complex, subepithelial capillary plexus [41]. Based on the RNA-Seq microarray data of the present study, *FGFR2* was relatively abundant in non-treated and E2-treated cells. However, its expression significantly decreased after treatment with P4 for 12 h. Insulin-like growth factor binding protein-5 (*IGFBP5*) was also significantly down-regulated after treatment with P4 for 12 h. This is consistent with a previous report that P4 inhibits the expression of *IGFBP5* during embryo implantation [42]. Furthermore, 98.8% and 98.6% of the genes in Ishikawa cells that were significantly up-regulated following treatment with E2 and P4, respectively, were also present in the human endometrial transcriptome during embryo implantation.

Significant changes in expression were not detected for several known endometrial biomarkers in this study. This may be due to the *in vitro* conditions assayed, or the duration of hormone and modulator treatments applied. For example, expression of leukaemia inhibitory factor (LIF) was not detected, which has previously been shown to be important during embryo implantation in both animal and human studies [43]. Moreover, expression of LIF should be the highest in the luminal and

Table 4. Selection of TAM and RU486 regulated gene products in Ishikawa cells related to reproductive system diseases.

Functions Annotation	p-Value	Molecules
Selection of TAM regulated gene products related to reproductive system diseases		
adenomyosis	2,12E-05	AIG1,ANXA2,CBX6,CXXC5,DST,IQGAP1,LDHA,MALAT1,MTHFD2,TCF4,THBS1,TSPAN12
genital tumor	4,90E-05	ABCB1,ABR,ALDH3A1,ALPP/ALPPL2,ANTXR1,ANXA2,AR,ASS1,ATP1A1,BMPR1B,C9orf5,CCND1,CD44,CDH1,CDH2,CLU,COL18A1, CXXC5,ECT2,EGFL7,EHF,ENO1,EP300,EPHA2,ERBB3,ETV1,FGFR1,FGFR2,FHL2,FN1,GPC1,GPRC5A,GSTP1,HDAC4, HDAC6,HSP90AA1,ING4,ITGB4,JAG2,KIF1B,KRT23,KRT7,LDHA,LETM1,LRP5,LRRN4,MAPK8,MECOM,MKI67,MTHFD2,MYC,NCOR2, NTRK2,PAX8,PDE11A,PGR,PLEKHB1,PRC1,PSMD4,PTAFR,RACGAP1,S100A2,SAT1,SLC12A6,SLC16A3,SLC2A1,SLIT2, SMC4,SNAP25,SORT1,SRSF5,STIP1,TCF4,TFPI2,TMPRSS2,TOP2A,TRADD,TUBA1A,TUBE1,TUSC3,WT1,XIAP,ZNF217
gonadal tumor	1,66E-04	ABCB1,ALDH3A1,ALPP/ALPPL2,AR,CD44,CDH1,CLU,COL18A1,CXXC5,ECT2,ENO1,EP300,EPHA2,ERBB3,FGFR1,FGFR2,FN1,GSTP1, HDAC4,HDAC6,HSP90AA1,JAG2,KRT23,LDHA,LETM1,LRRN4,MECOM,MKI67,MTHFD2,PAX8,PGR,PTAFR,RACGAP1, S100A2,SLC12A6,SLC16A3,SLIT2,SMC4,SORT1,SRSF5,STIP1,TFPI2, TOP2A,TUBA1A,TUBE1,WT1,ZNF217
ovarian cancer	1,94E-04	ABCB1,ALDH3A1,AR,CD44,CDH1,CLU,COL18A1,CXXC5,ECT2,ENO1,EP300,EPHA2,ERBB3,FGFR1,FGFR2,FN1, GSTP1,HDAC4,HDAC6,HSP90AA1,JAG2,KRT23,LETM1,LRRN4,MECOM,MTHFD2,PAX8,PGR,PTAFR, RACGAP1,S100A2,SLC12A6,SLC16A3,SLIT2,SMC4,SORT1,SRSF5,STIP1,TFPI2,TOP2A,TUBA1A,TUBE1,WT1, ZNF217
gynecological disorder	2,20E-04	ABCB1,AIG1,ALDH3A1,ANXA1,ANXA2,AR,ARL4D,ATRX,CBX6,CD44,CDH1,CDH2,CEP70,CLU,COL18A1,CTSF,CXXC5,DST, ECT2,ENO1,EP300,EPHA2,ERBB3,FGFR1,FGFR2,FN1,FOXM1,GLIPR1,GNG11,GSTP1,HDAC4,HDAC6,HSP90AA1, HSPB1,IGFBP5,IGFBP7,IQGAP1,ITGB8,JAG2,JUP,KIAA0664,KRT23,LDHA,LETM1,LRRN4,LTBP1,LTBP4, MALAT1,MECOM,MKI67,MR1,MTHFD2,MYC,NEK2,OLFM1,PAX8,PGR,PLD3,POLG,PTAFR,RACGAP1,RAD51B, RAPGEF3,S100A2,S100A4,SLC12A6,SLC16A3,SLIT2,SMC4,SORT1,SRSF5,STIP1,TAGLN,TCF4,TFPI2,TGFBR3,THBS1,TMSB10/ TMSB4X,TOP2A,TPM2,TSPAN12,TUBA1A,TUBE1,WT1,ZNF217,ZNF350
cervical cancer	1,91E-03	ANXA1,ANXA2,CDH1,CDH2,CTSF,ENO1,FGFR1,FGFR2,FOXM1,GSTP1,HSP90AA1,HSPB1,ITGB8,JUP,LETM1,MKI67,PGR, SLC12A6,TAGLN,TMSB10/TMSB4X,TOP2A,TPM2,TUBA1A,TUBE1,ZNF350
uterine cancer	1,03E-02	AIG1,ANXA1,ANXA2,AR,ARL4D,CDH1,CDH2,CTSF,DST,ENO1,FGFR1,FGFR2, FOXM1,GLIPR1,GNG11,GSTP1,HSP90AA1,HSPB1,IGFBP5,IGFBP7,IQGAP1,ITGB8,JUP,KIAA0664, LETM1,LTBP1,LTBP4,MALAT1,MKI67,MR1,MTHFD2,MYC,OLFM1,PGR,PLD3,RAD51B,RAPGEF3,S100A4,SLC12A6,TAGLN,TMSB10/ TMSB4X,TOP2A, TPM2,TSPAN12,TUBA1A,TUBE1,WT1,ZNF350
amenorrhea	1,30E-02	AR,PGR,TGFBR3
metrorrhagia	1,53E-02	AR,PGR
serous ovarian carcinoma	3,78E-02	CLU,CXXC5,JAG2,LRRN4,PGR,PTAFR,RACGAP1,S100A2,SLIT2,SMC4,SORT1,TFPI2
disorder of ovary	3,90E-02	AR,ATRX,CEP70,ECT2,NEK2,PGR,POLG,TGFBR3
polycystic ovary syndrome	4,90E-02	AR,ATRX,CEP70,ECT2,NEK2,PGR
Selection of RU486 regulated gene products related to reproductive system diseases		
gynecological disorder	5,96E-04	AGR2,ALDH3A1,AR,ARL4D,AURKB,C18orf1,CALCRL,CBX6,CDC7,CDH2,CDKN2A,CTNNAL1,CTNND1,EME1,ENO1, EPCAM,ERBB3,FGFR2,FN1,H2AFX,HDAC4,HDAC7,HDAC9,IQGAP1,JUP,KAT2B,KIAA0664,KRT23, LDHA,MALAT1,MCAM,MECOM,MYC,OLFM1,PAX8,PCM1,PDK4,PGR,RAD51B, RASSF9,RNF144B,SLC16A3,SYNC,TAGLN,TAX1BP1,TCF4,THBS1,TMSB10/TMSB4X,ZDHHC17,ZNF138,ZNF350
adenomyosis	8,84E-04	CBX6,IQGAP1,LDHA,MALAT1,SYNC,TCF4,THBS1
gonadal tumor	3,65E-03	AGR2,ALDH3A1,ALPP/ALPPL2,AR,CDKN2A,CTNNAL1,ENO1,EPCAM,ERBB3,FGFR2,FN1,H2AFX,HDAC4,HDAC7, HDAC9,KAT2B,KRT23,LDHA,MCAM,MECOM,PAX8,PGR,RASSF9,RNF144B,SLC16A3,ZNF138
metrorrhagia	4,64E-03	AR,PGR
uterine leiomyoma	4,74E-03	ARL4D,AURKB,C18orf1,CALCRL,CDC7,CTNND1,IQGAP1,KIAA0664,MALAT1,MYC,OLFM1,PDK4,PGR,RAD51B,ZDHHC17
ovarian cancer	5,10E-03	AGR2,ALDH3A1,AR,CDKN2A,CTNNAL1,ENO1,EPCAM,ERBB3,FGFR2,FN1,H2AFX,HDAC4,HDAC7, HDAC9, KAT2B,KRT23,MCAM,MECOM,PAX8,PGR,RASSF9,RNF144B, SLC16A3, ZNF138
polycystic ovary syndrome	1,45E-02	AR,EME1,PCM1,PGR,TAX1BP1
genital tumor	1,77E-02	AGR2,ALDH3A1,ALPP/ALPPL2,AR,ARG2,AURKB,CDH2,CDKN2A,CGN,CTNNAL1, EHF,ENO1,EPCAM,ERBB3,FGFR2,FHL2,FN1,H2AFX,HDAC4,HDAC7, HDAC9,KAT2B,KRT23,LDHA,MCAM,MECOM,MTA1,MYC,NCOR2,NTRK2,OAZ1,PAX8,PGR,RASSF9, RCAN2,RNF144B,SLC16A3,TCF4,TMPRSS2,ULK3,ZNF138
atypical endometrial hyperplasia	2,84E-02	PGR
metastasis of cervical cancer cell lines	2,84E-02	ZNF350
preterm birth	2,84E-02	PGR
primary hypogonadism	2,84E-02	AR

Table 4. Cont.

Selection of RU486 regulated gene products related to reproductive system diseases		
Functions Annotation	**p-Value**	**Molecules**
subfertility	2,84E-02	PGR
serous ovarian carcinoma process	3,07E-02	CTNNAL1,EPCAM,H2AFX,MCAM,PGR,RASSF9,RNF144B,ZNF138
amenorrhea	3,11E-02	AR,PGR
The categories related to male infertility and breast cancer are excluded		

doi:10.1371/journal.pone.0068907.t004

glandular epithelium during the luteal phase [44]. However, the *in vitro* conditions assayed in the present study did not imitate the physiological conditions of the luteal phase since E2 and P4 were applied separately. Expression of the receptor for LIF, LIFR, was detected, although it did not undergo significant changes in expression following hormone/antagonist treatments. On the other hand, expression of *IL6ST*, a co-receptor of LIFR, was significantly up-regulated after 3 h of P4 treatment. Interestingly, another well-known endometrial marker, vascular endothelial growth factor (VEGF), was not identified in our dataset, although *VEGFB* showed relatively high abundance in all experiments and was slightly (albeit not significantly) up-regulated after 12 h of P4

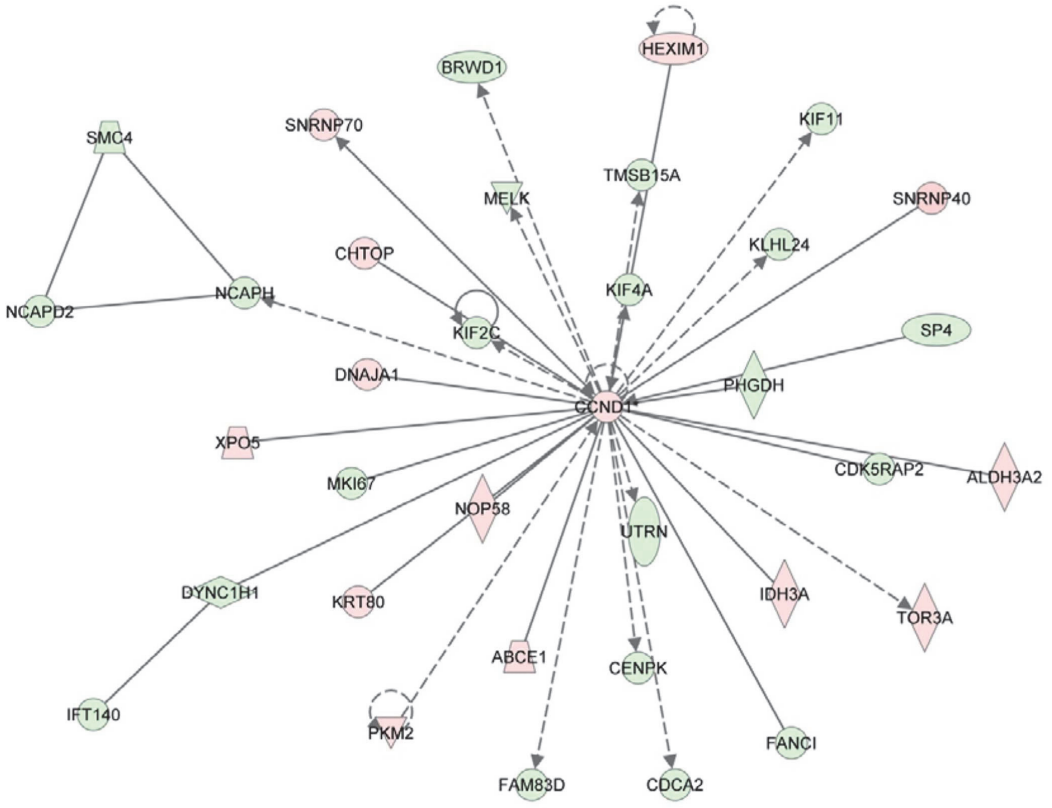

Figure 3. Top 1 network with TAM 12 h significant genes related to DNA replication, recombination and repair, cell cycle, cellular assembly and organization. Red molecules represent up-regulated and green down-regulated genes among TAM 12 h significant genes in Ishikawa cells. The networks were generated through the use of IPA (Ingenuity® Systems, www.ingenuity.com).
doi:10.1371/journal.pone.0068907.g003

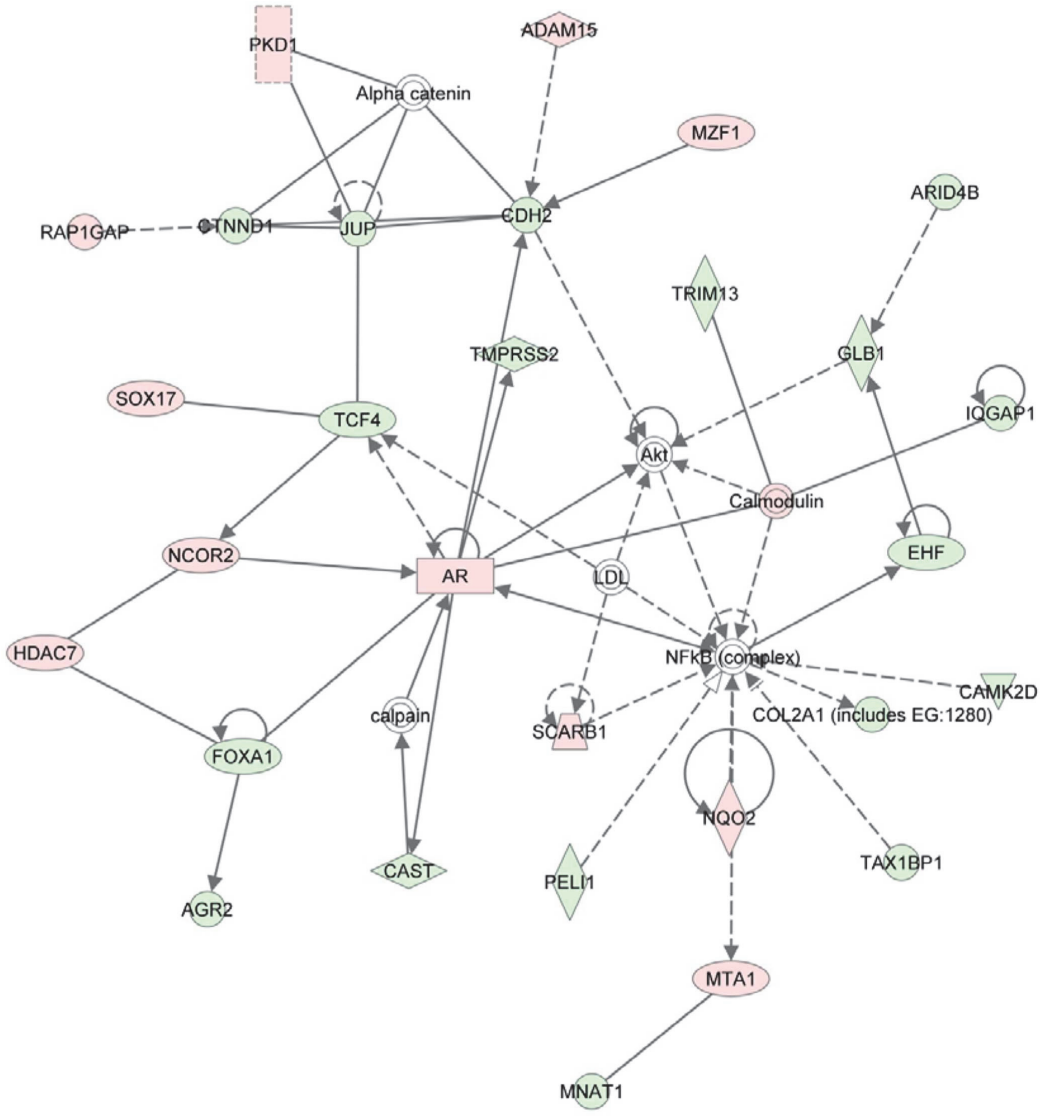

Figure 4. Top one network formed among RU486 significant genes related to gene expression, cell-to-cell signalling and interaction, and tissue development. The central molecules in the network were cadherin 2 (CDH2), AR and NFκB complex. The networks were generated through the use of IPA (Ingenuity® Systems, www.ingenuity.com).
doi:10.1371/journal.pone.0068907.g004

treatment. Based on these results, and those of a previous study that performed immunostaining of VEGF in both glandular epithelial cells and stromal cells during the mid-secretory phase of the human endometrium [45], epithelial Ishikawa cells may not be the best model for studying endometrial vascularization and *VEGF* expression. In addition, expression of epidermal growth factor (EGF) was relatively low, and did not change significantly following any treatment. Given that the endometrium is a complex tissue with different tissue components being important for signal transduction, epithelial cells alone do not sufficiently represent these complexities. In addition, during the natural cycles, endometrial tissue is longer exposed to ovarian steroid hormones. Therefore, some of the effects observed when using these tissues could be induced by stromal factors. In addition, the

changes in mRNA expression reported in the present study represent early changes that occurred following the administration of E2, P4, and their respective modulator treatments. Therefore, it is still possible that these changes could activate the cascade of molecular changes that are eventually needed to achieve a receptive endometrium and successful embryo implantation in humans.

An additional objective of this study was to examine the molecular effects of TAM and RU486 on Ishikawa cells. TAM transduces its signal by competing with E2 for receptor binding, and exhibits antagonistic, or agonistic, activity towards E2 in a tissue-specific manner [46,47]. Moreover, it has been well-documented that the use of TAM is associated with a 2-to-7-fold increase in the incidence of endometrial cancer in TAM-treated patients [48]. However, the molecular mechanisms responsible for the endometrial aberrations observed remain unclear. In a previous study that compared the estrogenic effect of TAM and E2 in Ishikawa cells, the reported gene expression profile was highly diverse and ligand specific. As a result, it was hypothesized that TAM influences the transcriptional response of a specific subset of genes in the uterus [49]. In another study where the endometrial cell line, ECC-1, was treated with TAM versus E2, it was also observed that TAM regulates a subset of specific genes distinct from E2. This result was further confirmed *in vivo* when 256 genes were specifically identified in TAM-treated patients [50,51]. In the current study, 1013 genes were observed to undergo significant changes in expression after 12 h of TAM treatment. Moreover, only 224 of these genes overlapped with genes that underwent changes following treatment with E2 for 12 h. In addition, a total of 168 of these TAM-specific genes were associated with various diseases of the reproductive system. Consistent with *in vivo* data obtained from postmenopausal women treated with TAM [50,51]), cyclin D1 (*CCND1*) was found to be significantly up-regulated 12 h after treatment with TAM in the Ishikawa cell line. CCND1 was also found to be a central molecule in a signalling network identified from the TAM responsive genes revealed in the current study. This network was associated with mediating early processes related to DNA replication, recombination and repair, as well as cell cycle progression, cellular assembly, and cellular organization. Amplification or overexpression of *CCND1* has been shown to play a pivotal role in the development of several human cancers, including parathyroid adenoma, breast cancer, colon cancer, lymphoma, melanoma, and prostate cancer [52]. In addition, several studies have been reported an abundance of CCND1 in endometrial carcinoma [53,54], which is hypothesized to be caused by altered protein degradation and nuclear export due to mutations present in threonine 286 of the *CCND1* coding region [55].

In most cases, TAM exhibited antagonistic activity towards E2 specific genes. However, a subset of genes was found to be similarly regulated (e.g., up-regulated or down-regulated) after both E2 and TAM treatments, thereby demonstrating agonistic activity. Specifically, TAM and E2 both up-regulated mRNAs of *AR, FGFR1, KIAA0664, MALAT1, OLFM1, TMSB10/TMSB4X, TPM2, JAG2, PAX8,* and *SRSF5*. Moreover, this set of genes is believed to be related to uterine, ovarian, and cervical cancers [56,57]. In addition, RNA-Seq data from the present study indicated that the mRNA of ferritin light chain (*FTL*) was present in very high abundance after 12 h of E2 treatment and TAM treatment. Ferritin is the major intracellular iron storage protein in cells and variations in ferritin subunit composition has the potential to affect the rates of iron uptake and release in different tissues [58].

The classical P4 antagonist, RU486, has been used for emergency contraception and the medical termination of pregnancies up to 49 days after gestation based on its ability to block the action of P4 by binding to its receptor expressed by the endometrium. As a result, impaired endometrial maturation leads to degeneration and shedding of the endometrial lining, thereby preventing or disrupting implantation of the conceptus [59,60]. In women, a single dose of mifepristone (200 mg) during the secretory phase of a cycle rapidly renders the endometrium unreceptive, and has been shown to alter gene expression in the uterus within 6 h of oral administration [61,62]. When considering these effects on the endometrium, it is also important to consider that RU486 has both antagonistic and agonistic activities towards PRs, yet exhibits additionally anti-glucocorticoid and anti-androgenic activities [63]. The results of the RNA-Seq analysis in the present study found that proline dehydrogenase 1 (*PRODH*) was down-regulated and thrombospondin 1 (*THBS1*) was up-regulated following RU486 administration, and these results are consistent with those described for the human endometrium [64]. In addition, the genes, follistatine-like 1 (*FSTL1*) and epidermal growth factor receptor (*ERBB3*), showed similar levels of down-regulation after 12 h of treatment with RU486, and these results are consistent with those previously reported for rhesus monkeys in response to treatment with RU486 for four days [65]. Previous studies have also shown that treatment with RU486 in the early luteal phase inhibits normal down-regulation of the PR gene (*PGR*) [66]. Correspondingly, expression of *PGR* was found to be significantly up-regulated after 12 h of treatment with RU486, in our analysis. However, the observation that *PGR* expression was also up-regulated after 12 h of P4 treatment is inconsistent with previous studies. Most likely, longer P4 treatments are needed for *PGR* suppression.

