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EFFECTS OF INSULIN ON THE DECIDUALIZATION OF HUMAN ENDOMETRIAL STROMAL CELLS – IN VITRO STUDIES

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Effects of insulin on the decidualization of human endometrial stromal cells – *in vitro* studies
THESIS FOR DOCTORAL DEGREE (PhD)

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To Amanda and Elly Estra

POPULAR SCIENCE SUMMARY OF THE THESIS

Infertility is common and research about its causes is important in order to find new treatments. Insulin is a hormone that regulates the body's energy supplies and high insulin levels are typical for several disorders that are associated with fertility problems, such as obesity and polycystic ovary syndrome (PCOS). PCOS is the most common hormonal disorder in women characterized by fertility problems and high levels of male hormones.

Decidualization is a hormone-driven process that prepares the uterus for pregnancy. It transforms endometrial stromal cells (ESCs), a specific cell type in the uterine lining, into specialized cells that protect, support and nourish the embryo. Improper decidualization is thought to lead to problems during pregnancy, such as miscarriage. The overall aim of this thesis was to study the effect of high insulin levels on decidualization.

Decidualization can be induced in a laboratory setting and its progress evaluated by measuring the activity of specific genes. Because decidualization makes the cells bigger, estimating changes in cell size is also useful for determining decidual progress. We showed that high insulin levels both enhance and suppress genes that are typically active during decidualization making it impossible to decide whether the overall effect is negative or positive. Furthermore, insulin does not affect the size of cells. Therefore, insulin changes the process of decidualization, but it is difficult to state whether it is a good or a bad change based on gene expression and cell size only. More information is needed.

In the beginning of pregnancy, both fetal and uterine cells have to be motile in order to set the ground for a healthy pregnancy. We showed that insulin could significantly suppress the movement of these cells, especially in combination with high levels of male hormones. We studied the combined effect of male hormones and insulin on decidualization, because high insulin levels often coexist with high levels of male hormones in PCOS. Decreased motility of cells might contribute to the development of pregnancy complications associated with high insulin levels, which might point towards insulin having a negative effect on decidualization.

In summary, we showed that insulin seems to affect the process of decidualization on several levels. The overall effect is a state of imbalance where different cell characteristics change in sometimes opposite directions. However, the negative effect on cellular movement generally points towards a negative direction, taking us one step closer to explaining the mechanisms behind pregnancy complications presenting together with high insulin levels.

ABSTRACT

Hyperinsulinemia is a known characteristic of obesity and polycystic ovary syndrome, two common clinical conditions associated with reproductive problems. However, the underlying mechanisms to reduced fertility are not fully elucidated, and the role of insulin in reproduction needs further investigation.

Decidualization is an extensive post-ovulatory reorganization of the endometrial stromal compartment in preparation for a pregnancy. Defective decidualization has been associated with implantation failure and various pregnancy complications. The overall aim of this thesis was to study the effect of high insulin levels on different aspects of decidualization *in vitro*.

Study I aimed to examine the role of forkhead box O (FOXO) 1 in mediating the effect of insulin on decidualizing endometrial stromal cells (ESCs) and relate changes in decidual markers to cell morphology. We found that insulin downregulates the expression of FOXO1-target genes by nuclear export of FOXO1. However, despite significant suppression of these decidual markers, no significant changes in morphological transformation characteristic to decidualization were detected.

Study II describes the role of insulin in the regulation of prokineticin (PROK) 1, a known regulator of placenta formation during the first trimester. We demonstrated a significant enhancement of PROK1 expression when ESCs were decidualized in the presence of insulin. Furthermore, PROK1 inhibited the migration of ESCs, and the migration and invasion of extravillous trophoblast cells.

Study III aimed to elucidate the effect of high insulin levels on decidual solute carrier family 2 member 1 (SLC2A1), the most abundant glucose transporter in decidual ESCs, and glucose uptake. SLC2A1 mRNA and protein were suppressed by insulin. Furthermore, suppression in decidual cell glucose uptake was detected.

Study IV investigated a potential interaction between insulin and androgens in the regulation of decidual gene expression, cell morphology and motility. We showed that the combination of insulin and dihydrotestosterone enhanced the expression of several decidual markers, increased decidual cell size and complexity, but at the same time inhibited the migration of decidual ESCs and invasion of trophoblast cells.

In summary, the results of this thesis suggest that high levels of insulin significantly modify several aspects of decidualization, including suppressing FOXO1 target genes and augmenting other decidual markers. Insulin also interacts with androgens in enhancing some decidual characteristics while inhibiting cell migration and trophoblast invasion. The overall effect seems to be a dysregulation of the process, which might have implications for the understanding of reproductive problems in conditions of hyperinsulinemia and hyperandrogenism.

LIST OF SCIENTIFIC PAPERS

I. Ujvari D*, Jakson I*, Babayeva S, Salamon D, Rethi B, Gidlöf S, Hirschberg AL. **Dysregulation of *in vitro* decidualization of human endometrial stromal cells by insulin via transcriptional inhibition of forkhead box protein O1**. *Plos One*. 2017; 12(1): e0171004
** Shared first authorship*

II. Ujvari D, Jakson I, Oldmark C, Attarha S, Gidlöf S, Hirschberg AL. **Prokineticin 1 is up-regulated by insulin in decidualizing human endometrial stromal cells.** *Journal of Cellular Molecular Medicine. 2018; 22(1): 163-17*

III. Jakson I, Ujvari D, Brusell Gidlöf S, Hirschberg AL. **Insulin regulation of solute carrier family 2 member 1 (glucose transporter 1) expression and glucose uptake in decidualizing human endometrial stromal cells: an *in vitro* study.** *Reproductive Biology and Endocrinology. 2020; 18(1): 117*

IV. Hirschberg AL, Jakson I, Graells Brugalla C, Salamon D, Ujvari D. **Interaction between insulin and androgen signalling in decidualization, cell migration and trophoblast invasion *in vitro.*** *Published online in Journal of Cellular Molecular Medicine*