A functional analysis of RU486 responsive genes in Ishikawa cells identified a signalling pathway associated with cell-to-cell signalling, cell-to-cell interactions, and tissue development. Central molecules of this signalling pathway include CDH2, AR, and NFκB. The early molecular effects of RU486 also appear to involve the down-regulation of adhesion molecules and the induction of molecules related to apoptosis. For example, calcium-dependent cadherin CDH2, which is responsible for cell-cell adhesion, was found to be significantly suppressed following treatment with RU486. Similar was seen for adhesion molecule JUP. Nuclear receptor AR and nuclear co-repressor, NCOR2, were both found to be up-regulated following RU486 treatment. Correspondingly, in a previous study their recruitment was shown to be enhanced by RU486 [67]. While the expression of NFκB did not directly change, it was directly, or indirectly, linked to molecules related to apoptosis. As a result, it could be responsible for endometrial tissue shedding and early abortions of the conceptus.

In conclusion, these results provide valuable insight into the mechanisms of early steroid hormone signalling and the consequences of antagonist/agonist action in the human endometrium. However, studies of other *in vitro* models, as well as an analysis of additional human samples, is needed to confirm the early endometrial changes observed to be mediated by E2 and P4 and their modulators in this study.

Supporting Information

Figure S1 E2 significant genes in Ishikawa cell line. 1691 known genes showed significantly changed mRNA expression after 3 h (first column) and 12 h (second column) E2 treatment. 12 h TAM treatment (third column) had antagonistic activity on most of the E2 significant genes instead of 61 genes, which showed similar up-regulated expression and 101 genes, which had similar down-regulative expression pattern after E2 and TAM treatments. For data visualization hierarchical clustering was used. Genes were

clustered by taking account E2 significant genes after 3 h and 12 h treatment and compared to 12 h TAM.
(TIF)

Figure S2 P4 significant genes in Ishikawa cell line. The expression of 1692 known genes was significantly changed after 3 h (first column) and 12 h (second column) P4 treatment. 12 h RU486 treatment (third column) had antagonistic activity on most of the P4 significant genes instead of 101 genes, which showed similar up-regulated expression and 55 genes, which had similar down-regulated expression pattern after P4 and RU486 treatments.
(TIF)

Figure S3 Genes up (red) – or down (green) regulated by ERα (ESR1) in Ishikawa cells after 12 h E2 treatment. The networks were generated through the use of IPA (Ingenuity® Systems, www.ingenuity.com).
(TIF)

Figure S4 Genes up (red) – or down (green) regulated by PRA (PGR) in Ishikawa cells after 12 h P4 treatment. The networks were generated through the use of IPA (Ingenuity® Systems, www.ingenuity.com).
(TIF)

Table S1 FPKM values of genes in active transcriptome of non-treated, E2, P4, TAM and RU486 treated Ishikawa cells and human endometrial biospy samples (n = 2).
(XLSX)

Table S2 Gene expression changes significantly changed after E2 treatment in Ishikawa cell line compared to non-treated cells.
(XLSX)

Table S3 Gene expressions significantly changed after P4 treatments of Ishikawa cells.
(XLSX)

Table S4 Biomarkers related to reproductive system diseases or endocrine system disorders among E2 and P4 significant genes in Ishikawa cell line.
(XLS)

Table S5 Gene expressions (lndiff) significantly changed after TAM treatment in Ishikawa cells.
(XLSX)

Table S6 Gene expressions (lndiff) significantly changed after RU486 treatment in Ishikawa cells.
(XLSX)

Acknowledgments

We are grateful to the participating women and medical staff from the Nova Vita Clinic, especially Dr. Talving who performed the endometrial biopsy procedures. We also thank Prof. Anneli Stavreus-Evers who kindly provided the Ishikawa cell line and Margus Rosenstein for his experimental assistance and manuscript review.

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work. Although Karin Tamm-Rosenstein is the employee of Nova Vita Clinic, this does not alter her adherence to all the PLOS One policies on sharing data and materials.

Author Contributions

Conceived and designed the experiments: MM. Performed the experiments: KTR MS. Analyzed the data: JS. Contributed reagents/materials/analysis tools: AS. Wrote the paper: KTR. Manuscript revision: KTR JS MS AS MM.

References

1. Graham JD, Clarke CL (1997) Physiological action of progesterone in target tissues. Endocr Rev 18: 502–519.
2. Carson DD, Lagow E, Thathiah A, Al-Shami R, Farach-Carson MC, et al. (2002) Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening. Mol Hum Reprod 8: 871–879.
3. Kao LC, Tulac S, Lobo S, Imani B, Yang JP, et al. (2002) Global gene profiling in human endometrium during the window of implantation. Endocrinology 143: 2119–2138.
4. Borthwick JM, Charnock-Jones DS, Tom BD, Hull ML, Teirney R, et al. (2003) Determination of the transcript profile of human endometrium. Mol Hum Reprod 9: 19–33.
5. Riesewijk A, Martin J, van Os R, Horcajadas JA, Polman J, et al. (2003) Gene expression profiling of human endometrial receptivity on days LH+2 versus LH+7 by microarray technology. Mol Hum Reprod 9: 253–264.
6. Horcajadas JA, Riesewijk A, Martin J, Cervero A, Mosselman S, et al. (2004) Global gene expression profiling of human endometrial receptivity. J Reprod Immunol 63: 41–49.
7. Mirkin S, Nikas G, Hsiu JG, Diaz J, Oehninger S (2004) Gene expression profiles and structural/functional features of the peri-implantation endometrium in natural and gonadotropin-stimulated cycles. J Clin Endocrinol Metab 89: 5742–5752.
8. Talbi S, Hamilton AE, Vo KC, Tulac S, Overgaard MT, et al. (2006) Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. Endocrinology 147: 1097–1121.
9. Altmae S, Martinez-Conejero JA, Salumets A, Simon C, Horcajadas JA, et al. (2010) Endometrial gene expression analysis at the time of embryo implantation in women with unexplained infertility. Mol Hum Reprod 16: 178–187.
10. Haouzi D, Assou S, Mahmoud K, Tondeur S, Reme T, et al. (2009) Gene expression profile of human endometrial receptivity: comparison between natural and stimulated cycles for the same patients. Hum Reprod 24: 1436–1445.
11. Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, et al. (1997) Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab 82: 4258–4265.
12. Kuiper GG, Gustafsson JA (1997) The novel estrogen receptor-beta subtype: potential role in the cell- and promoter-specific actions of estrogens and anti-estrogens. FEBS Lett 410: 87–90.
13. Wen DX, Xu YF, Mais DE, Goldman ME, McDonnell DP (1994) The A and B isoforms of the human progesterone receptor operate through distinct signaling pathways within target cells. Mol Cell Biol 14: 8356–8364.
14. Kastner P, Krust A, Turcotte B, Stropp U, Tora L, et al. (1990) Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J 9: 1603–1614.
15. Wang H, Critchley HO, Kelly RW, Shen D, Baird DT (1998) Progesterone receptor subtype B is differentially regulated in human endometrial stroma. Mol Hum Reprod 4: 407–412.
16. Lessey BA, Killam AP, Metzger DA, Haney AF, Greene GL, et al. (1988) Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle. J Clin Endocrinol Metab 67: 334–340.
17. Berrodin TJ, Jelinsky SA, Graciani N, Butera JA, Zhang Z, et al. (2009) Novel progesterone receptor modulators with gene selective and context-dependent partial agonism. Biochem Pharmacol 77: 204–215.
18. Buzdar AU, Hortobagyi GN (1998) Tamoxifen and toremifene in breast cancer: comparison of safety and efficacy. J Clin Oncol 16: 348–353.
19. Bergman L, Beelen ML, Gallee MP, Hollema H, Benraadt J, et al. (2000) Risk and prognosis of endometrial cancer after tamoxifen for breast cancer. Comprehensive Cancer Centres' ALERT Group. Assessment of Liver and Endometrial cancer Risk following Tamoxifen. Lancet 356: 881–887.
20. Cohen I (2004) Endometrial pathologies associated with postmenopausal tamoxifen treatment. Gynecol Oncol 94: 256–266.
21. Goldberg JR, Plescia MG, Anastasio GD (1998) Mifepristone (RU 486): current knowledge and future prospects. Arch Fam Med 7: 219–222.
22. Brogden RN, Goa KL, Faulds D (1993) Mifepristone. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential. Drugs 45: 384–409.
23. Croxtall JD, Elder MG, White JO (1990) Hormonal control of proliferation in the Ishikawa endometrial adenocarcinoma cell line. J Steroid Biochem 35: 665–669.
24. Lessey BA, Ilesanmi AO, Castelbaum AJ, Yuan L, Somkuti SG, et al. (1996) Characterization of the functional progesterone receptor in an endometrial adenocarcinoma cell line (Ishikawa): progesterone-induced expression of the alpha1 integrin. J Steroid Biochem Mol Biol 59: 31–39.

25. Nishida M, Kasahara K, Kaneko M, Iwasaki H, Hayashi K (1985) [Establishment of a new human endometrial adenocarcinoma cell line, Ishikawa cells, containing estrogen and progesterone receptors]. Nihon Sanka Fujinka Gakkai Zasshi 37: 1103–1111.
26. Trapnell C, Pachter L, Salzberg SL (2009) TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25: 1105–1111.
27. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, et al. (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol 28: 511–515.
28. Flicek P, Aken BL, Beal K, Ballester B, Caccamo M, et al. (2008) Ensembl 2008. Nucleic Acids Res 36: D707–714.
29. Golouh R, Cufer T, Sadikov A, Nussdorfer P, Usher PA, et al. (2008) The prognostic value of Stathmin-1, S100A2, and SYK proteins in ER-positive primary breast cancer patients treated with adjuvant tamoxifen monotherapy: an immunohistochemical study. Breast Cancer Res Treat 110: 317–326.
30. Casneuf T, Van de Peer Y, Huber W (2007) In situ analysis of cross-hybridisation on microarrays and the inference of expression correlation. BMC Bioinformatics 8: 461.
31. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods 5: 621–628.
32. O'Brien JS, Kishimoto Y (1991) Saposin proteins: structure, function, and role in human lysosomal storage disorders. FASEB J 5: 301–308.
33. Zhang X, Bocca S, Franchi A, Anderson S, Kaur M, et al. (2010) Do GnRH analogues directly affect human endometrial epithelial cell gene expression? Mol Hum Reprod 16: 347–360.
34. Grimaldi G, Christian M, Steel JH, Henriet P, Poutanen M, et al. (2011) Down-regulation of the histone methyltransferase EZH2 contributes to the epigenetic programming of decidualizing human endometrial stromal cells. Mol Endocrinol 25: 1892–1903.
35. Aplin JD, Hey NA, Graham RA (1998) Human endometrial MUC1 carries keratan sulfate: characteristic glycoforms in the luminal epithelium at receptivity. Glycobiology 8: 269–276.
36. Acosta AA, Elberger L, Borghi M, Calamera JC, Chemes H, et al. (2000) Endometrial dating and determination of the window of implantation in healthy fertile women. Fertil Steril 73: 788–798.
37. Horne AW, White JO, Lalani el N (2002) Adhesion molecules and the normal endometrium. BJOG 109: 610–617.
38. Horne AW, Lalani EN, Margara RA, Ryder TA, Mobberley MA, et al. (2005) The expression pattern of MUC1 glycoforms and other biomarkers of endometrial receptivity in fertile and infertile women. Mol Reprod Dev 72: 216–229.
39. Horne AW, Lalani EN, Margara RA, White JO (2006) The effects of sex steroid hormones and interleukin-1-beta on MUC1 expression in endometrial epithelial cell lines. Reproduction 131: 733–742.
40. Maruyama T, Yoshimura Y, Sabe H (1999) Tyrosine phosphorylation and subcellular localization of focal adhesion proteins during in vitro decidualization of human endometrial stromal cells. Endocrinology 140: 5982–5990.
41. Moller B, Rasmussen C, Lindblom B, Olovsson M (2001) Expression of the angiogenic growth factors VEGF, FGF-2, EGF and their receptors in normal human endometrium during the menstrual cycle. Mol Hum Reprod 7: 65–72.
42. Okada H, Nakajima T, Yoshimura T, Yasuda K, Kanzaki H (2003) Microarray analysis of genes controlled by progesterone in human endometrial stromal cells in vitro. Gynecol Endocrinol 17: 271–280.
43. Arici A, Engin O, Attar E, Olive DL (1995) Modulation of leukemia inhibitory factor gene expression and protein biosynthesis in human endometrium. J Clin Endocrinol Metab 80: 1908–1915.
44. Hsieh YY, Lin CS, Sun YL, Chang CC, Tsai HD, et al. (2002) In vivo gene transfer of leukemia inhibitory factor (LIF) into mouse endometrium. J Assist Reprod Genet 19: 79–83.
45. Sugino N, Kashida S, Karube-Harada A, Takiguchi S, Kato H (2002) Expression of vascular endothelial growth factor (VEGF) and its receptors in human endometrium throughout the menstrual cycle and in early pregnancy. Reproduction 123: 379–387.
46. Lonard DM, Smith CL (2002) Molecular perspectives on selective estrogen receptor modulators (SERMs): progress in understanding their tissue-specific agonist and antagonist actions. Steroids 67: 15–24.
47. Shang Y, Brown M (2002) Molecular determinants for the tissue specificity of SERMs. Science 295: 2465–2468.
48. McGonigle KF, Smith DD, Marx HF, Morgan RJ, Vasilev SA, et al. (2006) Uterine effects of tamoxifen: a prospective study. Int J Gynecol Cancer 16: 814–820.
49. Johnson SM, Maleki-Dizaji M, Styles JA, White IN (2007) Ishikawa cells exhibit differential gene expression profiles in response to oestradiol or 4-hydroxytamoxifen. Endocr Relat Cancer 14: 337–350.
50. Gielen SC, Kuhne LC, Ewing PC, Blok LJ, Burger CW (2005) Tamoxifen treatment for breast cancer enforces a distinct gene-expression profile on the human endometrium: an exploratory study. Endocr Relat Cancer 12: 1037–1049.
51. Gielen SC, Burger CW, Kuhne LC, Hanifi-Moghaddam P, Blok LJ (2005) Analysis of estrogen agonism and antagonism of tamoxifen, raloxifene, and ICI182780 in endometrial cancer cells: a putative role for the epidermal growth factor receptor ligand amphiregulin. J Soc Gynecol Investig 12: e55–67.
52. Fu M, Wang C, Li Z, Sakamaki T, Pestell RG (2004) Minireview: Cyclin D1: normal and abnormal functions. Endocrinology 145: 5439–5447.
53. Cao QJ, Einstein MH, Anderson PS, Runowicz CD, Balan R, et al. (2002) Expression of COX-2, Ki-67, cyclin D1, and P21 in endometrial endometrioid carcinomas. Int J Gynecol Pathol 21: 147–154.
54. Machin P, Catasus L, Pons C, Munoz J, Matias-Guiu X, et al. (2002) CTNNB1 mutations and beta-catenin expression in endometrial carcinomas. Hum Pathol 33: 206–212.
55. Moreno-Bueno G, Rodriguez-Perales S, Sanchez-Estevez C, Hardisson D, Sarrio D, et al. (2003) Cyclin D1 gene (CCND1) mutations in endometrial cancer. Oncogene 22: 6115–6118.
56. Wong YF, Cheung TH, Lo KW, Yim SF, Siu NS, et al. (2007) Identification of molecular markers and signaling pathway in endometrial cancer in Hong Kong Chinese women by genome-wide gene expression profiling. Oncogene 26: 1971–1982.
57. Guo F, Li Y, Liu Y, Wang J, Li Y, et al. (2010) Inhibition of metastasis-associated lung adenocarcinoma transcript 1 in CaSki human cervical cancer cells suppresses cell proliferation and invasion. Acta Biochim Biophys Sin (Shanghai) 42: 224–229.
58. Brown AJ, Leibold EA, Munro HN (1983) Isolation of cDNA clones for the light subunit of rat liver ferritin: evidence that the light subunit is encoded by a multigene family. Proc Natl Acad Sci U S A 80: 1265–1269.
59. Danielsson KG, Swahn ML, Bygdeman M (1997) The effect of various doses of mifepristone on endometrial leukaemia inhibitory factor expression in the midluteal phase–an immunohistochemical study. Hum Reprod 12: 1293–1297.
60. Lalitkumar PG, Sengupta J, Karande AA, Ghosh D (1998) Placental protein 14 in endometrium during menstrual cycle and effect of early luteal phase mifepristone administration on its expression in implantation stage endometrium in the rhesus monkey. Hum Reprod 13: 3478–3486.
61. Hapangama DK, Critchley HO, Henderson TA, Baird DT (2002) Mifepristone-induced vaginal bleeding is associated with increased immunostaining for cyclooxygenase-2 and decrease in prostaglandin dehydrogenase in luteal phase endometrium. J Clin Endocrinol Metab 87: 5229–5234.
62. Danielsson KG, Marions L, Bygdeman M (2003) Effects of mifepristone on endometrial receptivity. Steroids 68: 1069–1075.
63. Chwalisz K, Brenner RM, Fuhrmann UU, Hess-Stumpp H, Elger W (2000) Antiproliferative effects of progesterone antagonists and progesterone receptor modulators on the endometrium. Steroids 65: 741–751.
64. Catalano RD, Critchley HO, Heikinheimo O, Baird DT, Hapangama D, et al. (2007) Mifepristone induced progesterone withdrawal reveals novel regulatory pathways in human endometrium. Mol Hum Reprod 13: 641–654.
65. Ghosh D, Sharkey AM, Charnock-Jones DS, Smith SK, Sengupta J (2009) Effect of low-dose mifepristone administration on day 2 after ovulation on transcript profiles in implantation-stage endometrium of rhesus monkeys. Reproduction 138: 357–370.
66. Sun X, Christow A, Marions L, Gemzell-Danielsson K (2003) Progesterone receptor isoform B in the human fallopian tube and endometrium following mifepristone. Contraception 67: 319–326.
67. Hodgson MC, Astapova I, Cheng S, Lee LJ, Verhoeven MC, et al. (2005) The androgen receptor recruits nuclear receptor CoRepressor (N-CoR) in the presence of mifepristone via its N and C termini revealing a novel molecular mechanism for androgen receptor antagonists. J Biol Chem 280: 6511–6519.

PUBLICATION III

Altmäe S, **Tamm-Rosenstein K**, Esteban FJ, Simm J, Kolberg L, Peterson H, Metsis M, Haldre K, Horcajadas JA, Salumets A, and Stavreus-Evers A. Endometrial transcriptome analysis indicates superiorty of natural over artifical cycles in recurrent implantation failure patients undergoing frozen embryo transfer. Reproductive BioMedicine Online. In Press.

Reproductive BioMedicine Online (2016) ■■, ■■–■■

www.sciencedirect.com
www.rbmonline.com

ARTICLE

Endometrial transcriptome analysis indicates superiority of natural over artificial cycles in recurrent implantation failure patients undergoing frozen embryo transfer

Signe Altmäe [a,b,*], Karin Tamm-Rosenstein [c], Francisco J Esteban [d], Jaak Simm [c], Liis Kolberg [e], Hedi Peterson [e,f], Madis Metsis [a,g], Kai Haldre [h], José A Horcajadas [i], Andres Salumets [a,j,1], Anneli Stavreus-Evers [k,1]

[a] *Competence Centre on Health Technologies, Tartu, Estonia;* [b] *Department of Paediatrics, School of Medicine, University of Granada, Granada, Spain;* [c] *Department of Gene Technology, Tallinn University of Technology, Tallinn, Estonia;* [d] *Department of Experimental Biology, University of Jaén, Jaén, Spain;* [e] *Institute of Computer Science, University of Tartu, Estonia;* [f] *Quretec Ltd., Tartu, Estonia;* [g] *School of Natural Sciences and Health, Tallinn University, Tallinn, Estonia;* [h] *Centre for Reproductive Medicine, West Tallinn Central Hospital Women's Clinic, Tallinn, Estonia;* [i] *Araid at I + CS, Hospital Miguel Servet, Zaragoza, Spain;* [j] *Department of Obstetrics and Gynaecology, University of Tartu, Tartu, Estonia;* [k] *Department of Women's and Children's Health, Uppsala University, Uppsala, Sweden*

* Corresponding author. *E-mail address:* signealtmae@ugr.es (S Altmäe). [1] These authors contributed equally to the manuscript.

Abstract Little consensus has been reached on the best protocol for endometrial preparation for frozen embryo transfer (FET). It is not known how, and to what extent, hormone supplementation in artificial cycles influences endometrial preparation for embryo implantation at a molecular level, especially in patients who have experienced recurrent implantation failure. Transcriptome analysis of 15 endometrial biopsy samples at the time of embryo implantation was used to compare two different endometrial preparation protocols, natural versus artificial cycles, for FET in women who have experienced recurrent implantation failure compared with fertile women. IPA and DAVID were used for functional analyses of differentially expressed genes. The TRANSFAC database was used to identify oestrogen and progesterone response elements upstream of differentially expressed genes. Cluster analysis demonstrated that natural cycles are associated with a better endometrial receptivity transcriptome than artificial cycles. Artificial cycles seemed to have a stronger negative effect on expression of genes and pathways crucial for endometrial receptivity, including *ESR2*, *FSHR*, *LEP*, and several interleukins and matrix metalloproteinases. Significant overrepresentation of oestrogen response elements among the genes with deteriorated expression in artificial cycles ($P < 0.001$) was found; progesterone response elements predominated in genes with amended expression with artificial cycles ($P = 0.0052$).

http://dx.doi.org/10.1016/j.rbmo.2016.03.004
1472-6483/

Please cite this article in press as: Signe Altmäe, Karin Tamm-Rosenstein, Francisco J. Esteban, Jaak Simm, Liis Kolberg, Hedi Peterson, Madis Metsis, Kai Haldre, José A. Horcajadas, Andres Salumets, Anneli Stavreus-Evers, Endometrial transcriptome analysis indicates superiority of natural over artificial cycles in patients who have experienced recurrent implantation failure undergoing frozen embryo transfer, Reproductive BioMedicine Online (2016), doi: 10.1016/j.rbmo.2016.03.004

KEYWORDS: artificial cycle, endometrial receptivity, frozen embryo transfer, hormone response elements, recurrent implantation failure, unexplained female infertility

Introduction

The current trend towards cryopreservation of all embryos after IVF, with transfer of a thawed embryo in a subsequent cycle now contributes more substantially than ever to the cumulative live birth rates after IVF treatment. Together with the evidence of improved endometrial receptivity in cycles without ovarian stimulation (Roque et al., 2013; Shapiro et al., 2011, 2013), the increased success rate of frozen embryo transfer (FET) now equals or even betters that of IVF with fresh embryo transfer (Allersma et al., 2013; Roque et al., 2015; Shapiro et al., 2014; Wong et al., 2014). Furthermore, the better perinatal outcomes of preterm birth, low birth weight and being small for gestational age among singletons born after the transfer of frozen–thawed embryos compared with infants born after ovarian stimulation and IVF (Ishihara et al., 2014; Maheshwari et al., 2012; Pinborg et al., 2013; Wennerholm et al., 2013) have led to an important shift from fresh embryo transfers in IVF towards a freeze-all strategy (Evans et al., 2014; Wong et al., 2014).