CONTENTS

LIST OF ABBREVIATIONS

[3H]-2DG	Tritiated 2-deoxy-D-glucose
AR	Androgen receptor
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CTGF	Connective tissue growth factor
CX43	Connexin 43
db-cAMP	N^6 2`-O-dibutyryladenosine cAMP
DCN	Decorin
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
ESC	Endometrial stromal cell
EVT	Extravillous trophoblast
FBS	Fetal bovine serum
FSH	Follicle-stimulating hormone
FOXO	Forkhead box O
GDM	Gestational diabetes mellitus
GLUT1	Glucose transporter 1
GnRH	Gonadotropin releasing hormone
HI-FBS	Heat-inactivated fetal bovine serum
HIF1α	Hypoxia inducible factor 1 alpha
IGFBP1	Insulin-like growth factor binding protein 1
INSR	Insulin receptor
HPG	Hypothalamic-pituitary-gonadal
IGF1R	Insulin-like growth factor 1 receptor
IRS1	Insulin receptor substrate 1
IRS2	Insulin receptor substrate 2
LEFTY2	Left-right determination factor 2
LH	Luteinizing hormone

mRNA	Messenger RNA
MAPK	Mitogen activated protein kinase
MPA	Medroxyprogesterone-17-acetate
PBS	Phosphate-buffered saline
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PE	Preeclampsia
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PRL	Prolactin
PROK1	Prokineticin 1
RNA	Ribonucleic acid
RPL13a	Ribosomal protein L13a
SDS	Sodium dodecyl sulfate
SHBG	Sex hormone binding globulin
siRNA	Small interfering RNA
SLC2A1	Solute carrier family 2 member 1
SLC2A3	Solute carrier family 2 member 3
SLC2A4	Solute carrier family 2 member 4
SLC2A10	Solute carrier family 2 member 10
TF	Tissue factor
TIMP	Tissue inhibitor of metalloproteinase
TBS	Tris-buffered saline

1 LITERATURE REVIEW

1.1 HYPERINSULINEMIA AND REPRODUCTIVE IMPLICATIONS

Insulin is a peptide hormone with diverse biological functions. It stimulates cellular glucose uptake, regulates carbohydrate, lipid and protein metabolism and promotes cell growth and survival, mitogenesis and gene expression (Figure 1) (1). High levels of insulin are a sign of abundant exogenous energy and lead to energy storage and conservation. Insulin is secreted by the beta cells of the pancreatic islets of Langerhans in response to various stimuli with rising glucose levels considered to be the main stimulus. Insulin binding to the insulin receptor (INSR) present in target tissues activates its tyrosine kinase activity, which subsequently phosphorylates intracellular mediators thereby activating distinct pathways such as phosphatidylinositol-3-kinase (PI3K) and Akt, Ras/mitogen activated protein kinase (MAPK) and G proteins. The final effect is achieved via regulation of intracellular vesicle trafficking, changes in gene transcription and enzyme activity of various targets. The effect is dependent on the specific tissue, time of exposure and presence of other mediators (1,2).

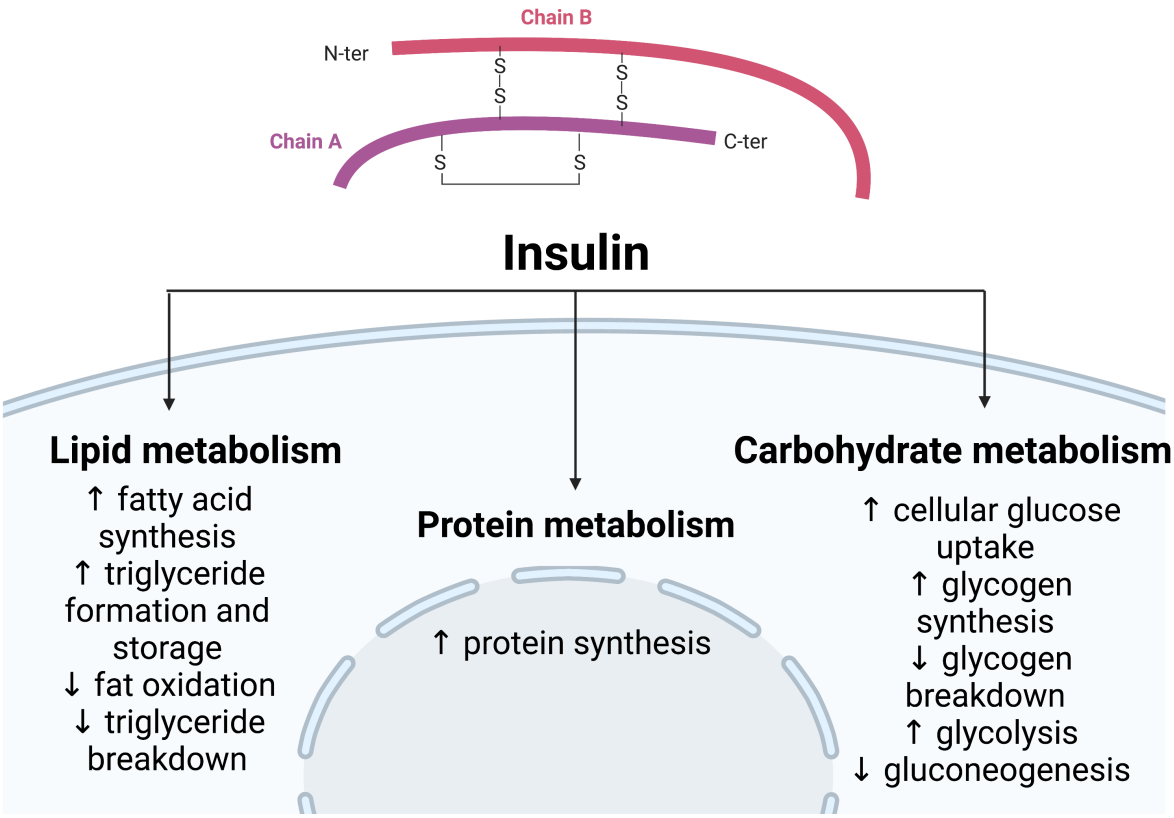

Figure 1 Biological functions of insulin. © Ivika Jakson. Created with BioRender.com.

Hyperinsulinemia may be defined as the presence of excess insulin in the blood relative to glucose levels (3). It is usually the result of insulin resistance where decreased insulin sensitivity of cells is compensated with increased insulin secretion by the pancreas (4). Studies with mouse models suggest that deviations in insulin signaling might affect the hypothalamic-pituitary-gonadal (HPG) axis, decidualization and angiogenesis during early pregnancy (5,6). In the ovaries, insulin signaling has been shown to stimulate follicle-stimulating hormone (FSH)-dependent steroidogenesis in granulosa cells and luteinizing hormone (LH)-dependent stroidogenesis in theca cells (7). Insulin has also been implicated in the central regulation of gonadotropin releasing hormone (GnRH) and LH secretion, however, the exact functions of insulin are often difficult to elucidate because of accompanying metabolic disturbances (8).

Much knowledge about the effect of insulin and hyperinsulinemia on reproductive function has originated from hyperinsulinemic disorders associated with subfertility and various pregnancy complications. Polycystic ovary syndrome (PCOS) and obesity are two examples of such disorders.

PCOS is the most common endocrine syndrome in women of reproductive age and is characterized by a combination of ovulatory dysfunction, hyperandrogenism and polycystic ovarian morphology. The prevalence is approximately 5-15%, depending on the diagnostic criteria used (9). The majority, but not all women with PCOS are obese and hyperinsulinemic (about 50% and 50-70%, respectively) (10–12). In PCOS, hyperinsulinemia, which is independent of body mass index (BMI), seems to be contributing to hyperandrogenism and ovulatory dysfunction (13).

In 2013, more than 20% of European women were obese, whereas in United States of America, North Africa and Middle East more than 30% of women had a BMI of more than 30 kg/m^2 and thereby classified as obese. Worldwide, the prevalence of overweight (BMI $\geq$ 25) among women increased from 30% in 1980 to 38% in 2013 and women are more affected than men in both developed and developing countries (14). There is a well-established connection between increasing body weight and hyperinsulinemia (15).

Obesity is a significant risk factor for infertility with a combination of disturbed HPG axis, deranged oocyte quality and poor reproductive outcomes, including miscarriage, gestational diabetes mellitus (GDM) and hypertensive disorders (16,17). Subfertility in PCOS, obesity and other hyperinsulinemic disorders cannot be explained by ovulatory dysfunction only. Even with ovulatory menstrual cycles, pregnancy and live birth rates may still be lower

(18,19). This means that endometrial aberrations may contribute to subfertility in these women. In support, several pregnancy complications, including miscarriage, GDM and preeclampsia (PE) could be explained by defective uterine receptivity, implantation failure and defective placentation (20–23). The prevalence of several pregnancy complications in obese women and women with PCOS is increased, including PE, pregnancy-induced hypertension, GDM and preterm birth (24).

1.2 THE MENSTRUAL CYCLE AND THE ENDOMETRIUM

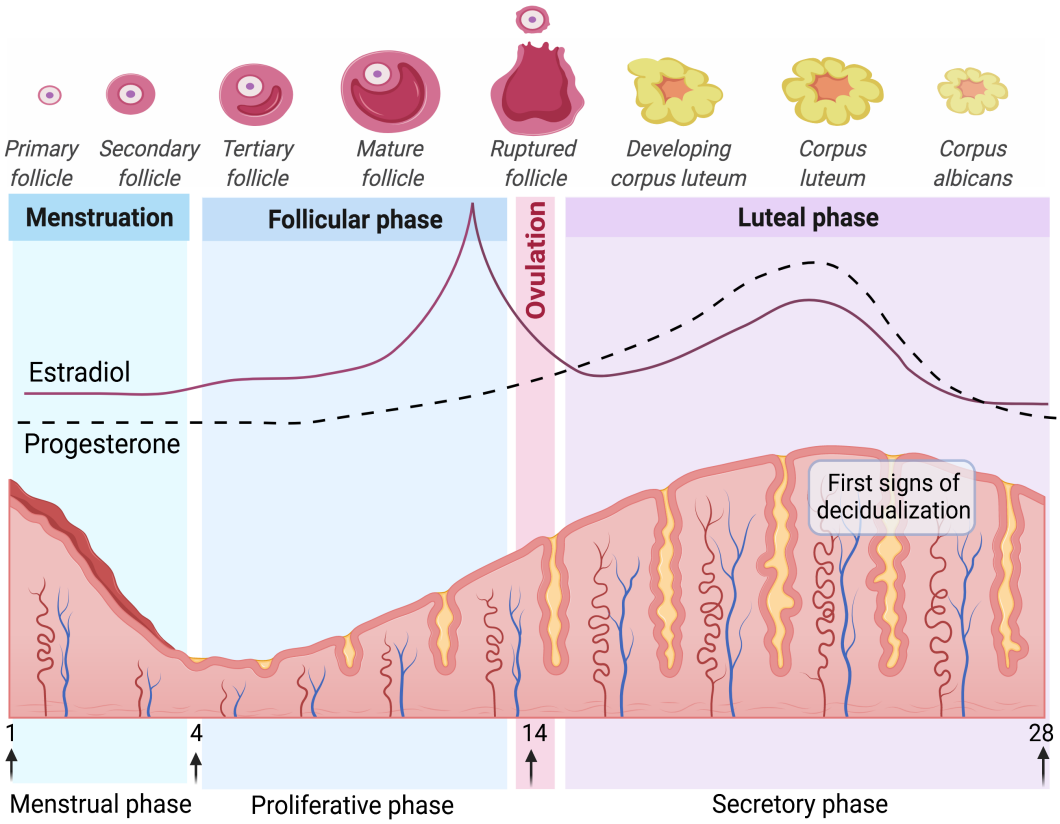

Figure 2 Menstrual cycle. Adapted from "Uterine Cycle", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates

The characteristic monthly pattern of ovarian hormones results in a normal predictable menstrual cycle. The endometrium is a highly active metabolic tissue that undergoes profound remodeling in response to the fluctuating ovarian hormones in preparation for a possible pregnancy. The menstrual cycle can be described from the ovarian perspective with follicular phase, ovulation and luteal phase indicating ovarian events, or the endometrial

perspective by proliferative phase, secretory phase and menstruation indicating endometrial events (Figure 2). During the follicular phase of the menstrual cycle, the growing dominant follicle in the ovary produces rising levels of circulating estradiol that stimulate endometrial proliferation (proliferative phase). Following an estradiol-induced surge in pituitary LH levels, the ovary releases a mature egg picked up by the fallopian tube after ovulation. Following ovulation, the remaining structures of the ruptured follicle transform into the corpus luteum that secretes estrogens and progesterone. Progesterone induces differentiation and secretory transformation of the estrogen-primed endometrium in preparation for a possible pregnancy (secretory phase). The changes include the growth of endometrial vasculature, secretory transformation of endometrial epithelial glands, recruitment of specific immune cells and decidualization of the endometrial stroma. In case pregnancy does not occur, declining ovarian hormone levels caused by a degeneration of corpus luteum result in shedding of the superficial endometrial lining during menstruation (25,26).