A crucial aspect of FET cycles is the timing and preparation of the endometrium to receive the transferred embryo(s). Protocols used in FET include natural cycle (NC-FET) and artificial cycle (AC-FET) with or without preceding pituitary down-regulation through GnRH agonist co-treatment (Hill et al., 2010). Patients undergoing AC-FET start with daily oestrogens, supplemented with progesterone when the endometrium has reached sufficient thickness (Groenewoud et al., 2012). Although the advantages of natural cycles FET (NC-FET) such as less medication and cheaper price somewhat counterbalance the need for more frequent ultrasonographic evaluation of the dominant follicle, the risk of unexpected ovulation and insufficient endometrial development in these cycles (Groenewoud et al., 2012), the clinical preference for the predictability and reliability of AC-FET has prevailed (Givens et al., 2009). So far, however, no clear data support one endometrial preparation method over another. In a recent systematic review and meta-analysis, it was concluded that there are no differences in the clinical pregnancy rate, ongoing pregnancy rate or live birth rate in connection with the different methods of endometrial preparation before FET (Groenewoud et al., 2013). Furthermore, despite active investigation of the endometrial transcriptome that clearly demonstrates an unfavourable endometrial gene expression profile during hormonal stimulation in ovarian stimulation (Haouzi et al., 2009a; Horcajadas et al., 2008; Ruiz-Alonso et al., 2012), there is no knowledge of how and to what extent hormonally supplemented cycles influence endometrial preparation for embryo implantation at a molecular level.

In the present study, endometrial gene expression profiles in infertile women undergoing two different endometrial preparation protocols (AC-FET and NC-FET) was compared with fertile women in a natural cycle. Our study group of women who had experienced recurrent implantation failure (RIF), also diagnosed as unexplained infertility, is especially intriguing as we have demonstrated altered endometrial receptivity in these women (Aghajanova et al., 2009; Altmäe et al., 2010). Therefore, we aimed to clarify whether AC-FET with oestrogen and progesterone improves endometrial maturation in our study group. To answer this question, we aimed to identify two groups of genes with opposite transcriptional behaviour in patients who have experienced RIF. First, we were interested in the genes, which show abnormal expression in the natural cycle, but are amended in artificial cycles and, second, the opposite case of genes showing normal expression in natural cycles, with deteriorated gene-activity after administration of steroid hormones in artificial cycles. By analysing the genes in both categories, the aim was to arrive at conclusions about the pros and cons of using artificial cycles in FET.

Endometrial tissue is one of a few tissues that are overwhelmingly controlled by steroid hormones, oestradiol and progesterone. These steroid hormones act as transcriptional regulators via ligand-bound receptor complexes interacting with the DNA consensus sequences in target genes, referred to as hormone response elements (HRE). Although major progress has been made in deciphering the HRE for oestrogen response elements and progesterone response elements, little is known about their roles in embryo implantation. Additionally we set out to analyse the oestrogen response elements and progesterone response element sequences –50 kb upstream of genes that were differentially expressed after artificial cycles or related to infertility.

The aforementioned aims are critical in obtaining a better understanding of the mechanisms of steroid hormone involvement in endometrial maturation, and in the long term this knowledge should help to devise better hormonal regimens for FET, even for patients with the complication of RIF.

Materials and methods

Study design and endometrial biopsy sample collection

In total fifteen endometrial biopsy samples were obtained from women with unexplained RIF in NC-FET ($n = 5$), women with unexplained RIF in AC-FET ($n = 5$), and from healthy women with proven fertility in natural cycles (NC-FC) ($n = 5$). The characteristics of the women are presented in **Table 1**. Two infertile women provided biopsy samples for both NC-FET and AC-FET.

The fertile control women were candidates for oocyte donation, and were recruited at the Instituto Valenciano de Infertilidad, Valencia, Spain. All women signed an informed consent document approved by the local Ethics Committee (15 May 2007).

Please cite this article in press as: Signe Altmäe, Karin Tamm-Rosenstein, Francisco J. Esteban, Jaak Simm, Liis Kolberg, Hedi Peterson, Madis Metsis, Kai Haldre, José A. Horcajadas, Andres Salumets, Anneli Stavreus-Evers, Endometrial transcriptome analysis indicates superiority of natural over artificial cycles in patients who have experienced recurrent implantation failure undergoing frozen embryo transfer, Reproductive BioMedicine Online (2016), doi: 10.1016/j.rbmo.2016.03.004

Table 1 Characteristics of the study groups.

	RIF, NC-FET (n = 5)	*RIF, AC-FET (n = 5)*	*NC-FC (n = 5)*
Age (years)	30.2 ± 4.3	32.4 ± 5.0	31.8 ± 3.8
Body mass index (kg/m^2)	20.7 ± 1.8	20.9 ± 2.0	23.5 ± 2.1
Cycle length (days)	28.2 ± 1.7	28.0 ± 1.4	28.4 ± 0.7
Menses duration (days)	4.4 ± 0.7	4.5 ± 0.7	4.0 ± 0.2
Previous implantation failures	3.4 ± 0.9	3.4 ± 0.6	0
Parity	0	0	1.5 ± 0.2
Endometrial thickness (mm)	9.3 ± 0.9	8.5 ± 1.7	n.a.
Biopsy sample taken	LH+7	Prog+6	LH+7

Results are mean ± SD.
AC-FET, artificial cycle-frozen embryo transfer; LH+, day since the luteinizing hormone (LH) surge; na, not assessed; NC-FC, natural cycle-fertile control; NC-FET, natural cycle-frozen embryo transfer; Prog+, progesterone administration in days; RIF, recurrent implantation failure.

The patient group of women with unexplained RIF attended the Department of Obstetrics and Gynaecology, Karolinska University Hospital Huddinge, Sweden. The Ethics Committee of Karolinska Institutet approved the study (10 March 2003, number 78/03) and signed informed consent was obtained from every patient. Unexplained RIF was diagnosed by a set of tests that included normal concentrations of thyroid-stimulating hormone (0.4–4.7 mU/L), prolactin (3–27 mg/L), oestradiol (follicular phase <600 pmol/L), LH (follicular phase 1.8–12 U/L), FSH (follicular phase 2.5–10 U/L) and progesterone (luteal phase >17 nmol/L). The infertile women had patent fallopian tubes as determined by hysterosalpingosonography, a normal mid-secretory endometrial thickness of 9.3 ± 0.5 mm (mean ± SD), and their partners had normal semen analysis results according to World Health Organization criteria (WHO, 1999). All patients had two to four previous embryo transfers without achieving pregnancy (RIF definition proposed recently by Polanski et al., 2014)).

One-half of the group of women who had experienced RIF underwent artificial endometrial stimulation for FET. These women received oestradiol valerate (Progynon®; Schering Nordiska, Berlin, Germany) orally, 6 mg from cycle day 1, 2 mg in the morning and 4 mg in the evening. The women were instructed to take their oestrogen tablets every 12 h, at 8 o'clock in the morning and at 8 o'clock in the evening ± 1 h. Micronized progesterone (APL, Stockholm, Sweden) was administered vaginally; 400 mg twice a day from the time the endometrium had reached a thickness of at least 7 mm, and absence of follicles with a diameter of 15 mm or more. Oral oestrogen support was simultaneously reduced to 2 mg twice a day. The embryos were not thawed and transferred in these treatments, and therefore, we cannot compare the pregnancy rates after using AC-FET or NC-FET.

Endometrial biopsy sampling

All samples were obtained during the mid-secretory phase, at the time of the "window of implantation" (WOI) (LH+7) for NC-FET patients and fertile women, and in AC-FET women on the 6th day of progesterone supplementation (Prog+6). The women with natural, non-stimulated cycles were not subject to any ovarian stimulation before entering the study. Endometrial biopsy samples were obtained from the anterior wall of the uterine cavity, without dilatation of the cervix, using a Pipelle catheter (Gynetics, Hamont-Achel, Belgium). The LH surge was detected in morning urine (Donacheck ovulación, Novalab Ibérica, S.A.L, Coslada, Madrid, Spain and Clearplan, Unipath Ltd., Bedford, UK).

The biopsy samples were prepared for histology, scanning electron microscopy (as described by Aghajanova et al., 2009), and microarray analysis and validation.

Total RNA isolation and microarray hybridization

For microarray and real-time polymerase chain reaction analyses, total RNA was extracted from the endometrial biopsy samples by using an RNeasy Mini-kit (Qiagen, Venlo, the Netherlands) or the "Trizol method" according to the protocol provided by the manufacturer (Life Technologies, Inc., Gaithersburg, MD, USA). RNA quality was assessed by loading 300 ng of total RNA onto an RNA LabChip and this was followed by analysis in a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). An RNA integrity value of over 8 was considered acceptable. Hybridization to the Whole Genome Oligo Microarray that comprises 44,000 gene targets (Agilent Technologies) was carried out as described previously (Altmäe et al., 2010).

Array data analyses

Pre-processing

GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, CA, USA) was used for microarray image analysis and

Please cite this article in press as: Signe Altmäe, Karin Tamm-Rosenstein, Francisco J. Esteban, Jaak Simm, Liis Kolberg, Hedi Peterson, Madis Metsis, Kai Haldre, José A. Horcajadas, Andres Salumets, Anneli Stavreus-Evers, Endometrial transcriptome analysis indicates superiority of natural over artificial cycles in patients who have experienced recurrent implantation failure undergoing frozen embryo transfer, Reproductive BioMedicine Online (2016), doi: 10.1016/j.rbmo.2016.03.004

calculation of spot intensities. Replicates of the gene transcripts were merged using the average of the hybridization values. The data were normalized by the mean of the spot subtracted by the median of the background. Further, densitometry values between arrays were normalized using the quantile normalization function to remove possible sources of variation of a non-biological origin between the arrays.

Sample size calculations

Following the guidelines for "omics" studies (Altmäe et al., 2014), sample size calculations were made. The acceptable amount of false positives was considered to be 0.05% and desired power was set at 90%. We had three study groups with sample size five in each group. For three or more group comparisons, seven samples or over in each group have been suggested (Savaris and Giudice, 2009); therefore, we increased the desired fold change difference in gene expression to three or over, which allows a smaller sample size (Liu and Hwang, 2007). An acceptable level of standard deviation in array studies is considered to be up to 0.7 (bioinformatics.mdanderson.org/MicroarraySampleSize/), but in order to strengthen the power in our study we used a standard deviation of 0.4.

Differential gene expression

R-statistical software (Free Software Foundation, Boston, USA) was used for data anlysis. Gene expression profiles were determined by comparing groups: NC-FET versus NC-FC; AC-FET versus NC-FC; and AC-FET versus NC-FET. Non-parametric tests (2×2 comparisons) were used. We set two criteria to define the genes that had altered mRNA abundance among the different sample sets: an absolute fold change of three or more and a proportion of false positives of less than 0.05.

Statistically significant differences between study groups were identified using the rank product non-parametric test in the Bioconductor RankProd package (Bioconductor, www.bioconductor.org). Because of the limited amount of samples, a non-parametric statistical test was conducted as a rough filter to narrow down the list of most relevant genes. In addition, we applied the rank product approach that includes a multiple hypothesis test for raw *P*-value correction to ascertain a false positive rate. A proportion of false postives of less than 0.05 was considered statistically significant.

Sample clustering and principal component analysis

To validate the results of a non-parametric method of analysing differentially expressed genes (DEGs), principal component analysis and hierarchical clustering were carried out using MeV 4.2.02 software (www.tm4.org) (Saeed et al., 2003). Principal component analysis projects high dimensional data into a lower dimensional span, where samples with a similar gene expression level tend to cluster together on a plot. A three-dimensional scatter plot was produced for visualizing differences between sample sets based on each sample's gene expression profile. In hierarchical clustering, the data were Z-normalized by gene, the Euclidean distance was selected as the similarity measure to cluster expression profiles, and linkage was conducted with a complete-linkage hierarchical clustering algorithm method.

Functional analysis of the results

Functional analysis of differentially regulated genes was explored by using the Database for Annotation, Visualization and Integrated Discovery (DAVID, v. 6.7) (Huang da et al., 2009), and Ingenuity Pathways Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com). DAVID searches blocks of functionally related genes according to different criteria such as the Gene Ontology (GO) terms for biological processes, cellular locations and molecular functions. We used GO FAT search that filters the broadest terms so that they do not overshadow the more specific terms (david.abcc.ncifcrf.gov). IPA was applied for canonical pathway and molecular network analyses. A False Discovery Rate of less than 5.0 and a *P*-value of less tha 0.05 were considered statistically significant.

Prediction of oestrogen response elements and progesterone response elements among differentially expressed genes

We used position weight matrices (PWM), which are widely used in computational molecular biology, in order to depict the DNA binding preferences of transcription factors (including steroid hormone receptors) in target genes. An *in silico* motif search for steroid hormone response elements, e.g. for oestrogen and progesterone response element sequences in proximal promoter region upstream of DEGs in the endometrium was conducted using the TRANSFAC professional database (Matys et al., 2006). All promoter sequences of DEGs in the present study were extracted from the Ensembl BioMart database (*Homo sapiens*, GRCh38.p2). For each gene, three upstream sequences were analysed relative to the major transcription start site, as following: from −1000 bp to +150 bp; from −10,000 bp to +150 bp; and from −50,000 bp to +150 bp. We analysed 17 different PWM from the TRANSFAC database in the oestrogen and progesterone element motif search: VER_Q6, VER_Q6_02, V$ERALPHA_01, V$ESR1_01, V$ERALPHA_Q6_01, V$ERALPHA_Q6_02, V$ESR1_03, V$ESR1_04, V$ESR1_05, V$ERALPHA_Q4, VPR_01, VPR_02, V$PR_Q2, and V$PR_Q6. As the progesterone response element is similar to the glucocorticoid response element, PWM for glucocorticoid response element were also included in the analysis: GR VGR_01, VGRE_C and V$GR_Q6.

Right-tailed Wilcoxon rank sum test was used in oestrogen and progesterone response element profile analysis (occurrences of respective PWM were summed) to compare the differentially expressed endometrial genes with other genes in the human genome ($n = 20{,}710$). To compare the studied subgroups (artificial cycle deteriorated, artificial cycle improved, RIF specific) Kruskal–Wallis test was used. In case of significant difference, Tukey test was used for post-hoc pairwise comparison. A standard hypergeometric test was used to assess the motif enrichment for each PWM separately. Fisher's exact test analysis was used to compare the observed proportion of occurrence of a particular PWM in the studied groups. In cases of significant difference, post-hoc analysis was applied to study the significance of pairwise differences between subgroups. To counteract the problem of multiple comparisons, Bonferroni correction was applied.

Please cite this article in press as: Signe Altmäe, Karin Tamm-Rosenstein, Francisco J. Esteban, Jaak Simm, Liis Kolberg, Hedi Peterson, Madis Metsis, Kai Haldre, José A. Horcajadas, Andres Salumets, Anneli Stavreus-Evers, Endometrial transcriptome analysis indicates superiority of natural over artificial cycles in patients who have experienced recurrent implantation failure undergoing frozen embryo transfer, Reproductive BioMedicine Online (2016), doi: 10.1016/j.rbmo.2016.03.004

Microarray validation

Total RNA (300 ng) was reverse-transcribed using Advantage RT-for-PCR kits (Clontech, Palo Alto, CA, USA) for all samples following the protocol as described in detail previously (Altmäe et al., 2010). The genes *HABP2*, *HLA-DOB*, *SPDEF* and *TRH* were selected for microarray validation. Forward and reverse primer sequences for each gene were (5′–3′): AGGAAGAGA ACACCAGTAGCA and TAGTAGGGAGGACTCTGGGTA for *HABP2*, AGGGCTCAGAAAGGATATGTGA and CTCAGAACACAGA GCTCCAGA for *HLA-DOB*, GTCCCGCCATGAACTACGA and CTGGAAGGTCAGAGCAGCA for *SPDEF*, TGGTTGCTGCTCGCTC TGGC and TCTGGGACGCGGAGTGCTCA for *TRH*, and CCCATCACCATCTTCCAGGA and CATCGCCCCACTTGATTTTG for the control *GAPDH* gene. The real-time polymerase chain reaction protocol has been described in detail elsewhere (Altmäe et al., 2010). Analysis of gene expression differences between the study groups was carried out by using the Mann–Whitney U-test; $P < 0.05$ was considered statistically significant.

Results

Evaluation of endometrial biopsies

Histological evaluation of the natural-cycle samples showed normal maturation in relation to the cycle day, according to Noyes et al. criteria (1975), thus eliminating a delay in endometrial maturation, which can be one reason for RIF (Ruiz-Alonso et al., 2013). Scanning electron microscopy was carried out to check for the presence of pinopodes, structural markers of uterine receptivity (Aghajanova et al., 2008a), in order to assess endometrial receptivity alterations among this patient group. In both infertile groups, pinopode formation differed from that in fertile women, thereby supporting the notion of aberrant endometrial receptivity among these women: in the NC-FET group, three women had no pinopodes, one had very few, and one woman presented normal formation of pinopodes; while in the AC-FET group three women had no pinopodes and two presented scarce pinopodes on the endometrial surface.

Cluster analysis of microarray data

Principal component analysis demonstrated a clear distinction in the endometrial gene expression patterns in the NC-FET, AC-FET and NC-FC groups (**Supplementary Figure S1**). Hierarchical clustering was then applied to the microarray data, and a similar pattern was observed: the study groups were in three clusters, where the NC-FET and NC-FC groups clustered closer than the AC-FET group (**Figure 1**). This clustering pattern indicates that, on the basis of whole endometrial transcriptome analysis at the time of embryo implantation, the NC-FET protocol yields a more similar endometrial pattern to fertile controls than the AC-FET protocol among women who have experienced RIF.

Differential gene expression analysis between NC-FET and AC-FET groups in men who have experienced RIF, and NC-FC women

Our primary microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-3713. In total we identified: 443 up-regulated and 446 down-regulated genes (three or more fold change; proportion of false positives <0.05) in infertile NC-FET versus fertile NC-FC women; 575 up-regulated and 335 down-regulated genes in infertile AC-FET compared with fertile NC-FC women; and 502 up-regulated and 201 down-regulated genes in infertile AC-FET compared with infertile NC-FET women.

These comparisons gave the means to focus on DEGs whose expression level improved (was more similar to fertile controls) with artificial cycles ($n = 620$) (i.e. DEGs unique for natural cycle RIF compared with NC-FC comparison: meaning that the initial gene expression profile in NC-RIF women differed from that in fertile controls, whereas these DEGs in the AC-RIF group versus NC-FC comparison demonstrated similar values to fertile controls); DEGs whose expression profile deteriorated (differed three or more fold compared with fertile controls) with artificial cycles ($n = 640$) (i.e. DEGs unique for AC-RIF versus NC-FC comparison: meaning that in the AC-RIF versus natural NC-FC comparison the gene expression profile was different, whereas the comparison of gene expression profiles in natural NC-RIF versus NC-FC demonstrated similar values) and genes specific to RIF ($n = 269$), i.e. DEGs whose expression was significantly different in infertile women in both natural cycle and artificial cycle when compared with fertile controls (see **Figure 2** for description of DEGs groups, and **Supplementary Table S1** for gene lists).

Functional profiling of differentially expressed genes that improved in artificial cycle in patients who have experienced RIF at the window of implantation or WOI

Biological functional analysis of DEGs that improved their expression pattern with artificial cycle in gene ontology terms showed that they were involved in various biological processes such as the G-protein-coupled receptor signalling pathway, defense response, potassium ion transport, cell surface receptor-mediated signal transduction, cell adhesion and the immune response; in cellular components of the extracellular matrix and region; and several molecular functions such as cytokine activity, G-protein-coupled receptor activity, ion channel activity, transmembrane transporter activity, carbohydrate binding and oxidoreductase activity (**Figure 3A** and **Supplementary Table S2**).

When investigating different biological pathways using IPA, we found that artificial endometrial stimulation in women with RIF influenced canonical pathways involved in G-protein-coupled receptor and cyclic adenosine monophosphate-mediated signalling, lipid signalling and defense responses (**Figure 3A**). Another analysis using DAVID indicated pathways involved in neuroactive ligand-receptor interactions (19 genes, $P < 0.001$), and cytokine–cytokine receptor interactions (16 genes, $P = 0.023$).

Please cite this article in press as: Signe Altmäe, Karin Tamm-Rosenstein, Francisco J. Esteban, Jaak Simm, Liis Kolberg, Hedi Peterson, Madis Metsis, Kai Haldre, José A. Horcajadas, Andres Salumets, Anneli Stavreus-Evers, Endometrial transcriptome analysis indicates superiority of natural over artificial cycles in patients who have experienced recurrent implantation failure undergoing frozen embryo transfer, Reproductive BioMedicine Online (2016), doi: 10.1016/j.rbmo.2016.03.004

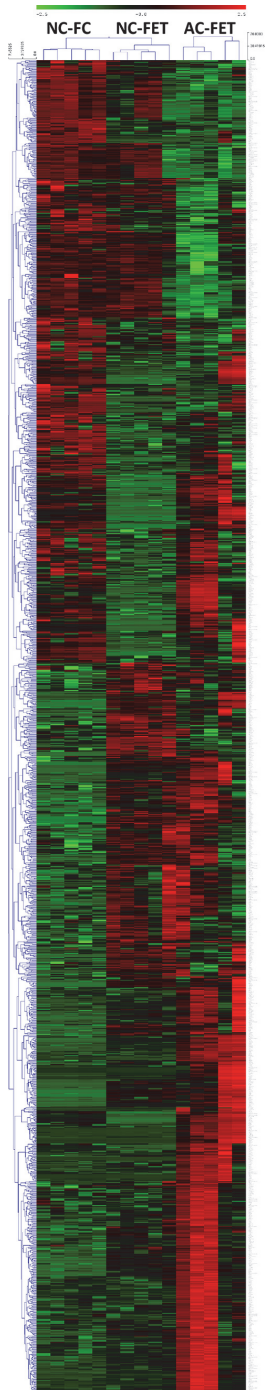

Figure 1 Cluster analysis of Z-scored gene expression values in the endometrium at the window of implantation in healthy women with proven fertility in natural cycles (NC-FC), in infertile women undergoing a natural cyle for frozen embryo transfer (NC-FET), and infertile women undergoing an artificial cycle for frozen embryo transfer (AC-FET). Red represents genes with a positive Z score and green, genes with negative Z score.