1.3 DECIDUALIZATION

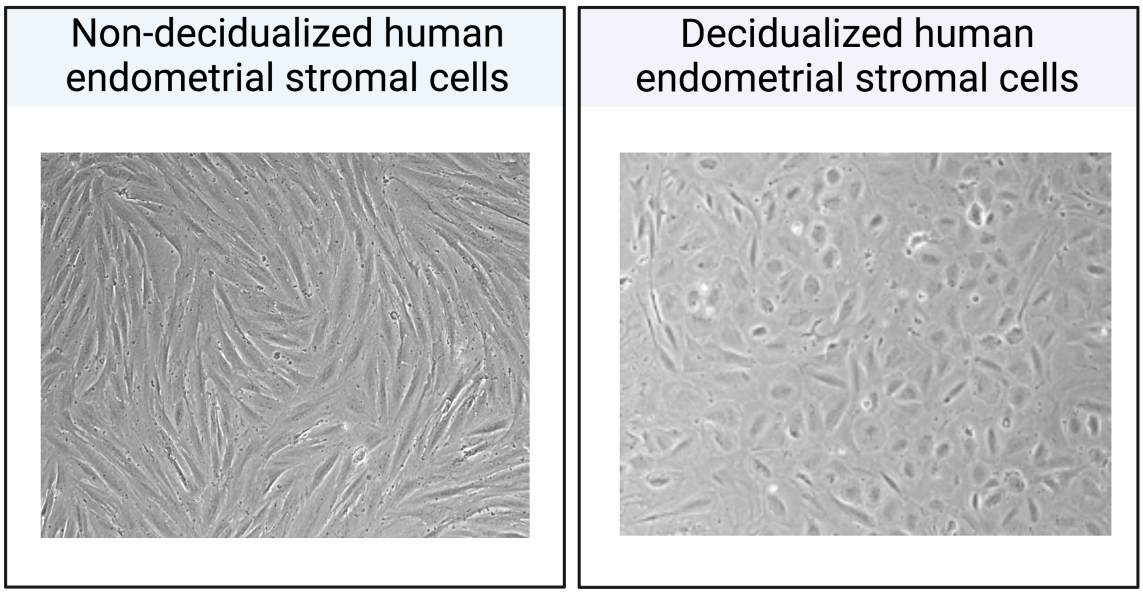

Figure 3 Morphology of decidualized and non-decidualized ESCs. © Ivika Jakson. Created with BioRender.com.

Decidualization indicates the morphological and biochemical transformation of the endometrial stroma into pregnancy specific secretory decidual tissue. Sustained decidualization is dependent on post-ovulatory increase in progesterone levels, however it is

the prior increase in cyclic adenosine monophosphate (cAMP) levels activating the protein kinase A (PKA) pathway that initiates decidualization and makes the endometrial stromal cells (ESCs) responsive to the effect of progesterone (27). Decidualized ESCs differ from non-decidualized stromal cells on a cellular and subcellular level. Decidual cells are characterized by increased circularity and cell size, as well as decreased cell stiffness and surface roughness compared to fibroblast-like non-decidualized ESCs (Figure 3) (28,29). Decidualized ESCs have more ribosomes, lysosomes, mitochondria, glycogen and lipids in the cytoplasm and a specific secretory profile that is distinct from non-decidualized stromal cells (30). Furthermore, decidual cells are considered to be important for controlling trophoblast proliferation, migration and invasion, nourishing the conceptus and protecting it from environmental and immunological assaults, mediating immune tolerance at the materno-fetal interface and possibly even recognizing low quality embryos (31–36). Defects in decidual transformation are therefore hypothesized to lead to early recurrent pregnancy loss, but also to late-pregnancy complications, such as preeclampsia (PE) and placenta accreta (33,37–39).

The following correlation seems to exist in various primate species: the deeper the trophoblastic invasion, the more extensive the alterations in the endometrium in preparation for pregnancy, and the more common the occurrence of menstruation in a species. This implies that menstruating species undergo profound pregnancy-related endometrial remodeling. Menstruation is a relatively rare event in animals, occurring in only simian primates and some bats. Spontaneous decidualization initiated by maternal hormones instead of an implanting blastocyst, such as in rats and mice, is a unifying characteristic in species experiencing menstrual shedding that renders the endometrium dependent on sufficient levels of progesterone (40). In humans, the first signs of decidualization are apparent about 10 days after ovulation in the vicinity of terminal spiral arterioles and capillaries subsequently spreading to other parts of the stroma (26). In case of pregnancy, the proportion of decidual cells rises throughout gestation reaching approximately 60% of ESCs at full term (41).

1.3.1 Decidual transcriptome

Deoxyribonucleic acid (DNA) microarray analyses and ribonucleic acid (RNA) sequencing data have identified genes that are up- or downregulated upon decidualization. Brar *et al* found that 4% of the 6918 genes of *in vitro* decidualized human uterine fibroblasts expressed 50% induction or reduction on microarray (42). Takano *et al* conducted a genome-wide microarray analysis and found 3307 genes altered during *in vitro* decidualization of human ESCs. Roughly 1/3 were upregulated and 2/3 downregulated (36). This shows that

decidualization is characterized by extensive alterations in gene expression. Genes involved in MAPK signaling pathway are activated early and followed by the phosphoinositide signaling pathway (43).

Many genes upregulated during stromal cell decidualization have been proposed as markers of the decidual transformation. Prolactin (PRL) and insulin-like growth factor binding protein 1 (IGFBP1) are two classical markers used to evaluate decidual progress. At least one of them is used in just about all decidualization studies, whereas the use of other markers varies widely and depends on the specific aim of the study (44–46). Any gene that is consistently upregulated during decidualization could theoretically be used as a marker for decidualization. However, ideally the expression pattern should change from very low or absent expression in ESCs to abundant expression in the decidual cells (43).

The exact function of most decidual proteins is unknown. However, presumed functions have been described for many of them. For example, decorin (DCN) and tissue inhibitors of metalloproteinase (TIMPs) appear to exhibit a regulatory function during embryo invasion (47,48). Tissue factor (TF) seems to initiate hemostasis during trophoblast invasion in the beginning of pregnancy (49).

1.3.2 Motility of decidual cells and the effect of decidualization on the motility of extravillous trophoblast cells

The endometrium was previously considered a relatively passive matrix waiting for an embryo to invade it. This concept has now been challenged because of data showing how crucial communication between the maternal environment and the invading embryo is (50). The ability to migrate and invade tissues is a characteristic of decidualized and non-decidualized ESCs and extravillous trophoblast (EVT) cells. The main difference between migration and invasion is the degradation of extracellular matrix during invasion (51). The importance of EVT motility has been studied for a long time whereas the dynamic properties of endometrial cells are a newer notion (52). Besides producing signals that modify trophoblast invasion, ESCs themselves also express motile capacity necessary for successful implantation (53). This capacity seems to be dependent on decidualization status and signals from the invading trophoblast. Decidualized cells are thought to be more invasive than non-decidualized ESCs and the presence of EVT cells enhances ESC motility, irrespective of decidualization status (Figure 4) (52,54,55). As well as responding to external signals and modifying their motility accordingly, ESCs also affect EVT invasion. However, the effect of ESC decidualization status on EVT invasion is unclear because different studies have

proposed a suppressing, enhancing and even neutral effect of decidualization on EVT invasion (Figure 4) (45,46,52). Altogether, it is evident that communication between ESCs and invading EVT cells is a characteristic of implantation and that motility is affected in a reciprocal manner. However, the exact nature of these interactions as well as the clinical importance is not clear.