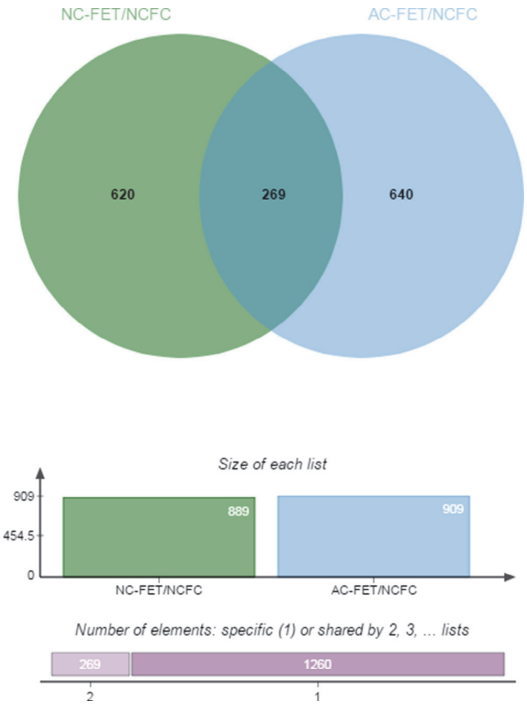

Figure 2 The three differentially expressed gene (DEG) groups analysed: DEGs whose expression improved with artificial cycle in infertile women (n = 620) (i.e. DEGs unique for women who have experienced recurrent implantation failure undergoing a natural cycle (NC-RIF) versus healthy women with proven fertility in natural cycles (NC-FC) comparison: meaning that the initial gene expression profile in NC-RIF women differed from that in fertile controls, whereas these DEGs in the AC-RIF (women who have experienced recurrent implantation failure undergoing an artificial cycle) group versus NC-FC comparison demonstrated similar values to fertile controls); DEGs whose expression profile deteriorated with artificial endometrium preparation in infertile women (n = 640) (i.e. DEGs unique for AC-RIF versus NC-FC comparison: meaning that in the AC-RIF versus natural NC-FC comparison the gene expression profile was different, whereas the comparison of gene expression profiles in natural NC-RIF versus NC-FC demonstrated similar values); and genes specific to RIF, n = 269 (i.e. DEGs whose expression was significantly different in infertile women in both natural cycle and artificial cycle when compared with fertile controls).

Please cite this article in press as: Signe Altmäe, Karin Tamm-Rosenstein, Francisco J. Esteban, Jaak Simm, Liis Kolberg, Hedi Peterson, Madis Metsis, Kai Haldre, José A. Horcajadas, Andres Salumets, Anneli Stavreus-Evers, Endometrial transcriptome analysis indicates superiority of natural over artificial cycles in patients who have experienced recurrent implantation failure undergoing frozen embryo transfer, Reproductive BioMedicine Online (2016), doi: 10.1016/j.rbmo.2016.03.004

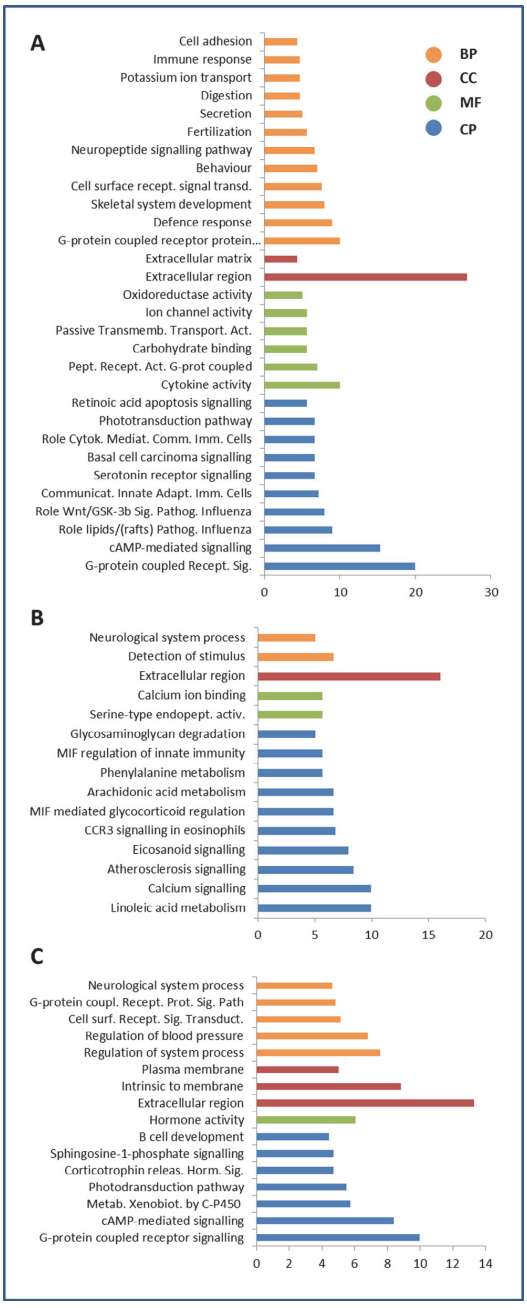

Figure 3 Functional enrichment analysis of the endometrial transcriptome at the window of implantation: genes – whose expression improved with artificial cycles (A), genes whose expression deteriorated with artificial cycles (B), and genes specific to recurrent implantation failure (C). Bar colour denotes different types of evidence from gene ontology (DAVID) and pathway (IPA) databases: BP, biological process (in orange); CC, cellular component (in red); CP, canonical pathways (in blue); MF, molecular function (in green). The X-axis denotes functional enrichment score, computed as $-\log_2$ of related *P*-values. Some abbreviations in the Y-axis are indicated in full text in **Supplementary Tables S2–S4**.

Analysis of the molecular relationships between DEGs that improved their expression patterns in artificial cycles (using the Ingenuity Pathways Knowledge Base) revealed one enriched gene network with high score (IPA score of 37) that united molecules involved in the inflammatory response, antigen presentation, cell-to-cell signalling and interaction where genes *IL31*, *IL21*, *MMP9*, *MUC5AC*/*MUC5B*, *POSTN* and *WISP1* intertwined (**Supplementary Figure S2A**).

To highlight, the DEGs that improved in artificial cycles that repeatedly were identified in different functional analyses using DAVID and IPA included *APOA1*, *AQP6*, *CD34*, *CEACAM1*, *CEACAM8*, *COMP*, *EDN3*, *HBA1*/*HBA2*, *IGFBP1*, *IL21*, *IL31*, *ITGA8*, *IFNB1*, *LHCGR*, *MMP9*, *MUC5B*, *POSTN*, *S100A2*, *SELP*, *SORD*, *TYR*, *WISP1*, *WNT16*, *WNT3A*, *WNT8B* and various chemokines, collagens, G protein-coupled receptors, immunoglobulins, interferons and olfactory receptors.

Functional profiling of DEGs that deteriorated in artificl cycles in RIF patients at the implantation or WOI

Functional analyses of the DEGs that deteriorated with artificial cycles in patients who have experienced RIF showed that they were involved in various biological processes such as detection of stimuli and neurological system processes; cellular components of the extracellular region; and molecular functions such as serine-type endopeptidase activity and calcium ion binding (**Figure 3B** and **Supplementary Table S3**).

The canonical pathways that were influenced negatively by artificial cycles were shown by IPA to be calcium signalling, linoleic acid, arachidonic acid and eicosanoid metabolism and signalling and macrophage migration inhibitory factor activity (**Figure 3B**). DAVID analysis of the pathways that were negatively influenced by artificial cycles involved neuroactive ligand–receptor interactions (17 genes, $P = 0.005$), and similarly to IPA showed the involvement of linoleic acid metabolism (five genes, $P = 0.05$).

Molecular interaction analysis of DEGs whose expression profile altered with artificial cycles revealed one enriched network with high score (IPA score of 38) that united molecules involved in cellular movement, immune cell trafficking, cellular growth and proliferation, where genes *CD4*, *CD22*, *ESR2*, *IL4*, *IL29*, *LEP*, *MMP3* and *SELE* interacted (**Supplementary Figure S2B**).

To sum up, top genes identified in all functional analyses among DEGs that were dysregulated in artificial cycles included *APOA2*, *APOC4*, *CALCRL*, *ESR2*, fibroblast growth factors *FGF17*, *FGF8*, *FGFBP2*; *FSHR*, *INHBC*, interleukins *IL1F6*, *IL27*, *IL29*, *IL4*, *IL9R*; *LEP*; matrix metallopeptidases *MMP17*, *MMP27*, *MMP3*; *NEUROG1*, *PPARD*, *PTGER3*, *SELE*, *WNT8A*, and kallikrein-related peptidases, olfactory receptors, phospholipases, and pregnancy-specific beta-1-glycoproteins.

Please cite this article in press as: Signe Altmäe, Karin Tamm-Rosenstein, Francisco J. Esteban, Jaak Simm, Liis Kolberg, Hedi Peterson, Madis Metsis, Kai Haldre, José A. Horcajadas, Andres Salumets, Anneli Stavreus-Evers, Endometrial transcriptome analysis indicates superiority of natural over artificial cycles in patients who have experienced recurrent implantation failure undergoing frozen embryo transfer, Reproductive BioMedicine Online (2016), doi: 10.1016/j.rbmo.2016.03.004

Functional profiling of differentially expressed genes specific to recurrent implantation failure at the window of implantation or WOI

Biological function analysis of DEGs specific to RIF in gene ontology terms indicated involvement in various biological processes such as cell surface (G-protein coupled) receptor-linked signal transduction (Figure 3C). A significant proportion of these RIF-specific genes were located in extracellular region or plasma membrane. Regarding molecular functions, the genes were mostly involved in hormone activity. IPA analysis revealed that RIF-specific DEGs were involved in several pathways such as G-protein-coupled receptor and cyclic adenosine monophosphate-mediated signalling, metabolism of xenobiotics by cytochrome P450, corticotropin-releasing hormone signaling and sphingosine-1-phosphate signalling (Figure 3C and Supplementary Table S4).

Analysis of the molecular relationships among RIF-specific DEGs revealed one enriched gene network with high score (IPA score of 37) that united genes involved in cellular movement, cell death and lipid metabolism, where molecules *ADRB2*, *CASP8*, *INHBA*, *LTF*, *MUC4*, *MUC5A/MUC5B*, *SERPINB3*, *TFF3*, and *WISP2* intertwined (Supplementary Figure S2C).

To sum up, top molecules identified in all functional analyses among genes specific to unexplained RIF included *ADRB2*, *ADM2*, *CRH*, *CYP3A4*, *DRD3*, *HABP2*, *HLA-DOB*, *IGH@*, *INHBA*, *IL28B*, *ITGA10*, *LTF*, *MMP8*, *MUC4*, *SPDEF*, *TRH*, *TFF3*, *WISP2*, G protein-coupled receptors, olfactory receptors and solute carrier family members.

Comparison of microarray data with the endometrial receptivity array gene list

A novel diagnostic transcriptomic tool concerning endometrial receptivity, the endometrial receptivity array (ERA) test, has been previously presented, where 238 genes serve as an endometrial receptivity biomarker cluster (Blesa et al., 2014; Diaz-Gimeno et al., 2011, 2013; Garrido-Gomez et al., 2013; Ruiz-Alonso et al., 2013). This test has proved to be accurate and consistent, enabling detection of personalized timing of the window of implantation among various patient groups (Díaz-Gimeno et al., 2014). To investigate these endometrial receptivity markers in the current study, the lists of DEGs with the ERA gene list were compared. Improved DEGs with artificial cycles shared 11 common genes with the ERA list: *CALB2*, *COL16A1*, *COMP*, *EDN3*, *IGFBP1*, *LRRC17*, *OLFM4*, *POSTN*, *SLC15A1*, *SORD*, and *TMEM16A* (Table 2). Deteriorated DEGs with artificial cycles shared four common genes with the ERA test: *CRISP3*, *C14orf161*, *HAL* and *HPSE*. DEGs specific to RIF shared four genes with the ERA list: *HABP2*, *HLA-DOB*, *SPDEF* and *TRH* (Table 2).

Analysis of hormone response elements in differentially expressed genes

In hormone response elements *in silico* analysis, we focused on three promoter regions (−1000 bp to +150 bp, −10,000 bp to +150 bp, and −50,000 bp to +150 bp from transcription start site) of DEGs and searched for oestrogen, progesterone and glucocorticoid response elements. Of 1529 DEGs identified via microarrays, 1273 were eligible for TRANSFAC analysis: 534 DEGs that improved with artificial cyces, 508 DEGs that deteriorated with artificial cycles, and 231 DEGs specific to RIF.

Importantly, all DEGs that were eligible for TRANSFAC analysis had at least one HRE in their promoter regions. If all oestrogen response elements (10 PWM) and progesterone/glucocorticoid response elements (seven PMW) matrices were summarized and respective HRE profiles created, progesterone/glucocorticoid response element motifs showed significantly higher prevalence ($P < 2.2e{-}16$ from −50,000 bp to +150 bp) in promoter regions of DEGs compared with other genes in the human genome. Progesterone/glucocorticoid response element motifs showed higher frequency compared with oestrogen response elements in all promoter regions of DEGs identified in this study (Figure 4). When different subgroups were compared, significantly lower frequency ($P = 0.0052$) of progesterone/glucocorticoid response element sites were determined in most distal promoter region (−50,000 bp to +150 bp) of DEGs deteriorated compared with genes whose expression was improved after artificial cycles. In contrast, deteoriated genes from artificial cycles had significantly higher frequency of oestrogen response elements compared with improved genes from artificial cycles ($P = 3.2e{-}06$) and RIF-specific genes ($P = 0.0045$) (Figure 4).

When each PWM (17 oestrogen, progesterone and glucocorticoid response element motifs) was analysed separately, two hormone response elements motifs (PWM PR_Q6 and ESR1_01) demonstrated significant differences in promoter areas between the study groups (Figure 5). Progesterone response element motif (V$PR_Q6) was present at a significantly higher rate upstream of genes specific to RIF ($P = 0.044$) in the most proximal promoter region (−1,000 bp to +150 bp). For instance, progesterone response element motif was found upstream of genes as *AFM*, *BRINP3*, *CNNM1*, *FAM151A*, and *IL12RB2*. A significantly higher prevalence of this progesterone response elemet motif was also observed in the farther (−10,000 to +150 bp) promoter region ($P = 0.033$) of RIF-specific genes. In addition, DEGs whose expression improved after artificial cycle also showed a high prevalence of the same progesterone response element in the farther promoter region (−10,000 bp to +150 bp) ($P = 0.040$) (Supplementary Table S5). Genes whose expression improved after artificial cycles and consisted progesterone response element in promoter region were for example *ADAD1*, *CALML5*, *FAM196A*, *IFNA5*, and *IL21*.

In contrast, DEGs that deteriorated in response to artificial cyces more frequently had oestrogen response element motif (V$ESR1_01) in their proximal promoters, but not at a significant level after applying *Bonferroni correction* (Supplementary Table S5). When comparing the relative frequency of hormone response elements between groups, the named oesrogen response element motif was significantly over-represented upstream of DEGs that deteriorated with artificial cycles compared with DEGs that improved with artificial cycles (adjusted $P = 0.023$) in the most proximal promoter region (−1000 bp to +150 bp). For example, genes like *IL9R*, *MMP17*, *PTGER3*, *ESR2*, and *GATA3* all included oestrogen response element motif in their promoter regions and their expression was deteriorated with artificial cycles. All 17 motifs used in *in silico* analysis and their occurrence in promoter

Please cite this article in press as: Signe Altmäe, Karin Tamm-Rosenstein, Francisco J. Esteban, Jaak Simm, Liis Kolberg, Hedi Peterson, Madis Metsis, Kai Haldre, José A. Horcajadas, Andres Salumets, Anneli Stavreus-Evers, Endometrial transcriptome analysis indicates superiority of natural over artificial cycles in patients who have experienced recurrent implantation failure undergoing frozen embryo transfer, Reproductive BioMedicine Online (2016), doi: 10.1016/j.rbmo.2016.03.004

Table 2 Comparison of array data with the endometrial receptivity array (ERA) gene list.

Genes whose expression improved with artificial cycles

Gene	ERA[a]	NC-FET versus NC-FC	AC-FET versus NC-FC	HREs
CALB2	−8.04	5.15	nd	ERE/PRE/GRE
COL16A1	−4.89	5.89	nd	ERE/PRE/GRE
COMP	30.95	−3.93	nd	ERE/PRE/GRE
EDN3	−5.03	3.48	nd	ERE/PRE/GRE
IGFBP1	5.35	−4.17	nd	ERE/PRE/GRE
LRRC17	−4.64	4.25	nd	ERE/PRE/GRE
OLFM4	−9.35	15.35	nd	ERE/PRE/GRE
POSTN	−6.04	5.21	nd	ERE/PRE/GRE
SLC15A1	5.59	−4.61	nd	ERE/PRE/GRE
SORD	−3.21	3.7	nd	ERE/PRE/GRE
TMEM16A	−3.13	3.73	nd	ERE/PRE/GRE

Genes whose expression deteriorated with artificial cycles

Gene	ERA[a]	NC-FET versus NC-FC	AC-FET versus NC-FC	HREs
CRISP3	5.09	nd	−13.46	ERE/PRE/GRE
C14orf161	5.07	nd	−3.38	ERE/PRE/GRE
HAL	3.37	nd	−3.93	ERE/PRE/GRE
HPSE	5.17	nd	−7.48	ERE/PRE/GRE

Genes specific to RIF

Gene	ERA[a]	NC-FET versus NC-FC	AC-FET vsersus NC-FC	HREs
HABP2	4.09	−4.48	−5.48	ERE/PRE/GRE
HLA-DOB	−11.06	5.17	12.86	ERE/PRE/GRE
SPDEF	−3.78	9.27	12.20	ERE/PRE/GRE
TRH	−21.69	7.56	9.02	ERE/PRE/GRE

AC-FET, artificial cycle-frozen embryo transfer; ERE, oestrogen response element; HRE, hormone response element; GRE, glucocorticoid response element; NC-FC, natural cycle-fertile control; NC-FET, natural cycle-frozen embryo transfer; nd, non-detectable, meaning that there was no gene expression difference between the patients who have experienced RIF and fertile controls; PRE, progesterone response element; RIF, recurrent implantation failure.
[a]ERA test values are fold changes obtained from comparisons of receptive vs. pre-receptive endometrium (Diaz-Gimeno et al., 2011).

regions of genes among DEGs are shown in **Supplementary Table S5**.

Comparison of differentially expressed genes with genes that responded to oestradiol and progesterone in the Ishikawa cell line

In studies with human endometrial biopsy samples, it is difficult to analyse retrospectively whether changes in the tissue are primary or secondary results of steroid hormone action. Therefore, endometrial gene expression after artificial cycles were compared with that in the recent study of ours where the oestradiol and progesterone responsive transcriptome was analysed in Ishikawa cancer cell line (Tamm-Rosenstein et al., 2013). The endometrial epithelial Ishikawa cell line expresses functional response for oestradiol and progesterone, and is therefore a good in-vitro model for studying the responses of the endometrial epithelium to oestradiol and progesterone (Croxtall et al., 1990; Lessey et al., 1996). Although the expression platforms of our current and previous studies were different (microarray versus RNA-sequencing), we found 50 genes for which expression was significantly changed after hormonal treatments in both studies (**Figure 6**). We identified 37 genes with significant regulation in response to oestradiol treatment and 42 genes were changed after progesterone treatment, where 29 genes were influenced by both hormonal treatments in our previous study. Among the DEGs that improved with artificial cycles, 18 were regulated by oestradiol and progeserone (75%), one by oestradiol (4%) and five genes solely by progesterone (21%). DEGs that were negatively influenced with artificial cycles responded more to oestradiol regulation, where nine genes were regulated by oestradiol and progesterone (47%), six genes solely by oestradiol (32%) and four genes by progesterone (21%) (**Figure 6**). In addition, 58% of the DEGs that improved with artificial cycles shared the same expression direction with the cell line experiments, whereas 58% of DEGs that

Please cite this article in press as: Signe Altmäe, Karin Tamm-Rosenstein, Francisco J. Esteban, Jaak Simm, Liis Kolberg, Hedi Peterson, Madis Metsis, Kai Haldre, José A. Horcajadas, Andres Salumets, Anneli Stavreus-Evers, Endometrial transcriptome analysis indicates superiority of natural over artificial cycles in patients who have experienced recurrent implantation failure undergoing frozen embryo transfer, Reproductive BioMedicine Online (2016), doi: 10.1016/j.rbmo.2016.03.004

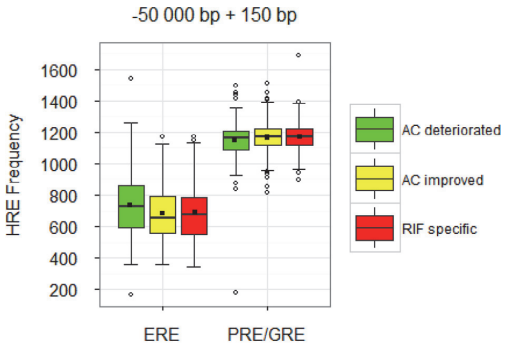

Figure 4 Tukey test for pairwise comparison of oestrogen receptor elements and progesterone receptor element profiles in promoter regions (−50,000 bp to +150 bp) of differentially expressed genes (DEGs) with artificial cycle deteriorated (green), artificial cycle improved (yellow) and recurrent implantation failure (RIF) specific (red). Box plot showing median and mean value. On the left side significant difference of the presence of oestrogen receptor element sites upstream of DEGs artificial cycle deteriorated compared with artificial cycle improved ($P = 3.2e{-}06$) and compared with RIF-specific genes (=0.0045) is shown. On the right significant difference of the presence of progesterone response element sites upstream of DEGs artificial cycle deteriorated compared with artificial cycle improved ($P = 0.0052$) is shown. AC, artificial cycle; ERE, oestrogen response element; HRE, hormone response element; GRE, glucocorticoid response element; PRE, progesterone response element; RIF, recurrent implantation failure.

deteriorated with artificial cycles demonstrated opposite expression direction with the Ishikawa cells (**Figure 6**).