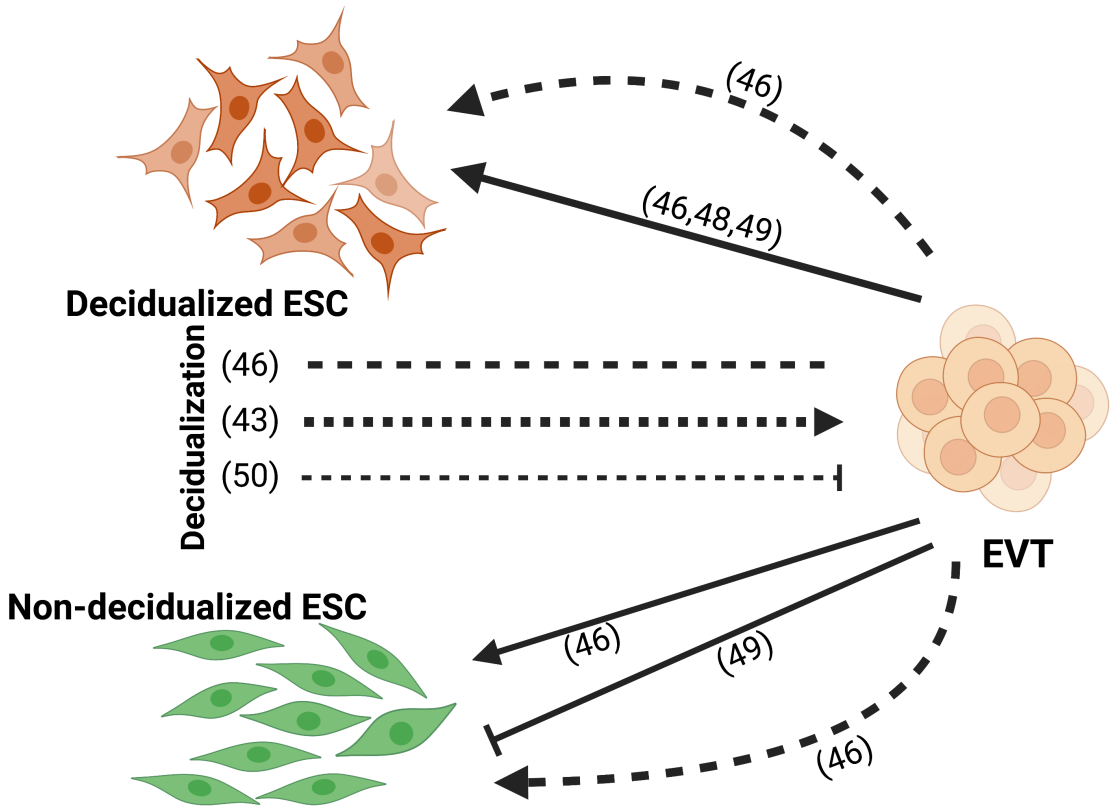

Figure 4 Promotion of migration ——>; inhibition of migration ——|; promotion of invasion – – –> ; inhibition of invasion – – –| ; no effect on invasion – – – . © Ivika Jakson. Created with BioRender.com.

1.3.3 Forkhead box O1 and decidualization

Forkhead box O (FOXO) 1 is one of many cAMP-induced transcription factors (56,57). FOXO1 is significantly upregulated very early during stromal cell decidualization. A study by Takano *et al* showed that of the 3307 genes altered upon decidualization, 15.8% were FOXO-dependent. Remarkably, a quarter of the 50 most abundant decidual gene transcripts were FOXO-regulated. A modest induction of FOXO1 results in a significantly higher induction of decidual gene expression, which suggests an involvement of co-activators that potentiate the effect of each other (36,43).

FOXO proteins belong to a highly conserved family of transcription factors. They regulate

genes responsible for proliferation, differentiation, and resistance to oxidative stress as well as apoptosis by acting as mediators for seemingly opposite decisions of cell fate (proliferation and differentiation versus cell death). Four FOXO members are present in mammals (FOXO1, FOXO3a, FOXO4 and FOXO6) with FOXO1 being the most researched in endometrial tissue because of its important role in regulating decidualization and menstruation (57). FOXO proteins are mainly regulated by reversible posttranslational modifications (phosphorylation, acetylation, ubiquitination) with or without changes in subcellular location. Transcriptional activity of FOXO proteins is highly affected by other regulatory proteins and virtually all environmental factors, which convey the ability to adapt to environmental changes.