In addition to overlapping genes with DEGs deteriorated and improved, seven oestradiol- and progesterone-regulated RIF specific DEGs were in common with Ishikawa cell line experiments. Among these, seven genes, two genes were regulated by oestradiol and progesterone (29%), one solely by oestradiol (14%) and four by progesterone (57%) (**Figure 6**).

Microarray validation

Real-time polymerase chain reaction was used for validating microarray results in genes *HABP2*, *HLA-DOB*, *SPDEF* and *TRH*, which were dysregulated in infertile women in both natural and artificial cycles (**Table 2**), and have been shown in a previous study to be endometrial receptivity genes (Diaz-Gimeno et al., 2011). Real-time polymerase chain reaction confirmed the array results: *HLA-DOB*, *SPDEF* and *TRH* were up-regulated, and *HABP2* was down-regulated in patients who had experienced recurrent implanatation failure in NC-FET and AC-FET compared with NC-FC control women (**Supplementary Figure S3**).

Discussion

Despite the growing importance of FET in the treatment of infertility there is little consensus on the best protocol for endometrial preparation (Groenewoud et al., 2013). To the best of our knowledge, this is the first study in which the effect of two different endometrial preparation protocols, NC-FET and AC-FET on the endometrial transcriptome among infertile patients who have experienced RIF has been analysed. We demonstrate that the whole endometrial gene expression pattern at the time of embryo implantation in the NC-FET protocol is more similar to the profile of fertile controls than is the AC-FET gene expression pattern. That means that, in this subgroup of infertile women, artificial endometrial preparation with oestradiol and progeserone alters more than improves the endometrial transcriptome, thus being disadvantageous, and the NC-FET protocol should be preferred.

Implantation failure remains an unsolved obstacle in reproductive medicine and is one of the major causes of infertility in otherwise healthy women (Margalioth et al., 2006; Simon and Laufer, 2012). Inadequate uterine receptivity is estimated to account for two-thirds of implantation failures, whereas the embryo itself is responsible for only one-third of failures (Lédée-Bataille et al., 2002). Compelling evidence demonstrates that there is endometrial receptivity alteration among patients with RIF (Koler et al., 2009; Koot et al., 2016; Lédée et al., 2011; Ruiz-Alonso et al., 2013, 2014; Tapia et al., 2008). To sum up, RIF is relatively common among those seeking infertility treatment, and the management of RIF is considered as one of the most complicated issues in assisted reproduction (Simon and Laufer, 2012).

In the artificial endometrial preparation cycle, oestradiol and progestrone administration is aimed at mimicking the endocrine exposure of the endometrium in normal cycles, where oestradiol initiates endometrial cellular proliferation and subsequent administration of progesterone leads to secretory changes (El-Toukhy et al., 2008). We therefore hypothesized that, among this patient group of infertile women, where aberrant endometrial maturation is suspected, artificial endometrial preparation would help to overcome or improve impaired uterine receptivity. Therefore, the result of AC-FET being disadvantageous versus NC-FET was surprising.

Our study results, in fact, demonstrate that the artificial cycle protocol favourably altered several transcripts of endometrial receptivity biomarkers implicated in previous independent transcriptome analyses on women with a fertile phenotype, such as *CALB2* (Chan et al., 2013; Diaz-Gimeno et al., 2011; Horcajadas et al., 2004), *COL16A1* (Borthwick et al., 2003; Diaz-Gimeno et al., 2011), *COMP* (Altmäe et al., 2012; Borthwick et al., 2003; Diaz-Gimeno et al., 2011; Haouzi et al., 2009b; Hu et al., 2014; Riesewijk et al., 2003; Talbi et al., 2006; Tapia et al., 2011), *EDN3* (Altmäe et al., 2012; Carson et al., 2002; Diaz-Gimeno et al., 2011; Horcajadas et al., 2004; Hu et al., 2014; Kao et al., 2002; Riesewijk et al., 2003; Talbi et al., 2006; Tapia et al., 2011), *IGFBP1* (Altmäe et al., 2010, 2012; Borthwick et al., 2003; Dassen et al., 2007; Diaz-Gimeno et al., 2011), *LRRC17* (Altmäe et al., 2012; Diaz-Gimeno et al., 2011), *OLFM4* (Diaz-Gimeno et al., 2011), *POSTN* (Altmäe et al., 2012; Diaz-Gimeno et al., 2011; Zhang et al., 2012a), *SLC15A1* (Chan et al., 2013; Diaz-Gimeno et al., 2011; Zhang et al., 2012a), *SORD* (Diaz-Gimeno et al., 2011; Horcajadas et al., 2007), and *TMEM16A* (Diaz-Gimeno et al., 2011). The improvement of insulin-like growth factor binding protein-1 expression levels with artificial cycles is interesting, as this protein is an established biomarker of decidualization of endometrial stroma. It is involved in the

Please cite this article in press as: Signe Altmäe, Karin Tamm-Rosenstein, Francisco J. Esteban, Jaak Simm, Liis Kolberg, Hedi Peterson, Madis Metsis, Kai Haldre, José A. Horcajadas, Andres Salumets, Anneli Stavreus-Evers, Endometrial transcriptome analysis indicates superiority of natural over artificial cycles in patients who have experienced recurrent implantation failure undergoing frozen embryo transfer, Reproductive BioMedicine Online (2016), doi: 10.1016/j.rbmo.2016.03.004

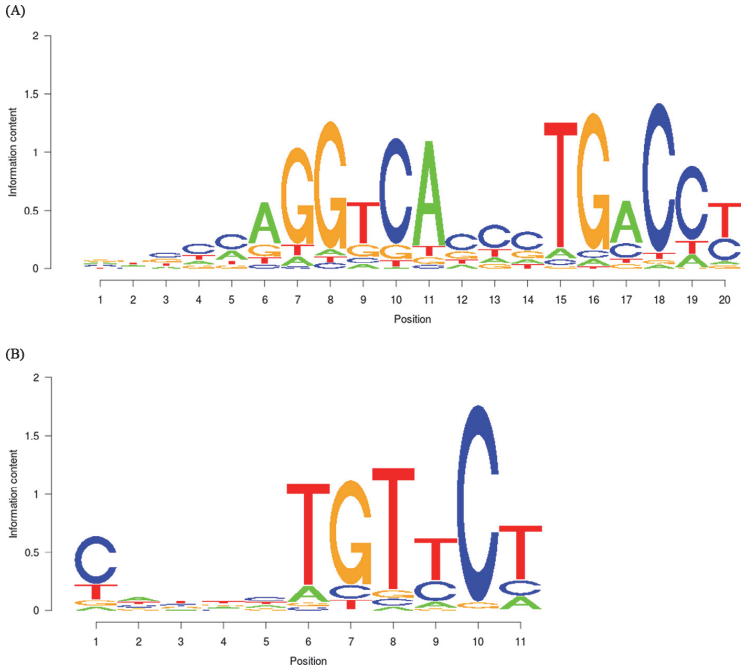

Figure 5 Two most prevalent hormone response elements found in promoter regions among differentially expressed genes identified in this study. Letters abbreviate the nucleotides (A, C, G, and T) in the images and are sized according to their relative occurrence. (A) ESR1 element (V$ESR1_01) was over represented upstream of differentially expressed genes (DEGs) deteriorated with artificial cycles (examples *IL9R*, *MMP17*, *PTGER3*, *ESR2*, and *GATA3*). This oestrogen receptor element motif in the most proximal promoter region (from −1,000 bp to +150 bp from transcription start sites) turned out to be significantly overrepresented in upstream of DEGs that deteriorated with artificial cycle compared with DEGs that improved with artificial cycle ($P = 0.023$); (B) progesterone response element (V$PR_Q6) was statistically more frequent in upstream of genes related to recurrent implantation failure (examples *AFM*, *BRINP3*, *CNNM1*, *FAM151A*, and *IL12RB2*) ($P = 0.04$) and among DEGs that improved with artificial cycle (examples *ADAD1*, *CALML5*, *FAM196A*, *IFNA5*, and *IL21*) ($P = 0.04$).

implantation process through regulating insulin-like growth factor actions in endometrial cells, and cellular proliferation and differentiation required for decidualization and maintenance of early pregnancy (Altmäe et al., 2010). A number of pathways important in endometrial receptivity were also improved with artificial cycles, including cytokine–cytokine receptor interaction, G-protein coupled receptor and cyclic adenosine monophosphate-mediated signalling, lipid signalling and defense responses (Altmäe et al., 2010, 2012; Giudice, 2006; Kao et al., 2002; Koler et al., 2009). Nevertheless, based on the whole transcriptome profile it seems that regardless of the "correction" of dysregulated genes with the AC-FET protocol, a negative effect of artificial cycles on the endometrial transcriptome prevailed.

The expression of various important genes known from previous studies to be involved in endometrial receptivity deteriorated with artificial cycles. These included *CRISP3* (Altmäe et al., 2012; Borthwick et al., 2003; Diaz-Gimeno et al., 2011; Zhang et al., 2012a), *HPSE* (Altmäe et al., 2012; Chan et al., 2013; Diaz-Gimeno et al., 2011; Zhang et al., 2012a), *HAL* (Altmäe et al., 2012; Carson et al., 2002; Diaz-Gimeno et al., 2011; Tapia et al., 2008) and *C14orf161* (Diaz-Gimeno et al., 2011). A number of relevant genes involved in implantation were also negatively altered with artificial cycles, including *ESR2*; fibroblast growth factors *FGF17*, *FGF8*, *FGFBP2*; *FSHR*; *INHBC*; interleukins *IL1F6*, *IL27*, *IL29*, *IL4*, *IL9R*; *LEP*; matrix metallopeptidases *MMP17*, *MMP27*, *MMP3*; *PPARD*; *PTGER3*, and *WNT8A* (Aghajanova, 2010; Aghajanova et al., 2008b; Altmäe et al., 2010, 2012; Bogacka et al., 2013; Gonzalez et al., 2000; Kao et al., 2002; McGowen et al., 2014; Osteen et al., 1999; van Mourik et al., 2009). Interestingly, the important hormone receptors, and follicle-stimulating hormone receptor were down-regulated in the endometrium with the AC-FET protocol. The crucial role of oestrogen receptor beta in the endometrium is gradually becoming more evident (summarized in Hapangama et al., 2014), and recently the presence of follicle-stimulating hormone receptor in human secretory endometrium was also demonstrated (Stilley et al., 2014). The importance of leptin in endometrial receptivity and implantation is well established (Gonzalez et al., 2000), and a recent study demonstrated abnormal leptin production in the endometrium among patients with RIF (Dos Santos et al., 2012). The most strongly artificial cyle-disrupted pathways among our patients were linoleic acid metabolism, and

Please cite this article in press as: Signe Altmäe, Karin Tamm-Rosenstein, Francisco J. Esteban, Jaak Simm, Liis Kolberg, Hedi Peterson, Madis Metsis, Kai Haldre, José A. Horcajadas, Andres Salumets, Anneli Stavreus-Evers, Endometrial transcriptome analysis indicates superiority of natural over artificial cycles in patients who have experienced recurrent implantation failure undergoing frozen embryo transfer, Reproductive BioMedicine Online (2016), doi: 10.1016/j.rbmo.2016.03.004

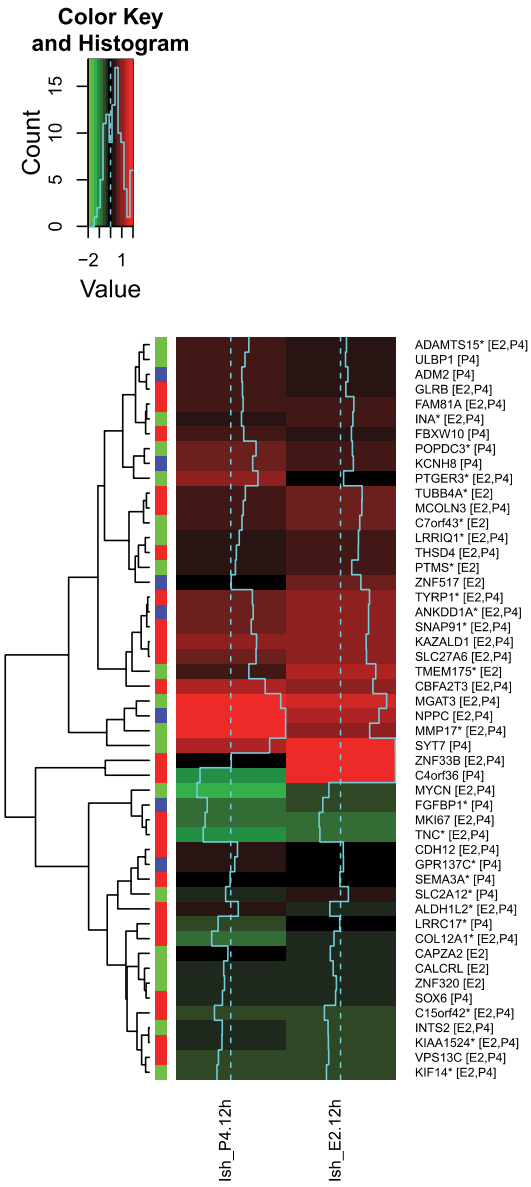

Figure 6 Genes regulated by oestradiol and progesterone in endometrium and Ishikawa cells. Fifty differentially expressed genes (DEGs) from the present study were also regulated by oestradiol and progesterone in our previous study using Ishikawa cells (Tamm-Rosenstein et al., 2013). Red denotes up-regulation and green down-regulation of gene expression in Ishikawa cells. The dotted line indicates zero and the variable line shows the expression change in response to hormone treatment (E2) – gene expression regulated by oestradiol; (P4) gene regulated by progesterone, and (E2, P4) – gene regulated by both oestradiol and progesterone. *Designates genes in the present study with an opposite expression direction versus Ishikawa cells. Red on the y-axis indicates genes whose expression improved with artificial cycle, green indicates genes whose expression deteriorated with artificial cycle, and blue highlights genes specific to recurrent implantation failure.

calcium signalling, both having important roles in endometrial receptivity (Aguilar and Mitchell, 2010; Altmäe et al., 2010; Brosens et al., 2014; Zhang et al., 2012b). To sum up, regardless of the similar number of genes whose expression improved or deteriorated in the endometrium in connection with AC-FET, endometrial stimulation with oestradiol and progesterone in artificial cycles seemed to have a stronger negative rather than an improving effect on the expression of genes and pathways crucial for endometrial receptivity.

The ovarian steroid hormones oestrogen and progesterone play pivotal roles in development of the human endometrium. With a high prevalence of steroid hormone receptors in different compartments of endometrial tissue, it is believed that gene expression of this tissue is highly controlled by respective receptors, which are master transcriptional regulators of downstream genetic activity. There are two types of oestrogen receptors, ERα and ERβ, which share approximately 97% similarity in their DNA-binding domains (Matthews and Gustafsson, 2003). Both oestrogen receptors interact with the same conserved ERE (5′-GGTCAnnnTGACC-3′) as either homodimers or α/β heterodimers (Cowley et al., 1997; Klinge et al., 2004). Progesterone also has two receptor isoforms, PRA and PRB, which differ only in that PRB contains an additional 164 amino acids in the N-terminal region. Both progesterone receptors bind to the same sites of DNA.

We carried out a comprehensive analysis of oestrogen response element and progesterone response elements sequences in genes involved in endometrial receptivity. Our in-silico analysis of hormone response element sites among DEGs that were influenced by artificial cycles demonstrated that the promoter regions of all genes contained response elements for oestrogen and progesterone receptors, and can thereby be directly modulated by oestradiol and progesterone. Summarized progesterone/glucocorticoid response element motifs showed median higher frequency compared with oestrogen receptors in all DEGs identified in this study. Those DEGs whose expression was deteriorated showed significantly less progesterone/glucocorticoid response elements and more oestrogen response element sites in their promoter region compared with genes whose expression was improved after artificial cycles. In addition, we detected a significantly higher number of one particular oestrogen response elements site among DEGs that deteriorated with artificial cycles, whereas one progesterone response element site was overrepresented in the proximal promoter region upstream of genes that improved with artificial cycles. These findings indicate that DEGs that deteriorated with artficival cycles are more responsive to oestradiol, whereas DEGs that improved with artificial cycles are more responsive to progesterone. Therefore, following the current artificial cycle protocol, oestradiol could have had stronger (negative) effect on the endometrial transcriptome than progesterone. In line with this, when we compared the DEGs with those in our previous study on Ishikawa cells, which are regulated by

Please cite this article in press as: Signe Altmäe, Karin Tamm-Rosenstein, Francisco J. Esteban, Jaak Simm, Liis Kolberg, Hedi Peterson, Madis Metsis, Kai Haldre, José A. Horcajadas, Andres Salumets, Anneli Stavreus-Evers, Endometrial transcriptome analysis indicates superiority of natural over artificial cycles in patients who have experienced recurrent implantation failure undergoing frozen embryo transfer, Reproductive BioMedicine Online (2016), doi: 10.1016/j.rbmo.2016.03.004

oestradiol and progesteronoe, we found that among DEGs that deteriorated with artificial cycle 32% were solely oestradiol-regulated and 47% were regulated by oestradiol together with progesterone, whereas, among the improved transcript group, only 4% were oestradiol-regulated and 75% were regulated jointly by oestradiol and progesterone. In addition, the fact that many DEGs that changed expression with artificial cycles (58%) demonstrated an opposite expression direction in comparison with cell line experiments, gives further support to the notion that artificial cycles unfavourably influenced endometrial gene expression in this group of infertile women, where oestradiol seems to have had a higher (negative) effect than progeserone.

Oestrogen is essential for endometrial proliferation, whereas its role in the secretory phase and in implantation is less clear (Young, 2013). Human endometrium seems to function normally with very low concentrations of oestrogen, and, in a recent systematic review, it was suggested that there was no overall benefit in clinical outcomes of luteal oestrogen supplementation in IVF (Fatemi et al., 2007). There is, however, an ongoing discussion about the influence of supraphysiological levels of oestradiol on endometrial quality (Groenewoud et al., 2013). As oestradiol levels are higher in artificial compared with natural cycles, one would expect a thicker endometrium in artificial cycles, but this has not been observed (Hancke et al., 2012; Tomás et al., 2012). In our study also, we did not detect any significant differences in endometrial thickness between the NC-FET and AC-FET patients. Elevated levels of oestradiol have been suggested to lead to alteration in endometrial features and timing of the WOI, which could result in lower pregnancy rates in artificial cycle FET (Groenewoud et al., 2013).

Progesterone is absolutely necessary for endometrial receptivity. Evidence from normally ovulating women suggests that only a very small amount of progesterone is required (Young, 2013), and it has been shown that elevated progesterone levels have a detrimental effect on the endometrium (Weinerman and Mainigi, 2014). In short, only small amounts of oestradiol and progesterone seem to be required in the secretory phase for full reproductive performance among women with normal endometrial function (Young, 2013). Recent research, however, has shown that too high or too low mid-luteal serum progesterone concentrations (<50 and >99 nmol/L) associate with decreased implantation rates in cryopreserved embryo transfers conducted under hormone replacement (Yovich et al., 2015). Women who have experienced RIF have a high probability of endometrial dysfunction, and hormonal endometrial stimulation could help to improve aberrant endometrial maturation. Indeed, we identified several important genes (and pathways) involved in endometrial receptivity whose expression improved with artificial cycles. Therefore, our results, in line with previous studies (Yovich et al., 2015), encourage reconsideration and improvement of artificial cycle protocols in order to find optimal regimens for favourably maximizing endometrial gene expression profiles for distinct patient subgroups.

An additional finding in our study was the identification of genes specific to unexplained RIF. These were a subgroup of genes that were similarly and abnormally expressed in both endometrial preparation protocols, NC-FET and AC-FET among infertile women. We identified several relevant molecules and molecular pathways in female infertility, including genes involved in G-protein-coupled receptor and cAMP-mediated signalling, metabolism of xenobiotics by cytochrome P450, hormone activity, and lipid metabolism (Altmäe et al., 2010, 2012; Koot et al., 2016; Lédée et al., 2011). The top molecules detected with hormonal activity were adrenomedullin 2, corticotropin-releasing hormone, inhibin beta A, and thyrotropin-releasing hormone. Furthermore, we identified four dysregulated genes that have been reported in endometrial transcriptome studies as being window of implementation-specific genes that could serve as biomarkers of RIF: *HABP2* (Altmäe et al., 2010, 2012; Horcajadas et al., 2007; McGowen et al., 2014), *HLA-DOB* (Borthwick et al., 2003; Diaz-Gimeno et al., 2011; Horcajadas et al., 2004, 2007), *SPDEF* (Diaz-Gimeno et al., 2011; Hu et al., 2014) and *TRH* (Altmäe et al., 2012; Carson et al., 2002; Diaz-Gimeno et al., 2011; Horcajadas et al., 2004; Hu et al., 2014; Riesewijk et al., 2003; Talbi et al., 2006; Tapia et al., 2011). The importance of thyrotropin and thyroid hormone action in implantation has recently been summarized (Colicchia et al., 2014). Reduced endometrial gene expression of hyaluronan-binding protein 2 (HABP2), an interesting biomarker of RIF that was down-regulated among patients who had experienced RIF, has also been associated with unexplained female infertility and recurrent pregnancy loss (Altmäe et al., 2010; Bersinger et al., 2008). Another important set of genes in our study are *CASK*, *CASP8*, *COG*, *FCGBP*, *GRIN3B*, *PPA2*, and *SIX1* that are part of the recently published RIF prediction model (Koot et al., 2016).

An additional interesting finding among RIF-specific transcripts was a significantly high number of progesterone response element sites among these genes, indicating that pogesterone can have a direct stimulatory effect on their regulation. This notion was supported by the fact that about 60% of the transcripts were solely progesterone-regulated (whereas about 30% of the genes were regulated by both oestradiol and progesterone), when we compared the microarray results with those in our previous cell-line study (Tamm-Rosenstein et al., 2013). These findings highlight the fact that the genes dysregulated among our RIF patients respond to progesterone, reflecting dysregulation of progesterone signalling among these women. It is known from previous studies that compromised progesterone signalling can lead to impaired endometrial function (Aghajanova et al., 2010). Indeed, aberrant expression of progesterone-regulated genes in the endometrium has been implicated in several gynaecological disorders, such as endometriosis, polycystic ovary syndrome and endometrial hyperplasia (Aghajanova et al., 2010). Furthermore, a recent endometrial transcriptome study demonstrated that women refractory to embryo implantation have compromised progesterone signaling (Tapia-Pizarro et al., 2014). Several signalling pathways are believed to be implicated in the pathogenesis of RIF, and they are of interest as regards identifying potential therapeutic targets and developing new therapies. Our findings provide additional information concerning the complex molecular regulation of endometrial receptivity in RIF patients, where dysregulation of progesterone signalling could have an important role.

To sum up, in a recent systematic review and meta-analysis, it was concluded that "it is not possible, based on the current published literature, to recommend one endometrial preparation method in FET over another" (Groenewoud et al., 2013). Our results suggest that NC-FET is preferable

Please cite this article in press as: Signe Altmäe, Karin Tamm-Rosenstein, Francisco J. Esteban, Jaak Simm, Liis Kolberg, Hedi Peterson, Madis Metsis, Kai Haldre, José A. Horcajadas, Andres Salumets, Anneli Stavreus-Evers, Endometrial transcriptome analysis indicates superiority of natural over artificial cycles in patients who have experienced recurrent implantation failure undergoing frozen embryo transfer, Reproductive BioMedicine Online (2016), doi: 10.1016/j.rbmo.2016.03.004

to AC-FET in the subgroup of women who have experienced RIF. Furthermore, they also indicate that oestradiol might have a stronger (negative) effect on the endometrial transcriptome than progesterone in artificial cycles, thus encouraging reconsideration of the doses of administered steroid hormones and thereby improving protocols in artificial cycles. Despite the relatively small patient group, we believe that our transcriptome data provide valuable insights into the potential biomarkers and molecular mechanisms related to endometrial receptivity and RIF, but most importantly we hope our data will provide a step forward towards personalized medicine in the subgroup of infertile patients who have experienced recurrent implantation failure.

Acknowledgements

We are grateful to all participants and study personnel who took part in this work. We thank Nick S Macklon from University of Southampton, UK for constructive comments and discussion, and Nicholas John Bolton for language revision.

Funding

The study was funded by grants from the European Science Foundation (ESF) for the activity 'Frontiers of Functional Genomics', a Marie Curie post-doctoral fellowship (FP7, number 329812, NutriOmics), Enterprise Estonia (grant EU30020 and EU48695), Estonian Ministry of Education and Research (grant IUT34-16), the EU-FP7 Eurostars Program (grant NOTED, EU41564) and the EU-FP7 IAPP Project (grant SARM, EU324509).

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rbmo.2016.03.004.

References

Aghajanova, L., 2010. Update on the role of leukemia inhibitory factor in assisted reproduction. Curr. Opin. Obstet. Gynecol. 22, 213–219. doi:10.1097/GCO.0b013e32833848e5.

Aghajanova, L., Hamilton, A.E., Giudice, L.C., 2008a. Uterine receptivity to human embryonic implantation: histology, biomarkers, and transcriptomics. Semin. Cell Dev. Biol. 19, 204–211. doi:10.1016/j.semcdb.2007.10.008; S1084-9521(07)00176-0 [pii].

Aghajanova, L., Simón, C., Horcajadas, J.A., 2008b. Are favorite molecules of endometrial receptivity still in favor? Expert Rev. Obstet. Gynecol. 3, 487–501.

Aghajanova, L., Altmäe, S., Bjuresten, K., Hovatta, O., Landgren, B.M., Stavreus-Evers, A., 2009. Disturbances in the LIF pathway in the endometrium among women with unexplained infertility. Fertil. Steril. 91, 2602–2610. doi:10.1016/j.fertnstert.2008.04.010; S0015-0282(08)00797-8 [pii].

Aghajanova, L., Velarde, M.C., Giudice, L.C., 2010. Altered gene expression profiling in endometrium: evidence for progesterone resistance. Semin. Reprod. Med. 28, 51–58. doi:10.1055/s-0029-1242994.

Aguilar, H.N., Mitchell, B.F., 2010. Physiological pathways and molecular mechanisms regulating uterine contractility. Hum. Reprod. Update 16, 725–744. doi:10.1093/humupd/dmq016.

Allersma, T., Farquhar, C., Cantineau, A.E.P., 2013. Natural cycle in vitro fertilisation (IVF) for subfertile couples. Cochrane Database Syst. Rev. (8), CD010550. doi:10.1002/14651858.CD010550.pub2.

Altmäe, S., Martinez-Conejero, J.A., Salumets, A., Simon, C., Horcajadas, J.A., Stavreus-Evers, A., 2010. Endometrial gene expression analysis at the time of embryo implantation in women with unexplained infertility. Mol. Hum. Reprod. 16, 178–187. doi:10.1093/molehr/gap102; gap102 [pii].

Altmäe, S., Reimand, J., Hovatta, O., Zhang, P., Kere, J., Laisk, T., Saare, M., Peters, M., Vilo, J., Stavreus-Evers, A., Salumets, A., 2012. Research resource: interactome of human embryo implantation: identification of gene expression pathways, regulation, and integrated regulatory networks. Mol. Endocrinol. 26, 203–217. doi:10.1210/me.2011-1196.

Altmäe, S., Esteban, F.J., Stavreus-Evers, A., Simón, C., Giudice, L., Lessey, B.A., Horcajadas, J.A., Macklon, N.S., D'Hooghe, T., Campoy, C., Fauser, B.C., Salamonsen, L.A., Salumets, A., 2014. Guidelines for the design, analysis and interpretation of "omics" data: focus on human endometrium. Hum. Reprod. Update 20, 12–28. doi:10.1093/humupd/dmt048.

Bersinger, N.A., Wunder, D.M., Birkhäuser, M.H., Mueller, M.D., 2008. Gene expression in cultured endometrium from women with different outcomes following IVF. Mol. Hum. Reprod. 14, 475–484. doi:10.1093/molehr/gan036.

Blesa, D., Ruiz-Alonso, M., Simón, C., 2014. Clinical management of endometrial receptivity. Semin. Reprod. Med. 32, 410–413. doi:10.1055/s-0034-1376360.

Bogacka, I., Bogacki, M., Wasielak, M., 2013. The effect of embryo presence on the expression of peroxisome proliferator activated receptor (PPAR) genes in the porcine reproductive system during periimplantation. Acta Vet. Hung. 61, 405–415. doi:10.1556/AVet.2013.013.

Borthwick, J.M., Charnock-Jones, D.S., Tom, B.D., Hull, M.L., Teirney, R., Phillips, S.C., Smith, S.K., 2003. Determination of the transcript profile of human endometrium. Mol. Hum. Reprod. 9, 19–33.

Brosens, J.J., Salker, M.S., Teklenburg, G., Nautiyal, J., Salter, S., Lucas, E.S., Steel, J.H., Christian, M., Chan, Y.-W., Boomsma, C.M., Moore, J.D., Hartshorne, G.M., Sućurović, S., Mulac-Jericevic, B., Heijnen, C.J., Quenby, S., Koerkamp, M.J.G., Holstege, F.C.P., Shmygol, A., Macklon, N.S., 2014. Uterine selection of human embryos at implantation. Sci. Rep. 4, 3894. doi:10.1038/srep03894.

Carson, D.D., Lagow, E., Thathiah, A., Al-Shami, R., Farach-Carson, M.C., Vernon, M., Yuan, L., Fritz, M.A., Lessey, B., 2002. Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening. Mol. Hum. Reprod. 8, 871–879.

Chan, C., Virtanen, C., Winegarden, N.A., Colgan, T.J., Brown, T.J., Greenblatt, E.M., 2013. Discovery of biomarkers of endometrial receptivity through a minimally invasive approach: a validation study with implications for assisted reproduction. Fertil. Steril. 100, 810–817. doi:10.1016/j.fertnstert.2013.04.047.

Colicchia, M., Campagnolo, L., Baldini, E., Ulisse, S., Valensise, H., Moretti, C., 2014. Molecular basis of thyrotropin and thyroid hormone action during implantation and early development. Hum. Reprod. Update 0, 1–21. doi:10.1093/humupd/dmu028.

Cowley, S.M., Hoare, S., Mosselman, S., Parker, M.G., 1997. Estrogen receptors alpha and beta form heterodimers on DNA. J. Biol. Chem. 272, 19858–19862.

Croxtall, J.D., Elder, M.G., White, J.O., 1990. Hormonal control of proliferation in the Ishikawa endometrial adenocarcinoma cell line. J. Steroid Biochem. 35, 665–669.

Please cite this article in press as: Signe Altmäe, Karin Tamm-Rosenstein, Francisco J. Esteban, Jaak Simm, Liis Kolberg, Hedi Peterson, Madis Metsis, Kai Haldre, José A. Horcajadas, Andres Salumets, Anneli Stavreus-Evers, Endometrial transcriptome analysis indicates superiority of natural over artificial cycles in patients who have experienced recurrent implantation failure undergoing frozen embryo transfer, Reproductive BioMedicine Online (2016), doi: 10.1016/j.rbmo.2016.03.004

Dassen, H., Punyadeera, C., Kamps, R., Klomp, J., Dunselman, G., Dijcks, F., de Goeij, A., Ederveen, A., Groothuis, P., 2007. Progesterone regulation of implantation-related genes: new insights into the role of oestrogen. Cell. Mol. Life Sci. 64, 1009–1032. doi:10.1007/s00018-007-6553-9.

Díaz-Gimeno, P., Ruíz-Alonso, M., Blesa, D., Simón, C., 2014. Transcriptomics of the human endometrium. Int. J. Dev. Biol. 58, 127–137. doi:10.1387/ijdb.130340pd.

Diaz-Gimeno, P., Horcajadas, J.A., Martinez-Conejero, J.A., Esteban, F.J., Alama, P., Pellicer, A., Simon, C., 2011. A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature. Fertil. Steril. 95, 50–60, 60.e1-60.15. doi:10.1016/j.fertnstert.2010.04.063; S0015-0282(10)00701-6 [pii].

Diaz-Gimeno, P., Ruiz-Alonso, M., Blesa, D., Bosch, N., Martinez-Conejero, J.A., Alama, P., Garrido, N., Pellicer, A., Simon, C., 2013. The accuracy and reproducibility of the endometrial receptivity array is superior to histology as a diagnostic method for endometrial receptivity. Fertil. Steril. 99, 508–517. doi:10.1016/j.fertnstert.2012.09.046.

Dos Santos, E., Serazin, V., Morvan, C., Torre, A., Wainer, R., De Mazancourt, P., Dieudonné, M.N., 2012. Adiponectin and leptin systems in human endometrium during window of implantation. Fertil. Steril. 97, 771–778. doi:10.1016/j.fertnstert.2011.12.042.

El-Toukhy, T., Coomarasamy, A., Khairy, M., Sunkara, K., Seed, P., Khalaf, Y., Braude, P., 2008. The relationship between endometrial thickness and outcome of medicated frozen embryo replacement cycles. Fertil. Steril. 89, 832–839. doi:10.1016/j.fertnstert.2007.04.031.

Evans, J., Hannan, N.J., Edgell, T.A., Vollenhoven, B.J., Lutjen, P.J., Osianlis, T., Salamonsen, L.A., Rombauts, L.J.F., 2014. Fresh versus frozen embryo transfer: backing clinical decisions with scientific and clinical evidence. Hum. Reprod. Update 20, 808–821. doi:10.1093/humupd/dmu027.

Fatemi, H.M., Camus, M., Kolibianakis, E.M., Tournaye, H., Papanikolaou, E.G., Donoso, P., Devroey, P., 2007. The luteal phase of recombinant follicle-stimulating hormone/gonadotropin-releasing hormone antagonist in vitro fertilization cycles during supplementation with progesterone or progesterone and estradiol. Fertil. Steril. 87, 504–508. doi:10.1016/j.fertnstert.2006.07.1521.

Garrido-Gomez, T., Ruiz-Alonso, M., Blesa, D., Diaz-Gimeno, P., Vilella, F., Simon, C., 2013. Profiling the gene signature of endometrial receptivity: clinical results. Fertil. Steril. 99, 1078–1085. doi:10.1016/j.fertnstert.2012.12.005.

Giudice, L.C., 2006. Application of functional genomics to primate endometrium: insights into biological processes. Reprod. Biol. Endocrinol. 4 (Suppl. 1), S4. doi:10.1186/1477-7827-4-S1-S4; 1477-7827-4-S1-S4 [pii].

Givens, C.R., Markun, L.C., Ryan, I.P., Chenette, P.E., Herbert, C.M., Schriock, E.D., 2009. Outcomes of natural cycles versus programmed cycles for 1677 frozen-thawed embryo transfers. Reprod. Biomed. Online 19, 380–384.

Gonzalez, R.R., Simon, C., Caballero-Campo, P., Norman, R., Chardonnens, D., Devoto, L., Bischof, P., 2000. Leptin and reproduction. Hum. Reprod. Update 6, 290–300.

Groenewoud, E.R., Macklon, N.S., Cohlen, B.J., 2012. Cryo-thawed embryo transfer: natural versus artificial cycle. A non-inferiority trial (ANTARCTICA trial). BMC Womens Health 12, 27. doi:10.1186/1472-6874-12-27.

Groenewoud, E.R., Cantineau, A.E.P., Kollen, B.J., Macklon, N.S., Cohlen, B.J., 2013. What is the optimal means of preparing the endometrium in frozen-thawed embryo transfer cycles? A systematic review and meta-analysis. Hum. Reprod. Update 19, 458–470. doi:10.1093/humupd/dmt030.

Hancke, K., More, S., Kreienberg, R., Weiss, J.M., 2012. Patients undergoing frozen-thawed embryo transfer have similar live birth rates in spontaneous and artificial cycles. J. Assist. Reprod. Genet. 29, 403–407. doi:10.1007/s10815-012-9724-z.

Haouzi, D., Assou, S., Mahmoud, K., Tondeur, S., Reme, T., Hedon, B., De Vos, J., Hamamah, S., 2009a. Gene expression profile of human endometrial receptivity: comparison between natural and stimulated cycles for the same patients. Hum. Reprod. 24, 1436–1445. doi:10.1093/humrep/dep039.

Haouzi, D., Mahmoud, K., Fourar, M., Bendhaou, K., Dechaud, H., De Vos, J., Reme, T., Dewailly, D., Hamamah, S., 2009b. Identification of new biomarkers of human endometrial receptivity in the natural cycle. Hum. Reprod. 24, 198–205. doi:10.1093/humrep/den360; den360 [pii].

Hapangama, D.K., Kamal, A.M., Bulmer, J.N., 2014. Estrogen receptor : the guardian of the endometrium. Hum. Reprod. Update 21, 174–193. doi:10.1093/humupd/dmu053.

Hill, M.J., Miller, K.A., Frattarelli, J.L., 2010. A GnRH agonist and exogenous hormone stimulation protocol has a higher live-birth rate than a natural endogenous hormone protocol for frozen-thawed blastocyst-stage embryo transfer cycles: an analysis of 1391 cycles. Fertil. Steril. 93, 416–422. doi:10.1016/j.fertnstert.2008.11.027.

Horcajadas, J.A., Riesewijk, A., Martin, J., Cervero, A., Mosselman, S., Pellicer, A., Simon, C., 2004. Global gene expression profiling of human endometrial receptivity. J. Reprod. Immunol. 63, 41–49. doi:10.1016/j.jri.2004.02.003; S0165037804000671 [pii].

Horcajadas, J.A., Pellicer, A., Simon, C., 2007. Wide genomic analysis of human endometrial receptivity: new times, new opportunities. Hum. Reprod. Update 13, 77–86. doi:10.1093/humupd/dml046; dml046 [pii].

Horcajadas, J.A., Minguez, P., Dopazo, J., Esteban, F.J., Dominguez, F., Giudice, L.C., Pellicer, A., Simon, C., 2008. Controlled ovarian stimulation induces a functional genomic delay of the endometrium with potential clinical implications. J. Clin. Endocrinol. Metab. 93, 4500–4510. doi:10.1210/jc.2008-0588; jc.2008-0588 [pii].

Hu, S., Yao, G., Wang, Y., Xu, H., Ji, X., He, Y., Zhu, Q., Chen, Z., Sun, Y., 2014. Transcriptomic changes during the pre-receptive to receptive transition in human endometrium detected by RNA-Seq. J. Clin. Endocrinol. Metab. 99, E2744–E2753. doi:10.1210/jc.2014-2155.

Huang da, W., Sherman, B.T., Lempicki, R.A., 2009. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 37, 1–13. doi:10.1093/nar/gkn923.

Ishihara, O., Araki, R., Kuwahara, A., Itakura, A., Saito, H., Adamson, G.D., 2014. Impact of frozen-thawed single-blastocyst transfer on maternal and neonatal outcome: an analysis of 277,042 single-embryo transfer cycles from 2008 to 2010 in Japan. Fertil. Steril. 101, 128–133. doi:10.1016/j.fertnstert.2013.09.025.

Kao, L.C., Tulac, S., Lobo, S., Imani, B., Yang, J.P., Germeyer, A., Osteen, K., Taylor, R.N., Lessey, B.A., Giudice, L.C., 2002. Global gene profiling in human endometrium during the window of implantation. Endocrinology 143, 2119–2138.

Klinge, C.M., Jernigan, S.C., Mattingly, K.A., Risinger, K.E., Zhang, J., 2004. Estrogen response element-dependent regulation of transcriptional activation of estrogen receptors alpha and beta by coactivators and corepressors. J. Mol. Endocrinol. 33, 387–410. doi:10.1677/jme.1.01541.

Koler, M., Achache, H., Tsafrir, A., Smith, Y., Revel, A., Reich, R., 2009. Disrupted gene pattern in patients with repeated in vitro fertilization (IVF) failure. Hum. Reprod. 24, 2541–2548. doi:10.1093/humrep/dep193; dep193 [pii].

Koot, Y.E.M., van Hooff, S.R., Boomsma, C.M., van Leenen, D., Groot Koerkamp, M.J.A., Goddijn, M., Eijkemans, M.J.C., Fauser, B.C.J.M., Holstege, F.C.P., Macklon, N.S., 2016. An endometrial gene expression signature accurately predicts recurrent implantation failure after IVF. Sci. Rep. 6, 19411. doi:10.1038/srep19411.

Lédée, N., Munaut, C., Aubert, J., Sérazin, V., Rahmati, M., Chaouat, G., Sandra, O., Foidart, J.M., 2011. Specific and extensive

Please cite this article in press as: Signe Altmäe, Karin Tamm-Rosenstein, Francisco J. Esteban, Jaak Simm, Liis Kolberg, Hedi Peterson, Madis Metsis, Kai Haldre, José A. Horcajadas, Andres Salumets, Anneli Stavreus-Evers, Endometrial transcriptome analysis indicates superiority of natural over artificial cycles in patients who have experienced recurrent implantation failure undergoing frozen embryo transfer, Reproductive BioMedicine Online (2016), doi: 10.1016/j.rbmo.2016.03.004

endometrial deregulation is present before conception in IVF/ICSI repeated implantation failures (IF) or recurrent miscarriages. J. Pathol. 225, 554–564. doi:10.1002/path.2948.

Lédée-Bataille, N., Laprée-Delage, G., Taupin, J.-L., Dubanchet, S., Frydman, R., Chaouat, G., 2002. Concentration of leukaemia inhibitory factor (LIF) in uterine flushing fluid is highly predictive of embryo implantation. Hum. Reprod. 17, 213–218.

Lessey, B.A., Ilesanmi, A.O., Castelbaum, A.J., Yuan, L., Somkuti, S.G., Chwalisz, K., Satyaswaroop, P.G., 1996. Characterization of the functional progesterone receptor in an endometrial adenocarcinoma cell line (Ishikawa): progesterone-induced expression of the alpha1 integrin. J. Steroid Biochem. Mol. Biol. 59, 31–39.

Liu, P., Hwang, J.T., 2007. Quick calculation for sample size while controlling false discovery rate with application to microarray analysis. Bioinformatics 23, 739–746. doi:10.1093/bioinformatics/btl664.

Maheshwari, A., Pandey, S., Shetty, A., Hamilton, M., Bhattacharya, S., 2012. Obstetric and perinatal outcomes in singleton pregnancies resulting from the transfer of frozen thawed versus fresh embryos generated through in vitro fertilization treatment: a systematic review and meta-analysis. Fertil. Steril. 98, 368–377, e1-e9. doi:10.1016/j.fertnstert.2012.05.019.

Margalioth, E.J., Ben-Chetrit, A., Gal, M., Eldar-Geva, T., 2006. Investigation and treatment of repeated implantation failure following IVF-ET. Hum. Reprod. 21, 3036–3043. doi:10.1093/humrep/del305.

Matthews, J., Gustafsson, J.A., 2003. Estrogen signaling: a subtle balance between ER alpha and ER beta. Mol. Interv. 3, 281–292. doi:10.1124/mi.3.5.281.

Matys, V., Kel-Margoulis, O.V., Fricke, E., Liebich, I., Land, S., Barre-Dirrie, A., Reuter, I., Chekmenev, D., Krull, M., Hornischer, K., Voss, N., Stegmaier, P., Lewicki-Potapov, B., Saxel, H., Kel, A.E., Wingender, E., 2006. TRANSFAC and its module TRANSCompel: transcriptional gene regulation in eukaryotes. Nucleic Acids Res. 34, D108–D110. doi:10.1093/nar/gkj143.

McGowen, M.R., Erez, O., Romero, R., Wildman, D.E., 2014. The evolution of embryo implantation. Int. J. Dev. Biol. 58, 155–161. doi:10.1387/ijdb.140020dw.

Noyes, R.W., Hertig, A.T., Rock, J., 1975. Dating the endometrial biopsy. Am. J. Obstet. Gynecol. 122, 262–263.

Osteen, K.G., Keller, N.R., Feltus, F.A., Melner, M.H., 1999. Paracrine regulation of matrix metalloproteinase expression in the normal human endometrium. Gynecol. Obstet. Invest. 48 (Suppl. 1), 2–13. goi8a002 [pii].

Pinborg, A., Wennerholm, U.B., Romundstad, L.B., Loft, A., Aittomaki, K., Söderström-Anttila, V., Nygren, K.G., Hazekamp, J., Bergh, C., 2013. Why do singletons conceived after assisted reproduction technology have adverse perinatal outcome? Systematic review and meta-analysis. Hum. Reprod. Update 19, 87–104. doi:10.1093/humupd/dms044.

Polanski, L.T., Baumgarten, M.N., Quenby, S., Brosens, J., Campbell, B.K., Raine-Fenning, N.J., 2014. What exactly do we mean by "recurrent implantation failure"? A systematic review and opinion. Reprod. Biomed. Online 28, 409–423. doi:10.1016/j.rbmo.2013.12.006.

Riesewijk, A., Martin, J., van Os, R., Horcajadas, J.A., Polman, J., Pellicer, A., Mosselman, S., Simon, C., 2003. Gene expression profiling of human endometrial receptivity on days LH+2 versus LH+7 by microarray technology. Mol. Hum. Reprod. 9, 253–264.

Roque, M., Lattes, K., Serra, S., Solà, I., Geber, S., Carreras, R., Checa, M.A., 2013. Fresh embryo transfer versus frozen embryo transfer in in vitro fertilization cycles: a systematic review and meta-analysis. Fertil. Steril. 99, 156–162. doi:10.1016/j.fertnstert.2012.09.003.

Roque, M., Valle, M., Guimarães, F., Sampaio, M., Geber, S., 2015. Freeze-all policy: fresh vs. frozen-thawed embryo transfer. Fertil. Steril. 103, 1190–1193. doi:10.1016/j.fertnstert.2015.01.045.