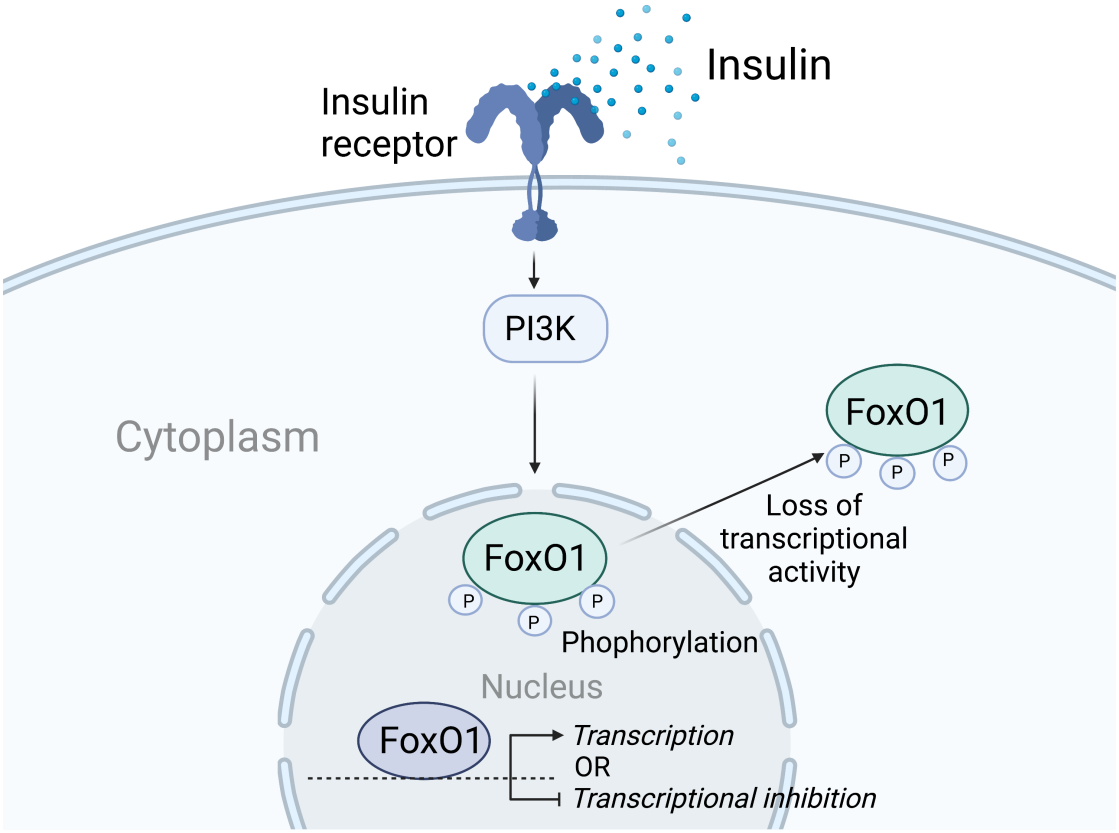

Figure 5 Insulin regulation of FOXO1. © Ivika Jakson. Created with BioRender.com.

One example of FOXO regulation is the activation of the PI3K/Akt pathway by insulin and other mitogens leading to FOXO1 phosphorylation which directs the active nuclear protein to the cytosol where it loses its transcriptional activity after binding to chaperone proteins (Figure 5) (57,58). *In vitro* studies have shown that high cAMP levels are associated with nuclear (transcriptionally active) FOXO1 and apoptosis, whereas progestins are associated with cytosolic (inactive) FOXO1 and proliferation. The subcellular location is dynamic

during the menstrual cycle and depends on the balance between different regulatory substances. FOXO1 protein is not present in follicular phase endometrial stromal cells (59). In secretory phase and first-trimester pregnancy, it is mainly located in the cytosol with the exception of pre-menstrual late secretory phase where nuclear FOXO1 prevails (60). The role of FOXO1 in mediating the effects of insulin on decidualization in endometrial tissue has not been elucidated.

1.3.4 Glucose uptake during decidualization

Sufficient glucose levels are a prerequisite for decidual transformation with decidualization hypothesized to be a highly energy-dependent process. This is based on three observations. First, progesterone-driven glycogen accumulation characterizes endometrial decidual cells meaning that assuring glucose availability is imperative (61). Second, decidualization under low-glucose culture conditions results in significantly lower levels of PRL and IGFBP1 (62,63). Third, the knockdown of the most abundant glucose transporter in the ESCs, solute carrier family 2 member 1 (SLC2A1), previously known as glucose transporter 1 (GLUT1), results in decreased expression of PRL and IGFBP1 during decidualization. At least 8 glucose transporters have been described in the uterus and endometrium. SLC2A1 is the most abundant glucose transporter in the endometrium (64).

SLC2A1 is not a classical decidualization marker because it is highly expressed in both decidualized and non-decidualized endometrial stromal cells, but the increase of this transporter can be considered as a characteristic of decidual transformation. Solute carrier family 2 member 10 (SLC2A10) and solute carrier family 2 member 3 (SLC2A3) are second and third most abundant glucose transporters in the stromal compartment of the endometrium (64). The expression of SLC2A1 is upregulated during decidualization and is associated with increased glucose uptake in the endometrial stroma (65,66). Its knockdown results in a 2-fold reduction of PRL and IGFBP1 expression (62). SLC2A1 is classically described as a non-insulin-responsive glucose transporter despite the fact that insulin has a role in regulating its expression and cellular location (67–69). This is because SLC2A1 is responsible for constitutive glucose transport in the majority of cells as opposed to solute carrier family 2 member 4 (SLC2A4) that is highly expressed in mainly insulin sensitive tissues, such as muscle and fat. The expression of insulin-responsive SLC2A4 in the endometrium is very low or non-detectable in the stroma, in contrast to SLC2A1, SLC2A10 and SLC2A3 (64,70). Our group has previously demonstrated that lifestyle intervention aiming for weight loss upregulated insulin receptor substrate 1 (IRS1) and SLC2A1 expression in the endometrium

in the proliferative phase and decreased blood insulin levels in overweight/obese women with PCOS (70).

1.4 PLACENTA FORMATION AND PROKINETICIN 1

Trophoblasts, cells from the outer layer of the blastocyst, play a pivotal role in establishing and maintaining a healthy pregnancy. During pregnancy, they differentiate into cytotrophoblasts and syncytiotrophoblasts (Figure 6). In a normal early pregnancy, cytotrophoblast cells cover the maternal-fetal interface, proliferate and form plugs that occlude endometrial spiral arteries creating a hypoxic milieu in the early placenta-fetal unit. This is necessary to protect the embryo from oxidative stress, which it is not equipped to handle yet. Syncytiotrophoblasts are a trophoblastic cell lineage responsible for nutrient and gas exchange in the placenta, as well as hormone and growth factor secretion. During the first trimester, cytotrophoblast cells switch from a proliferative to an invasive phenotype, invade the maternal decidua and spiral arteries as EVT cells and transform the utero-placental circulation into a well-oxygenated low-resistance and high-flow system with wider flaccid vessels that characterize a normal pregnancy. Failure of these changes to take place is thought to lead to adverse pregnancy outcomes (71,72).