Ruiz-Alonso, M., Blesa, D., Simon, C., 2012. The genomics of the human endometrium. Biochim. Biophys. Acta 1822, 1931–1942. doi:10.1016/j.bbadis.2012.05.004.

Ruiz-Alonso, M., Blesa, D., Díaz-Gimeno, P., Gómez, E., Fernández-Sánchez, M., Carranza, F., Carrera, J., Vilella, F., Pellicer, A., Simón, C., 2013. The endometrial receptivity array for diagnosis and personalized embryo transfer as a treatment for patients with repeated implantation failure. Fertil. Steril. 100, 818–824. doi:10.1016/j.fertnstert.2013.05.004.

Ruiz-Alonso, M., Galindo, N., Pellicer, A., Simón, C., 2014. What a difference two days make: "personalized" embryo transfer (pET) paradigm: a case report and pilot study. Hum. Reprod. 29, 1244–1247. doi:10.1093/humrep/deu070.

Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezantsev, A., Popov, D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavich, A., Trush, V., Quackenbush, J., 2003. TM4: a free, open-source system for microarray data management and analysis. Biotechniques 34, 374–378.

Savaris, R.F., Giudice, L.C., 2009. Genomics analysis: endometrium. Methods Mol. Biol. 590, 91–113. doi:10.1007/978-1-60327-378-7_6.

Shapiro, B.S., Daneshmand, S.T., Garner, F.C., Aguirre, M., Hudson, C., Thomas, S., 2011. Evidence of impaired endometrial receptivity after ovarian stimulation for in vitro fertilization: a prospective randomized trial comparing fresh and frozen-thawed embryo transfer in normal responders. Fertil. Steril. 96, 344–348. doi:10.1016/j.fertnstert.2011.05.050.

Shapiro, B.S., Daneshmand, S.T., Restrepo, H., Garner, F.C., Aguirre, M., Hudson, C., 2013. Matched-cohort comparison of single-embryo transfers in fresh and frozen-thawed embryo transfer cycles. Fertil. Steril. 99, 389–392. doi:10.1016/j.fertnstert.2012.09.044.

Shapiro, B.S., Daneshmand, S.T., Garner, F.C., Aguirre, M., Hudson, C., 2014. Freeze-all can be a superior therapy to another fresh cycle in patients with prior fresh blastocyst implantation failure. Reprod. Biomed. Online 29, 286–290. doi:10.1016/j.rbmo.2014.04.009.

Simon, A., Laufer, N., 2012. Repeated implantation failure: clinical approach. Fertil. Steril. 97, 1039–1043. doi:10.1016/j.fertnstert.2012.03.010.

Stilley, J.A.W., Christensen, D.E., Dahlem, K.B., Guan, R., Santillan, D.A., England, S.K., Al-Hendy, A., Kirby, P.A., Segaloff, D.L., 2014. FSH Receptor (FSHR) expression in human extragonadal reproductive tissues and the developing placenta, and the impact of its deletion on pregnancy in mice. Biol. Reprod. 91, 74. doi:10.1095/biolreprod.114.118562.

Talbi, S., Hamilton, A.E., Vo, K.C., Tulac, S., Overgaard, M.T., Dosiou, C., Le Shay, N., Nezhat, C.N., Kempson, R., Lessey, B.A., Nayak, N.R., Giudice, L.C., 2006. Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. Endocrinology 147, 1097–1121. doi:10.1210/en.2005-1076.

Tamm-Rosenstein, K., Simm, J., Suhorutshenko, M., Salumets, A., Metsis, M., 2013. Changes in the transcriptome of the human endometrial Ishikawa cancer cell line induced by estrogen, progesterone, tamoxifen, and mifepristone (RU486) as detected by RNA-sequencing. PLoS ONE 8, e68907. doi:10.1371/journal.pone.0068907.

Tapia, A., Gangi, L.M., Zegers-Hochschild, F., Balmaceda, J., Pommer, R., Trejo, L., Pacheco, I.M., Salvatierra, A.M., Henriquez, S., Quezada, M., Vargas, M., Rios, M., Munroe, D.J., Croxatto, H.B., Velasquez, L., 2008. Differences in the endometrial transcript profile during the receptive period between women who were refractory to implantation and those who achieved pregnancy.

Please cite this article in press as: Signe Altmäe, Karin Tamm-Rosenstein, Francisco J. Esteban, Jaak Simm, Liis Kolberg, Hedi Peterson, Madis Metsis, Kai Haldre, José A. Horcajadas, Andres Salumets, Anneli Stavreus-Evers, Endometrial transcriptome analysis indicates superiority of natural over artificial cycles in patients who have experienced recurrent implantation failure undergoing frozen embryo transfer, Reproductive BioMedicine Online (2016), doi: 10.1016/j.rbmo.2016.03.004

Hum. Reprod. 23, 340–351. doi:10.1093/humrep/dem319; dem319 [pii].
Tapia, A., Vilos, C., Marin, J.C., Croxatto, H.B., Devoto, L., 2011. Bioinformatic detection of E47, E2F1 and SREBP1 transcription factors as potential regulators of genes associated to acquisition of endometrial receptivity. Reprod. Biol. Endocrinol. 9, 14. doi:10.1186/1477-7827-9-14.
Tapia-Pizarro, A., Figueroa, P., Brito, J., Marín, J.C., Munroe, D.J., Croxatto, H.B., 2014. Endometrial gene expression reveals compromised progesterone signaling in women refractory to embryo implantation. Reprod. Biol. Endocrinol. 12, 92. doi:10.1186/1477-7827-12-92.
Tomás, C., Alsbjerg, B., Martikainen, H., Humaidan, P., 2012. Pregnancy loss after frozen-embryo transfer – a comparison of three protocols. Fertil. Steril. 98, 1165–1169. doi:10.1016/j.fertnstert.2012.07.1058.
van Mourik, M.S., Macklon, N.S., Heijnen, C.J., 2009. Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. J. Leukoc. Biol. 85, 4–19. doi:10.1189/jlb.0708395; jlb.0708395 [pii].
Weinerman, R., Mainigi, M., 2014. Why we should transfer frozen instead of fresh embryos: the translational rationale. Fertil. Steril. 102, 10–18. doi:10.1016/j.fertnstert.2014.05.019.
Wennerholm, U.-B., Henningsen, A.-K.A., Romundstad, L.B., Bergh, C., Pinborg, A., Skjaerven, R., Forman, J., Gissler, M., Nygren, K.G., Tiitinen, A., 2013. Perinatal outcomes of children born after frozen-thawed embryo transfer: a Nordic cohort study from the CoNARTaS group. Hum. Reprod. 28, 2545–2553. doi:10.1093/humrep/det272.
WHO, 1999. Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction, fourth ed. Cambridge University Press, Cambridge.
Wong, K.M., Mastenbroek, S., Repping, S., 2014. Cryopreservation of human embryos and its contribution to in vitro fertilization success rates. Fertil. Steril. 102, 19–26. doi:10.1016/j.fertnstert.2014.05.027.
Young, S.L., 2013. Oestrogen and progesterone action on endometrium: a translational approach to understanding endometrial receptivity. Reprod. Biomed. Online 27, 497–505. doi:10.1016/j.rbmo.2013.06.010.
Yovich, J.L., Conceicao, J.L., Stanger, J.D., Hinchliffe, P.M., Keane, K.N., 2015. Mid-luteal serum progesterone concentrations govern implantation rates for cryopreserved embryo transfers conducted under hormone replacement. Reprod. Biomed. Online 31, 180–191. doi:10.1016/j.rbmo.2015.05.005.
Zhang, D., Sun, C., Ma, C., Dai, H., Zhang, W., 2012a. Data mining of spatial-temporal expression of genes in the human endometrium during the window of implantation. Reprod. Sci. 19, 1085–1098. doi:10.1177/1933719112442248.
Zhang, R.-J., Zou, L.-B., Zhang, D., Tan, Y.-J., Wang, T.-T., Liu, A.-X., Qu, F., Meng, Y., Ding, G.-L., Lu, Y.-C., Lv, P.-P., Sheng, J.-Z., Huang, H.-F., 2012b. Functional expression of large-conductance calcium-activated potassium channels in human endometrium: a novel mechanism involved in endometrial receptivity and embryo implantation. J. Clin. Endocrinol. Metab. 97, 543–553. doi:10.1210/jc.2011-2108.

Declaration: The authors report no financial or commercial conflicts of interest.

Received 26 November 2015; refereed 14 March 2016; accepted 15 March 2016.

Please cite this article in press as: Signe Altmäe, Karin Tamm-Rosenstein, Francisco J. Esteban, Jaak Simm, Liis Kolberg, Hedi Peterson, Madis Metsis, Kai Haldre, José A. Horcajadas, Andres Salumets, Anneli Stavreus-Evers, Endometrial transcriptome analysis indicates superiority of natural over artificial cycles in patients who have experienced recurrent implantation failure undergoing frozen embryo transfer, Reproductive BioMedicine Online (2016), doi: 10.1016/j.rbmo.2016.03.004

CURRICULUM VITAE

Personal data

Name: Karin Rosenstein (maiden name Tamm)
Date and place of birth: 04.03.1981, Ollepa, Estonia
Nationality: Estonian
E-mail: karin.tamm@ttu.ee

Education

2006 - ...	Tallinn University of Technology, PhD studies in chemistry and gene technology
2003 - 2005	University of Tartu, Faculty of Biology and Geography, MSc in developmental biology
1999 - 2003	University of Tartu, Faculty of Biology and Geography, BSc in gene technology
1996 - 1999	Pärnu-Jaagupi Secondary School, secondary education

Language competence

Estonian - native speaker
English - fluent

Professional employment

2015 - ...	Cellin Technologies, Project manager
2010 - ...	Nova Vita Clinic, Quality manager and Research coordinator
2011 - 2012	Competence Centre on Health Technologies,
2009 - 2010	CRM London Clinic, embryologist
2009 - 2009	Create Health Clinic, embryologist
2008 - 2012	Tallinn University of Technology, Centre for Biology of Integrated Systems
2005 - 2009	Nova Vita Clinic, embryologist

Supervised dissertations

2013 - Marina Suhorutšenko, master's thesis, “Gene Regulation by Natural Modulators of AhR: Their Role in Male Infertility and Prostate Cancer”.
2008 - Miia Rõõm, master's thesis, “Estrogen signalling in mammary gland and endometrium derived cell lines”.

International conferences

2011 - ESHRE (European Society of Human Reproduction and Embryology) 27th Annual Meeting. Stockholm, Sweden. Poster presentation.
2010 - ESHRE Workshop: The Maternal Embryonic Interface. Valencia, Spain. Oral presentation.
2010 - WARM: 5th International Congress World Association of Reproductive Medicine. Russia, Moscow. Poster presentation.

2010 - Illumina Workshop: Sequencing Simplified, Haartman Institute, Helsinki, Finland.
2009 - Spetses summer school. Nuclear Receptor Signalling: From Molecular Mechanisms to Integrative Physiology. Greek, Spetses. Poster presentation.
2008 - CSHL (Cold Spring Harbor Laboratory) conference. Nuclear Receptors: Bench to Bedside. Cold Spring Harbor, New York, USA. Poster presentation.
2008 - Keystone conference. Nuclear Receptors: Steroid Sisters. Canada, Whistler. Poster presentation.

Publications

1. Altmäe S, **Tamm-Rosenstein K**, Esteban FJ, Simm J, Kolberg L, Peterson H, Metsis M, Haldre K, Horcajadas JA, Salumets A, and Stavreus-Evers A. Endometrial transcriptome analysis indicates superiorty of natural over artifical cycles in recurrent implantation failure patients undergoing frozen embryo transfer. Reproductive BioMedicine Online. In Press.
2. Ahelik A, Mändar R, Korrovits P, Karits P, Talving E, **Rosenstein K**, Jaagura M, Salumets A, Kullisaar T. Systemic oxidative stress could predict assisted reproductive technique outcome. J Assist Reprod Genet. 2015 May; 32(5):699-704. doi: 10.1007/s10815-015-0466-6. Epub 2015 Mar 28.
3. **Tamm-Rosenstein K**, Simm J, Suhorutšenko M, Salumets A, Metsis M. (2013). Changes in the Transcriptome of the Human Endometrial Ishikawa Cancer Cell Line Induced by Estrogen, Progesterone, Tamoxifen, and Mifepristone (RU486) as Detected by RNA-Sequencing. PLoS ONE, 8, e68907.
4. **Tamm K**, Suhorutshenko M, Rõõm M, Simm J, Metsis M. (2012). The Tissue Specific Role of Estrogen and Progesterone in Human Endometrium and Mammary Gland. Abduljabbar, H. (Toim.). Steroids - Basic Science (35 - 64). InTech - Open Access Publisher.
5. **Tamm K**, Suhorutšenko M, Talving E, Kaljas A, Metsis M. (2012). Suguhormoonide östradiooli ja progesterooni koespetsiifiline roll inimese endomeetriumis ja rinnanäärmes. Eesti Arst, 91(4), 182 - 189.
6. Piirsoo M, Kaljas A, **Tamm K**, Timmusk T. (2010). Expression of NGF and GDNF family members and their receptors during peripheral nerve development and differentiation of Schwann cells in vitro. Neuroscience Letters, 469(1), 135 - 140.
7. **Tamm K**, Rõõm M, Salumets A, Metsis M. (2009). Genes targeted by the estrogen and progesterone receptors in the human endometrial cell lines HEC1A and RL95-2. Reproductive biology and endocrinology, 7(170), 1 - 12.

ELULOOKIRJELDUS

Isikuandmed
Nimi: Karin Rosenstein (endine Tamm)
Sünniaeg ja -koht: 04.03.1981, Ollepa, Eesti
Kodakondsus: eestlane
E-posti aadress: karin.tamm@ttu.ee

Hariduskäik

2006 - ...	Tallinna Tehnikaülikool, keemia ja geenitehnoloogia doktorantuur
2003 - 2005	Tartu Ülikool, Bioloogia-Geograafia teaduskond, MSc arengubioloogias
1999 - 2003	Tartu Ülikool, Bioloogia-Geograafia teaduskond, BSc geenitehnoloogias
1996 - 1999	Pärnu-Jaagupi Keskkool, keskharidus

Keelteoskus
Eesti keel - emakeel
Inglise keel - kõrgtase

Teenistuskäik

2015 - ...	Cellin Technologies, projektijuht
2010 - ...	Nova Vita Kliinik, kvaliteedijuht ja teadusprojektide koordinaator
2011 - 2012	Tervisetehnoloogiate Arenduskeskus, teadur
2009 - 2010	CRM London Clinic, embrüoloog
2009 - 2009	Create Health Clinic, embrüoloog
2008 - 2012	Tallinna Tehnikaülikool, Integreeritud Süsteemide Bioloogia Keskus, teadur
2005 - 2009	Nova Vita Kliinik, embrüoloog

Juhendatud lõputööd
2013 - Marina Suhorutšenko, magistritöö, "Tuumaretseptori AhR looduslike modulaatorite mõju geeniregulatsioonile: roll meeste viljatuses ja eesnäärme vähis".
2008 - Miia Rõõm, magistritöö, "Östradiooli sihtmärkgeenid rinnakoe ja endomeetriumi rakuliinides".

Konverentsid
2011 - ESHRE (*European Society of Human Reproduction and Embryology*) aastakonverents, Stockholm, Sweden. Posterettekanne.
2010 - ESHRE töötuba teemal: *"The Maternal Embryonic Interface"*. Valencia, Spain. Suuline ettekanne.

2010 - WARM *(World Association of Reproductive Medicine).* Moskva, Venemaa. Posterettekanne.
2010 - Illumina töötuba teemal: *"Sequencing Simplified"*. Haartman Instituut, Helsingi, Soome.
2009 - Spetses suvekool. *Nuclear Receptor Signalling: From Molecular Mechanisms to Integrative Physiology.* Kreeka, Spetses. Posterettekanne.
2008 - CSHL (Cold Spring Harbor Laboratory) konverents. *Nuclear Receptors: Bench to Bedside.* Cold Spring Harbor, USA. Posterettekanne
2008 - Keystone konverents: *Nuclear Receptors: Steroid Sisters.* Kanada, Whistler. Poster Posterettekanne.

Publikatsioonid

1. Altmäe S, **Tamm-Rosenstein K**, Esteban FJ, Simm J, Kolberg L, Peterson H, Metsis M, Haldre K, Horcajadas JA, Salumets A, and Stavreus-Evers A. Endometrial transcriptome analysis indicates superiorty of natural over artifical cycles in recurrent implantation failure patients undergoing frozen embryo transfer. Reproductive BioMedicine Online. In Press.
2. Ahelik A, Mändar R, Korrovits P, Karits P, Talving E, **Rosenstein K**, Jaagura M, Salumets A, Kullisaar T. Systemic oxidative stress could predict assisted reproductive technique outcome. J Assist Reprod Genet. 2015 May; 32(5):699-704. doi: 10.1007/s10815-015-0466-6. Epub 2015 Mar 28.
3. **Tamm-Rosenstein K**, Simm J, Suhorutšenko M, Salumets A, Metsis M. (2013). Changes in the Transcriptome of the Human Endometrial Ishikawa Cancer Cell Line Induced by Estrogen, Progesterone, Tamoxifen, and Mifepristone (RU486) as Detected by RNA-Sequencing. PLoS ONE, 8, e68907.
4. **Tamm K**, Suhorutshenko M, Rõõm M, Simm J, Metsis M. (2012). The Tissue Specific Role of Estrogen and Progesterone in Human Endometrium and Mammary Gland. Abduljabbar, H. (Toim.). Steroids - Basic Science (35 - 64).InTech - Open Access Publisher.
5. **Tamm K**, Suhorutšenko M, Talving E, Kaljas A, Metsis M. (2012). Suguhormoonide östradiooli ja progesterooni koespetsiifiline roll inimese endomeetriumis ja rinnanäärmes. Eesti Arst, 91(4), 182 - 189.
6. Piirsoo M, Kaljas A, **Tamm K**, Timmusk T. (2010). Expression of NGF and GDNF family members and their receptors during peripheral nerve development and differentiation of Schwann cells in vitro. Neuroscience Letters, 469(1), 135 - 140.
7. **Tamm K**, Rõõm M, Salumets A, Metsis M. (2009). Genes targeted by the estrogen and progesterone receptors in the human endometrial cell lines HEC1A and RL95-2. Reproductive biology and endocrinology, 7(170), 1 - 12.

DISSERTATIONS DEFENDED AT TALLINN UNIVERSITY OF TECHNOLOGY ON *NATURAL AND EXACT SCIENCES*

1. **Olav Kongas**. Nonlinear Dynamics in Modeling Cardiac Arrhytmias. 1998.

2. **Kalju Vanatalu**. Optimization of Processes of Microbial Biosynthesis of Isotopically Labeled Biomolecules and Their Complexes. 1999.

3. **Ahto Buldas**. An Algebraic Approach to the Structure of Graphs. 1999.

4. **Monika Drews**. A Metabolic Study of Insect Cells in Batch and Continuous Culture: Application of Chemostat and Turbidostat to the Production of Recombinant Proteins. 1999.

5. **Eola Valdre**. Endothelial-Specific Regulation of Vessel Formation: Role of Receptor Tyrosine Kinases. 2000.

6. **Kalju Lott**. Doping and Defect Thermodynamic Equilibrium in ZnS. 2000.

7. **Reet Koljak**. Novel Fatty Acid Dioxygenases from the Corals *Plexaura homomalla* and *Gersemia fruticosa.* 2001.

8. **Anne Paju**. Asymmetric oxidation of Prochiral and Racemic Ketones by Using Sharpless Catalyst. 2001.

9. **Marko Vendelin**. Cardiac Mechanoenergetics *in silico*. 2001.

10. **Pearu Peterson**. Multi-Soliton Interactions and the Inverse Problem of Wave Crest. 2001.

11. **Anne Menert**. Microcalorimetry of Anaerobic Digestion. 2001.

12. **Toomas Tiivel**. The Role of the Mitochondrial Outer Membrane in *in vivo* Regulation of Respiration in Normal Heart and Skeletal Muscle Cell. 2002.

13. **Olle Hints**. Ordovician Scolecodonts of Estonia and Neighbouring Areas: Taxonomy, Distribution, Palaeoecology, and Application. 2002.

14. **Jaak Nõlvak**. Chitinozoan Biostratigrapy in the Ordovician of Baltoscandia. 2002.

15. **Liivi Kluge**. On Algebraic Structure of Pre-Operad. 2002.

16. **Jaanus Lass**. Biosignal Interpretation: Study of Cardiac Arrhytmias and Electromagnetic Field Effects on Human Nervous System. 2002.

17. **Janek Peterson**. Synthesis, Structural Characterization and Modification of PAMAM Dendrimers. 2002.

18. **Merike Vaher**. Room Temperature Ionic Liquids as Background Electrolyte Additives in Capillary Electrophoresis. 2002.

19. **Valdek Mikli**. Electron Microscopy and Image Analysis Study of Powdered Hardmetal Materials and Optoelectronic Thin Films. 2003.

20. **Mart Viljus**. The Microstructure and Properties of Fine-Grained Cermets. 2003.

21. **Signe Kask**. Identification and Characterization of Dairy-Related *Lactobacillus.* 2003

22. **Tiiu-Mai Laht**. Influence of Microstructure of the Curd on Enzymatic and Microbiological Processes in Swiss-Type Cheese. 2003.

23. **Anne Kuusksalu**. 2–5A Synthetase in the Marine Sponge *Geodia cydonium*. 2003.

24. **Sergei Bereznev**. Solar Cells Based on Polycristalline Copper-Indium Chalcogenides and Conductive Polymers. 2003.

25. **Kadri Kriis**. Asymmetric Synthesis of C_2-Symmetric Bimorpholines and Their Application as Chiral Ligands in the Transfer Hydrogenation of Aromatic Ketones. 2004.

26. **Jekaterina Reut**. Polypyrrole Coatings on Conducting and Insulating Substracts. 2004.

27. **Sven Nõmm**. Realization and Identification of Discrete-Time Nonlinear Systems. 2004.

28. **Olga Kijatkina**. Deposition of Copper Indium Disulphide Films by Chemical Spray Pyrolysis. 2004.

29. **Gert Tamberg**. On Sampling Operators Defined by Rogosinski, Hann and Blackman Windows. 2004.

30. **Monika Übner**. Interaction of Humic Substances with Metal Cations. 2004.

31. **Kaarel Adamberg**. Growth Characteristics of Non-Starter Lactic Acid Bacteria from Cheese. 2004.

32. **Imre Vallikivi**. Lipase-Catalysed Reactions of Prostaglandins. 2004.

33. **Merike Peld**. Substituted Apatites as Sorbents for Heavy Metals. 2005.

34. **Vitali Syritski**. Study of Synthesis and Redox Switching of Polypyrrole and Poly(3,4-ethylenedioxythiophene) by Using *in-situ* Techniques. 2004.

35. **Lee Põllumaa**. Evaluation of Ecotoxicological Effects Related to Oil Shale Industry. 2004.

36. **Riina Aav**. Synthesis of 9,11-Secosterols Intermediates. 2005.

37. **Andres Braunbrück**. Wave Interaction in Weakly Inhomogeneous Materials. 2005.

38. **Robert Kitt**. Generalised Scale-Invariance in Financial Time Series. 2005.

39. **Juss Pavelson**. Mesoscale Physical Processes and the Related Impact on the Summer Nutrient Fields and Phytoplankton Blooms in the Western Gulf of Finland. 2005.

40. **Olari Ilison**. Solitons and Solitary Waves in Media with Higher Order Dispersive and Nonlinear Effects. 2005.

41. **Maksim Säkki**. Intermittency and Long-Range Structurization of Heart Rate. 2005.

42. **Enli Kiipli**. Modelling Seawater Chemistry of the East Baltic Basin in the Late Ordovician–Early Silurian. 2005.

43. **Igor Golovtsov**. Modification of Conductive Properties and Processability of Polyparaphenylene, Polypyrrole and polyaniline. 2005.

44. **Katrin Laos**. Interaction Between Furcellaran and the Globular Proteins (Bovine Serum Albumin β-Lactoglobulin). 2005.

45. **Arvo Mere**. Structural and Electrical Properties of Spray Deposited Copper Indium Disulphide Films for Solar Cells. 2006.

46. **Sille Ehala**. Development and Application of Various On- and Off-Line Analytical Methods for the Analysis of Bioactive Compounds. 2006.

47. **Maria Kulp**. Capillary Electrophoretic Monitoring of Biochemical Reaction Kinetics. 2006.

48. **Anu Aaspõllu.** Proteinases from *Vipera lebetina* Snake Venom Affecting Hemostasis. 2006.

49. **Lyudmila Chekulayeva**. Photosensitized Inactivation of Tumor Cells by Porphyrins and Chlorins. 2006.

50. **Merle Uudsemaa**. Quantum-Chemical Modeling of Solvated First Row Transition Metal Ions. 2006.

51. **Tagli Pitsi**. Nutrition Situation of Pre-School Children in Estonia from 1995 to 2004. 2006.

52. **Angela Ivask**. Luminescent Recombinant Sensor Bacteria for the Analysis of Bioavailable Heavy Metals. 2006.

53. **Tiina Lõugas**. Study on Physico-Chemical Properties and Some Bioactive Compounds of Sea Buckthorn (*Hippophae rhamnoides* L.). 2006.

54. **Kaja Kasemets**. Effect of Changing Environmental Conditions on the Fermentative Growth of *Saccharomyces cerevisae* S288C: Auxo-accelerostat Study. 2006.

55. **Ildar Nisamedtinov**. Application of ^{13}C and Fluorescence Labeling in Metabolic Studies of *Saccharomyces* spp. 2006.

56. **Alar Leibak**. On Additive Generalisation of Voronoï's Theory of Perfect Forms over Algebraic Number Fields. 2006.

57. **Andri Jagomägi**. Photoluminescence of Chalcopyrite Tellurides. 2006.

58. **Tõnu Martma**. Application of Carbon Isotopes to the Study of the Ordovician and Silurian of the Baltic. 2006.

59. **Marit Kauk**. Chemical Composition of $CuInSe_2$ Monograin Powders for Solar Cell Application. 2006.

60. **Julia Kois**. Electrochemical Deposition of $CuInSe_2$ Thin Films for Photovoltaic Applications. 2006.

61. **Ilona Oja Açik**. Sol-Gel Deposition of Titanium Dioxide Films. 2007.

62. **Tiia Anmann**. Integrated and Organized Cellular Bioenergetic Systems in Heart and Brain. 2007.

63. **Katrin Trummal**. Purification, Characterization and Specificity Studies of Metalloproteinases from *Vipera lebetina* Snake Venom. 2007.

64. **Gennadi Lessin**. Biochemical Definition of Coastal Zone Using Numerical Modeling and Measurement Data. 2007.

65. **Enno Pais**. Inverse problems to determine non-homogeneous degenerate memory kernels in heat flow. 2007.

66. **Maria Borissova**. Capillary Electrophoresis on Alkylimidazolium Salts. 2007.

67. **Karin Valmsen**. Prostaglandin Synthesis in the Coral *Plexaura homomalla*: Control of Prostaglandin Stereochemistry at Carbon 15 by Cyclooxygenases. 2007.

68. **Kristjan Piirimäe**. Long-Term Changes of Nutrient Fluxes in the Drainage Basin of the Gulf of Finland – Application of the PolFlow Model. 2007.

69. **Tatjana Dedova**. Chemical Spray Pyrolysis Deposition of Zinc Sulfide Thin Films and Zinc Oxide Nanostructured Layers. 2007.

70. **Katrin Tomson**. Production of Labelled Recombinant Proteins in Fed-Batch Systems in *Escherichia coli*. 2007.

71. **Cecilia Sarmiento**. Suppressors of RNA Silencing in Plants. 2008.

72. **Vilja Mardla**. Inhibition of Platelet Aggregation with Combination of Antiplatelet Agents. 2008.

73. **Maie Bachmann**. Effect of Modulated Microwave Radiation on Human Resting Electroencephalographic Signal. 2008.

74. **Dan Hüvonen**. Terahertz Spectroscopy of Low-Dimensional Spin Systems. 2008.

75. **Ly Villo**. Stereoselective Chemoenzymatic Synthesis of Deoxy Sugar Esters Involving *Candida antarctica* Lipase B. 2008.

76. **Johan Anton**. Technology of Integrated Photoelasticity for Residual Stress Measurement in Glass Articles of Axisymmetric Shape. 2008.

77. **Olga Volobujeva**. SEM Study of Selenization of Different Thin Metallic Films. 2008.

78. **Artur Jõgi**. Synthesis of 4'-Substituted 2,3'-dideoxynucleoside Analogues. 2008.

79. **Mario Kadastik**. Doubly Charged Higgs Boson Decays and Implications on Neutrino Physics. 2008.

80. **Fernando Pérez-Caballero**. Carbon Aerogels from 5-Methylresorcinol-Formaldehyde Gels. 2008.

81. **Sirje Vaask**. The Comparability, Reproducibility and Validity of Estonian Food Consumption Surveys. 2008.

82. **Anna Menaker**. Electrosynthesized Conducting Polymers, Polypyrrole and Poly(3,4-ethylenedioxythiophene), for Molecular Imprinting. 2009.

83. **Lauri Ilison**. Solitons and Solitary Waves in Hierarchical Korteweg-de Vries Type Systems. 2009.

84. **Kaia Ernits**. Study of In_2S_3 and ZnS Thin Films Deposited by Ultrasonic Spray Pyrolysis and Chemical Deposition. 2009.

85. **Veljo Sinivee**. Portable Spectrometer for Ionizing Radiation "Gammamapper". 2009.

86. **Jüri Virkepu**. On Lagrange Formalism for Lie Theory and Operadic Harmonic Oscillator in Low Dimensions. 2009.

87. **Marko Piirsoo**. Deciphering Molecular Basis of Schwann Cell Development. 2009.

88. **Kati Helmja**. Determination of Phenolic Compounds and Their Antioxidative Capability in Plant Extracts. 2010.

89. **Merike Sõmera**. Sobemoviruses: Genomic Organization, Potential for Recombination and Necessity of P1 in Systemic Infection. 2010.

90. **Kristjan Laes**. Preparation and Impedance Spectroscopy of Hybrid Structures Based on $CuIn_3Se_5$ Photoabsorber. 2010.

91. **Kristin Lippur**. Asymmetric Synthesis of 2,2'-Bimorpholine and its 5,5'-Substituted Derivatives. 2010.

92. **Merike Luman**. Dialysis Dose and Nutrition Assessment by an Optical Method. 2010.

93. **Mihhail Berezovski**. Numerical Simulation of Wave Propagation in Heterogeneous and Microstructured Materials. 2010.

94. **Tamara Aid-Pavlidis**. Structure and Regulation of BDNF Gene. 2010.

95. **Olga Bragina**. The Role of Sonic Hedgehog Pathway in Neuro- and Tumorigenesis. 2010.

96. **Merle Randrüüt**. Wave Propagation in Microstructured Solids: Solitary and Periodic Waves. 2010.

97. **Marju Laars**. Asymmetric Organocatalytic Michael and Aldol Reactions Mediated by Cyclic Amines. 2010.

98. **Maarja Grossberg**. Optical Properties of Multinary Semiconductor Compounds for Photovoltaic Applications. 2010.

99. **Alla Maloverjan**. Vertebrate Homologues of Drosophila Fused Kinase and Their Role in Sonic Hedgehog Signalling Pathway. 2010.

100. **Priit Pruunsild**. Neuronal Activity-Dependent Transcription Factors and Regulation of Human *BDNF* Gene. 2010.

101. **Tatjana Knjazeva**. New Approaches in Capillary Electrophoresis for Separation and Study of Proteins. 2011.

102. **Atanas Katerski**. Chemical Composition of Sprayed Copper Indium Disulfide Films for Nanostructured Solar Cells. 2011.

103. **Kristi Timmo.** Formation of Properties of $CuInSe_2$ and $Cu_2ZnSn(S,Se)_4$ Monograin Powders Synthesized in Molten KI. 2011.

104. **Kert Tamm**. Wave Propagation and Interaction in Mindlin-Type Microstructured Solids: Numerical Simulation. 2011.

105. **Adrian Popp**. Ordovician Proetid Trilobites in Baltoscandia and Germany. 2011.

106. **Ove Pärn**. Sea Ice Deformation Events in the Gulf of Finland and This Impact on Shipping. 2011.

107. **Germo Väli**. Numerical Experiments on Matter Transport in the Baltic Sea. 2011.

108. **Andrus Seiman**. Point-of-Care Analyser Based on Capillary Electrophoresis. 2011.

109. **Olga Katargina**. Tick-Borne Pathogens Circulating in Estonia (Tick-Borne Encephalitis Virus, *Anaplasma phagocytophilum*, *Babesia* Species): Their Prevalence and Genetic Characterization. 2011.

110. **Ingrid Sumeri**. The Study of Probiotic Bacteria in Human Gastrointestinal Tract Simulator. 2011.

111. **Kairit Zovo**. Functional Characterization of Cellular Copper Proteome. 2011.

112. **Natalja Makarytsheva**. Analysis of Organic Species in Sediments and Soil by High Performance Separation Methods. 2011.

113. **Monika Mortimer**. Evaluation of the Biological Effects of Engineered Nanoparticles on Unicellular Pro- and Eukaryotic Organisms. 2011.

114. **Kersti Tepp**. Molecular System Bioenergetics of Cardiac Cells: Quantitative Analysis of Structure-Function Relationship. 2011.

115. **Anna-Liisa Peikolainen**. Organic Aerogels Based on 5-Methylresorcinol. 2011.

116. **Leeli Amon**. Palaeoecological Reconstruction of Late-Glacial Vegetation Dynamics in Eastern Baltic Area: A View Based on Plant Macrofossil Analysis. 2011.

117. **Tanel Peets**. Dispersion Analysis of Wave Motion in Microstructured Solids. 2011.

118. **Liina Kaupmees**. Selenization of Molybdenum as Contact Material in Solar Cells. 2011.

119. **Allan Olspert**. Properties of VPg and Coat Protein of Sobemoviruses. 2011.

120. **Kadri Koppel**. Food Category Appraisal Using Sensory Methods. 2011.

121. **Jelena Gorbatšova**. Development of Methods for CE Analysis of Plant Phenolics and Vitamins. 2011.

122. **Karin Viipsi**. Impact of EDTA and Humic Substances on the Removal of Cd and Zn from Aqueous Solutions by Apatite. 2012.

123. **David Schryer**. Metabolic Flux Analysis of Compartmentalized Systems Using Dynamic Isotopologue Modeling. 2012.

124. **Ardo Illaste**. Analysis of Molecular Movements in Cardiac Myocytes. 2012.

125. **Indrek Reile**. 3-Alkylcyclopentane-1,2-Diones in Asymmetric Oxidation and Alkylation Reactions. 2012.

126. **Tatjana Tamberg**. Some Classes of Finite 2-Groups and Their Endomorphism Semigroups. 2012.

127. **Taavi Liblik**. Variability of Thermohaline Structure in the Gulf of Finland in Summer. 2012.

128. **Priidik Lagemaa**. Operational Forecasting in Estonian Marine Waters. 2012.

129. **Andrei Errapart**. Photoelastic Tomography in Linear and Non-linear Approximation. 2012.

130. **Külliki Krabbi**. Biochemical Diagnosis of Classical Galactosemia and Mucopolysaccharidoses in Estonia. 2012.

131. **Kristel Kaseleht**. Identification of Aroma Compounds in Food using SPME-GC/MS and GC-Olfactometry. 2012.

132. **Kristel Kodar**. Immunoglobulin G Glycosylation Profiling in Patients with Gastric Cancer. 2012.

133. **Kai Rosin**. Solar Radiation and Wind as Agents of the Formation of the Radiation Regime in Water Bodies. 2012.

134. **Ann Tiiman**. Interactions of Alzheimer's Amyloid-Beta Peptides with Zn(II) and Cu(II) Ions. 2012.

135. **Olga Gavrilova**. Application and Elaboration of Accounting Approaches for Sustainable Development. 2012.

136. **Olesja Bondarenko**. Development of Bacterial Biosensors and Human Stem Cell-Based *In Vitro* Assays for the Toxicological Profiling of Synthetic Nanoparticles. 2012.

137. **Katri Muska**. Study of Composition and Thermal Treatments of Quaternary Compounds for Monograin Layer Solar Cells. 2012.

138. **Ranno Nahku**. Validation of Critical Factors for the Quantitative Characterization of Bacterial Physiology in Accelerostat Cultures. 2012.

139. **Petri-Jaan Lahtvee**. Quantitative Omics-level Analysis of Growth Rate Dependent Energy Metabolism in *Lactococcus lactis*. 2012.

140. **Kerti Orumets**. Molecular Mechanisms Controlling Intracellular Glutathione Levels in Baker's Yeast *Saccharomyces cerevisiae* and its Random Mutagenized Glutathione Over-Accumulating Isolate. 2012.

141. **Loreida Timberg**. Spice-Cured Sprats Ripening, Sensory Parameters Development, and Quality Indicators. 2012.

142. **Anna Mihhalevski**. Rye Sourdough Fermentation and Bread Stability. 2012.

143. **Liisa Arike**. Quantitative Proteomics of *Escherichia coli*: From Relative to Absolute Scale. 2012.

144. **Kairi Otto**. Deposition of In_2S_3 Thin Films by Chemical Spray Pyrolysis. 2012.

145. **Mari Sepp**. Functions of the Basic Helix-Loop-Helix Transcription Factor TCF4 in Health and Disease. 2012.

146. **Anna Suhhova**. Detection of the Effect of Weak Stressors on Human Resting Electroencephalographic Signal. 2012.

147. **Aram Kazarjan**. Development and Production of Extruded Food and Feed Products Containing Probiotic Microorganisms. 2012.

148. **Rivo Uiboupin**. Application of Remote Sensing Methods for the Investigation of Spatio-Temporal Variability of Sea Surface Temperature and Chlorophyll Fields in the Gulf of Finland. 2013.

149. **Tiina Kriščiunaite**. A Study of Milk Coagulability. 2013.

150. **Tuuli Levandi**. Comparative Study of Cereal Varieties by Analytical Separation Methods and Chemometrics. 2013.

151. **Natalja Kabanova**. Development of a Microcalorimetric Method for the Study of Fermentation Processes. 2013.

152. **Himani Khanduri**. Magnetic Properties of Functional Oxides. 2013.

153. **Julia Smirnova**. Investigation of Properties and Reaction Mechanisms of Redox-Active Proteins by ESI MS. 2013.

154. **Mervi Sepp**. Estimation of Diffusion Restrictions in Cardiomyocytes Using Kinetic Measurements. 2013.

155. **Kersti Jääger**. Differentiation and Heterogeneity of Mesenchymal Stem Cells. 2013.

156. **Victor Alari**. Multi-Scale Wind Wave Modeling in the Baltic Sea. 2013.

157. **Taavi Päll**. Studies of CD44 Hyaluronan Binding Domain as Novel Angiogenesis Inhibitor. 2013.

158. **Allan Niidu**. Synthesis of Cyclopentane and Tetrahydrofuran Derivatives. 2013.

159. **Julia Geller**. Detection and Genetic Characterization of *Borrelia* Species Circulating in Tick Population in Estonia. 2013.

160. **Irina Stulova**. The Effects of Milk Composition and Treatment on the Growth of Lactic Acid Bacteria. 2013.

161. **Jana Holmar**. Optical Method for Uric Acid Removal Assessment During Dialysis. 2013.

162. **Kerti Ausmees**. Synthesis of Heterobicyclo[3.2.0]heptane Derivatives *via* Multicomponent Cascade Reaction. 2013.

163. **Minna Varikmaa**. Structural and Functional Studies of Mitochondrial Respiration Regulation in Muscle Cells. 2013.

164. **Indrek Koppel**. Transcriptional Mechanisms of BDNF Gene Regulation. 2014.

165. **Kristjan Pilt**. Optical Pulse Wave Signal Analysis for Determination of Early Arterial Ageing in Diabetic Patients. 2014.

166. **Andres Anier**. Estimation of the Complexity of the Electroencephalogram for Brain Monitoring in Intensive Care. 2014.

167. **Toivo Kallaste**. Pyroclastic Sanidine in the Lower Palaeozoic Bentonites – A Tool for Regional Geological Correlations. 2014.

168. **Erki Kärber**. Properties of ZnO-nanorod/In_2S_3/$CuInS_2$ Solar Cell and the Constituent Layers Deposited by Chemical Spray Method. 2014.

169. **Julia Lehner**. Formation of Cu_2ZnSnS_4 and $Cu_2ZnSnSe_4$ by Chalcogenisation of Electrochemically Deposited Precursor Layers. 2014.

170. **Peep Pitk**. Protein- and Lipid-rich Solid Slaughterhouse Waste Anaerobic Co-digestion: Resource Analysis and Process Optimization. 2014.

171. **Kaspar Valgepea**. Absolute Quantitative Multi-omics Characterization of Specific Growth Rate-dependent Metabolism of *Escherichia coli*. 2014.

172. **Artur Noole**. Asymmetric Organocatalytic Synthesis of 3,3'-Disubstituted Oxindoles. 2014.

173. **Robert Tsanev**. Identification and Structure-Functional Characterisation of the Gene Transcriptional Repressor Domain of Human Gli Proteins. 2014.

174. **Dmitri Kartofelev**. Nonlinear Sound Generation Mechanisms in Musical Acoustic. 2014.

175. **Sigrid Hade**. GIS Applications in the Studies of the Palaeozoic Graptolite Argillite and Landscape Change. 2014.

176. **Agne Velthut-Meikas**. Ovarian Follicle as the Environment of Oocyte Maturation: The Role of Granulosa Cells and Follicular Fluid at Pre-Ovulatory Development. 2014.

177. **Kristel Hälvin**. Determination of B-group Vitamins in Food Using an LC-MS Stable Isotope Dilution Assay. 2014.

178. **Mailis Päri**. Characterization of the Oligoadenylate Synthetase Subgroup from Phylum Porifera. 2014.

179. **Jekaterina Kazantseva**. Alternative Splicing of *TAF4*: A Dynamic Switch between Distinct Cell Functions. 2014.

180. **Jaanus Suurväli**. Regulator of G Protein Signalling 16 (RGS16): Functions in Immunity and Genomic Location in an Ancient MHC-Related Evolutionarily Conserved Synteny Group. 2014.

181. **Ene Viiard**. Diversity and Stability of Lactic Acid Bacteria During Rye Sourdough Propagation. 2014.

182. **Kristella Hansen**. Prostaglandin Synthesis in Marine Arthropods and Red Algae. 2014.

183. **Helike Lõhelaid**. Allene Oxide Synthase-lipoxygenase Pathway in Coral Stress Response. 2015.

184. **Normunds Stivrinš**. Postglacial Environmental Conditions, Vegetation Succession and Human Impact in Latvia. 2015.

185. **Mary-Liis Kütt**. Identification and Characterization of Bioactive Peptides with Antimicrobial and Immunoregulating Properties Derived from Bovine Colostrum and Milk. 2015.

186. **Kazbulat Šogenov**. Petrophysical Models of the CO_2 Plume at Prospective Storage Sites in the Baltic Basin. 2015.

187. **Taavi Raadik**. Application of Modulation Spectroscopy Methods in Photovoltaic Materials Research. 2015.

188. **Reio Põder**. Study of Oxygen Vacancy Dynamics in Sc-doped Ceria with NMR Techniques. 2015.

189. **Sven Siir**. Internal Geochemical Stratification of Bentonites (Altered Volcanic Ash Beds) and its Interpretation. 2015.

190. **Kaur Jaanson**. Novel Transgenic Models Based on Bacterial Artificial Chromosomes for Studying BDNF Gene Regulation. 2015.

191. **Niina Karro**. Analysis of ADP Compartmentation in Cardiomyocytes and Its Role in Protection Against Mitochondrial Permeability Transition Pore Opening. 2015.

192. **Piret Laht**. B-plexins Regulate the Maturation of Neurons Through Microtubule Dynamics. 2015.

193. **Sergei Žari**. Organocatalytic Asymmetric Addition to Unsaturated 1,4-Dicarbonyl Compounds. 2015.

194. **Natalja Buhhalko**. Processes Influencing the Spatio-temporal Dynamics of Nutrients and Phytoplankton in Summer in the Gulf of Finland, Baltic Sea. 2015.

195. **Natalia Maticiuc**. Mechanism of Changes in the Properties of Chemically Deposited CdS Thin Films Induced by Thermal Annealing. 2015.

196. **Mario Öeren**. Computational Study of Cyclohexylhemicucurbiturils. 2015.

197. **Mari Kalda**. Mechanoenergetics of a Single Cardiomyocyte. 2015.

198. **Ieva Grudzinska**. Diatom Stratigraphy and Relative Sea Level Changes of the Eastern Baltic Sea over the Holocene. 2015.

199. **Anna Kazantseva**. Alternative Splicing in Health and Disease. 2015.

200. **Jana Kazarjan**. Investigation of Endogenous Antioxidants and Their Synthetic Analogues by Capillary Electrophoresis. 2016.

201. **Maria Safonova**. SnS Thin Films Deposition by Chemical Solution Method and Characterization. 2016.

202. **Jekaterina Mazina**. Detection of Psycho- and Bioactive Drugs in Different Sample Matrices by Fluorescence Spectroscopy and Capillary Electrophoresis. 2016.