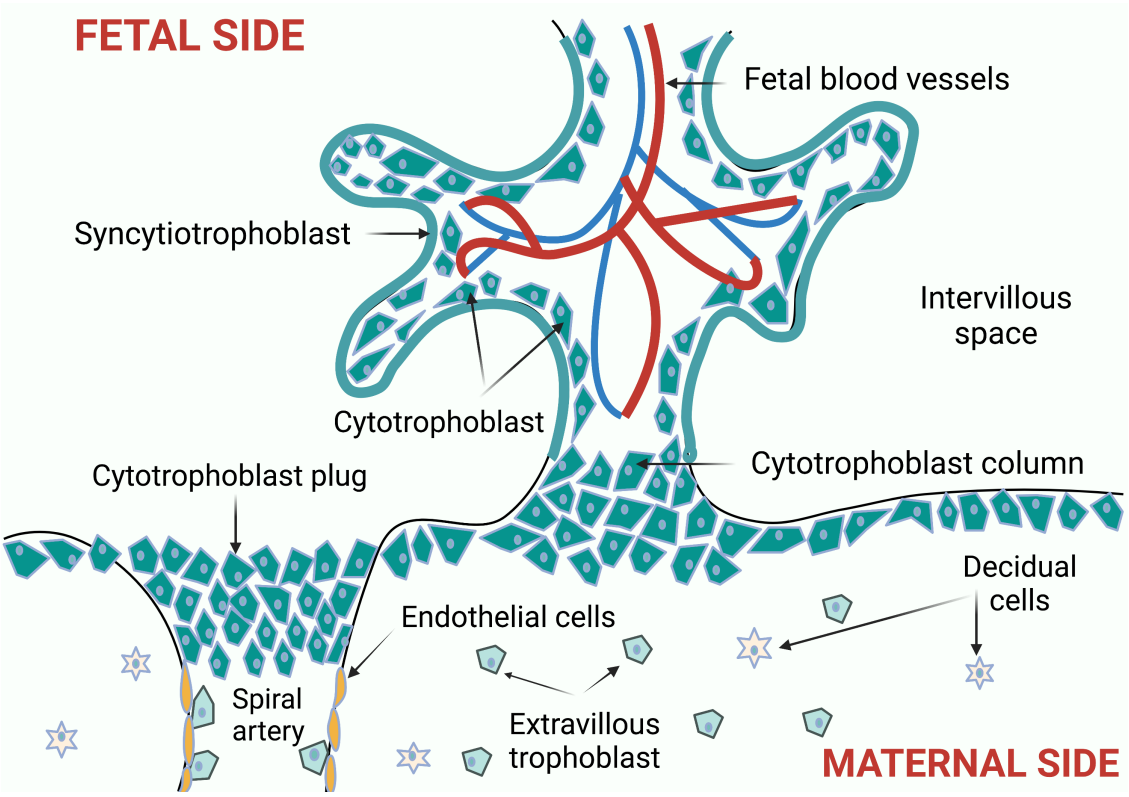

Figure 6 First trimester feto-maternal interface. © Ivika Jakson. Created with BioRender.com.

Prokineticin 1 (PROK1), a glandoendothelial growth factor produced by decidual cells and syncytiotrophoblasts, has been shown to promote trophoblast proliferation and inhibit EVT migration and invasion. Its expression is upregulated by hypoxia and human chorionic gonadotropin. Falling PROK1 levels in the end of the first trimester of normal pregnancies coincide with the invasion of EVT into the decidua and uterine spiral arteries. The declining levels of PROK1 are thought to mediate the transition from proliferative to invasive phenotype of EVT. PROK1 and its receptors have been associated with various pregnancy complications. Higher circulating levels of PROK1 have been measured in second and third trimester blood samples of preeclamptic women (73). Polymorphisms in PROK1 gene and its receptor are associated with higher risk for recurrent pregnancy loss (74). Placental levels of PROK1 and its receptors and circulating levels of PROK1 have been shown to be higher in pregnancies with fetal growth restriction (72)**.** Hyperinsulinemia is associated with various placentation disorders, but its effect on PROK1 has not been studied.

1.5 IN VITRO MODELS

1.5.1 Decidualization

In vitro models are used to study molecular mechanisms governing decidualization. Initiation of decidual transformation requires elevated levels of cAMP and sustained activation of PKA (26). cAMP is the main activator of PKA. Elevated cAMP levels can be achieved by adding prostaglandin E, relaxin, corticotropin-releasing hormone, gonadotropins or cAMP analogues to the *in vitro* system (75). Progestins are needed to sustain the decidual phenotype long-term and cAMP is thought to sensitize ESCs to progestins. Progestins alone are very weak inducers of decidual reaction and cAMP cannot sustain the decidual phenotype long-term (26). Different protocols are used to induce *in vitro* decidualization, for example combinations of a cAMP analogue and either progesterone or medroxyprogesterone-17-acetate (MPA) (51).

1.5.2 Cellular motility

Migration and invasion are two main types of cellular motility. Migration can further be divided into chemokinesis and chemotaxis. Chemotaxis denotes cellular movement towards a specific chemoattractant, whereas chemokinesis is stimulated cell motility without specific direction. What distinguishes migration from invasion is the need to degrade extracellular matrix in order to move within tissues in the latter. *In vitro*, chemokinesis is studied using

monolayer wound healing assays where migration into an artificially created cell-free zone is evaluated after a certain time period (24 hours). Transwell migration assays, where cells need to migrate towards a specific chemoattractant through a porous membrane, measure chemotaxis. In case the porous membrane is covered with artificial extracellular matrix in the same setting, cellular invasion can be evaluated (35).

1.5.3 Implantation

In vivo experiments studying molecular and cellular events in humans occurring around the time of implantation are hampered by ethical and practical complexities. Therefore, *in vitro* model systems have been developed to study implantation. Because the process involves several stages, different models may be utilized according to the purpose of the study. After the initial paracrine communication between the embryo and endometrial epithelium, the embryo attaches to the luminal epithelium and subsequently penetrates it reaching the endometrial stroma. To represent the maternal tissues taking part in implantation the following cells have been used: primary human endometrial epithelial and stromal cells and cell lines mimicking the properties of the primary cells. For the embryonic side of implantation models, human embryos or embryo surrogates can be used. While mouse blastocysts can be used as human embryo surrogates, usually trophoblast cell lines represent a better alternative because of considerable inter-species differences between early pregnancy events of mice and humans and easier access to cell lines. These cell types can be combined in implantation models in various forms. In case a human embryo is not used, trophoblast spheroids are shown to mimic the *in vivo* situation better than non-organized EVT cells. Alternatively, multilayer co-culture systems consisting of several cell types have been developed to better study the combined effects of several cell types (51).

There are many trophoblast cell lines available commercially and the choice depends on the intended experiment and also on previous studies in the field to make the results more comparable. Three often-used cell lines are HTR-8/SVneo, AC‑1M88 and JEG-3. HTR-8/SVneo is derived from first trimester villous explants and intended for adhesion, migration, invasion and proliferation studies. JEG-3 stems from choriocarcinoma explants and is best used in invasion experiments whereas AC‑1M88, a fusion of JEG-3 and term trophoblasts, is suitable for adhesion and migration experiments. The type of cell line for a specific experiment depends on the presence or absence of necessary cell adhesion molecules, proteases, cytokines, growth factors, chemokines and other factors. No cell line completely mimics all aspects of trophoblast cells *in vivo* (76).

1.6 INSULIN AND ENDOMETRIAL FUNCTION

Reports on the effect of insulin on decidual markers are contradictory. The INSR is present in the endometrium with increasing levels from follicular to luteal phase and highly expressed in the decidua, which suggests that insulin and the INSR likely regulate endometrial function before and during pregnancy (43,77). Thrailkill *et al* showed that insulin stimulates the synthesis and release of PRL from decidual cells (78). Others reported that insulin downregulates IGFBP1 by activating the PI3K pathway and, with increasing insulin doses, even the MAPK pathway (79,80). In the mouse model, hyperinsulinemia has been shown to attenuate endometrial angiogenesis (6). A few publications have suggested that insulin inhibits decidualization based on these results (81–83).

However, a recent study by Neff *et al* demonstrated the importance of insulin signaling in decidual gene expression and glucose metabolism by showing that downregulation of insulin receptor substrate 2 (IRS2) leads to decreased phosphorylation of ERK (extracellular signal-regulated kinase) 1/2 and AKT (also known as protein kinase B), reduced levels of SLC2A1 and SLC2A4 and decreased glucose uptake. Furthermore, the knockdown of IRS2 and INSR, important elements of the insulin signaling pathway, seems to suppress the expression of several decidualization markers and also the morphological transformation characteristic to decidualization (66). These notions support the importance of insulin signaling during decidualization. Furthermore, the cellular actions of insulin in the form of increased glucose uptake and glycogen, lipid and protein synthesis are all characteristics of decidual transformation making it lucrative to attribute a role to insulin in the regulation of decidualization (30).

1.7 ANDROGENS AND ENDOMETRIAL FUNCTION

Hyperinsulinemia often co-exists with hyperandrogenemia, as in women with PCOS. High insulin levels stimulate the synthesis of ovarian androgens together with LH and suppress hepatic sex hormone binding globulin (SHBG) production leading to increased bioavailable androgen levels. At the same time, hyperandrogenemia may cause free fatty acid-induced hepatic insulin resistance by stimulating visceral fat lipolysis (13). Therefore, studying the interaction between androgens and insulin is of great interest.

The androgen receptor (AR) is expressed primarily in the stromal compartment of the endometrium with highest levels observed in the early proliferative phase and declining

expression during rest of the menstrual cycle. Epithelial cells only express minimal levels of AR occasionally (84,85). AR is not present in any compartment of the endometrium in late secretory phase but becomes evident again in the decidual stromal cells during pregnancy (84,86). Circulating androgen levels do not appear to correlate with tissue androgen levels with the latter showing increased levels during the secretory phase, whereas circulating levels peak around ovulation with relatively lower levels afterwards. The apparent discrepancy between decreasing AR expression and high levels of tissue androgens seems to be explained by enhanced sensitivity of decidualizing ESCs to androgen stimulation (87). The endometrium expresses necessary enzymes for androgen synthesis from prohormones to biologically active testosterone and dihydrotestosterone (DHT) that can bind to the AR, making it a target of intracrine hormonal regulation (87,88). Androgens stimulate the synthesis of PRL and IGFBP1 and enhance cellular transformation characteristic to decidualization meaning that they seem to augment the decidual remodeling of the endometrium (88,89). However, in ovulatory women with PCOS, stromal AR levels have been found to be negatively correlated with endometrial thickness which might indicate an unfavorable effect on the secretory endometrium (90). Defective androgen regulation has been associated with reproductive dysfunction. PCOS is an example of a condition where hyperandrogenism could be involved in implantation failure and the increased risk of pregnancy complications (87).

1.8 GAP OF KNOWLEDGE

Conditions associated with chronic hyperinsulinemia and insulin resistance are becoming more common in the population, including in women of fertile age (14). The effects of chronic hyperinsulinemia on peripheral tissues have been extensively studied, as opposed to its effect on reproduction, which is less investigated. The main focus has been on the effect of hyperinsulinemia on ovarian function. However, the ovarian perspective alone does not fully explain reproductive dysfunction in women with hyperinsulinemia (18,19). Although endometrial factors and defective decidualization are known to play a role in many pregnancy complications, information about the effect of high insulin levels on decidualization is sparse. Because of the associations between hyperinsulinemia and reproductive disorders, we hypothesized that hyperinsulinemia inhibits decidualization. As FOXO1 has been shown to be a key transcription factor during decidualization, we decided to evaluate the effect of high insulin levels on FOXO1 target genes. To evaluate the decidual transformation from different perspectives we wanted to assess several properties of decidual cells. We therefore studied

cell size, glucose uptake and cellular migration and invasion in response to high insulin levels hypothesizing that all these properties might be inhibited by insulin. Furthermore, we hypothesized that PROK1, an angiogenic factor exhibiting a specific pattern of expression during placentation, might be regulated by high insulin levels in decidualizing ESCs. Because hyperinsulinemia often co-exists with hyperandrogenemia in specific patient groups, we hypothesized that these two factors together might interact in the regulation of decidual transformation and possibly potentiate each other's effects. These hypotheses were tested in the four studies included in this thesis. In 2013, when the work for this doctoral thesis began, there was very little information about the effect of insulin on decidualization *in vitro* in the literature. Thus, all our hypotheses aimed to fill a significant gap of knowledge.

2 THESIS AT A GLANCE

Study	Aim	Materials and methods	Conclusions
I	To gain insights into how insulin affects decidualization and the molecular mechanisms behind this.	Endometrial cells were obtained from healthy non-obese women with regular menstrual cycles. Methods used were cell culture, RNA extraction, reverse transcription, Real- time PCR and immunofluorescence.	Insulin inhibits the expression of FOXO1 target genes during decidualization through transcriptional and post-translational regulation of FOXO1 via the PI3K pathway without inhibiting the morphological transformation of cells.
II	To study how insulin affects PROK1 expression and early placental formation *in vitro.*	Human endometrial cells were obtained as in Study I. Methods used were cell culture, RNA extraction, reverse transcription, Real-time PCR, transwell migration assay, wound healing assay and spheroid invasion assay.	Insulin strongly upregulates PROK1 during decidualization, which can dysregulate the migration of ESCs and migration and invasion of trophoblast cells potentially affecting implantation.
III	To evaluate the effect of high levels of insulin on SLC2A1 expression and glucose uptake in decidualizing endometrial stromal cells *in vitro.*	Methods used were cell culture of human endometrial cells, RNA extraction, reverse transcription, Real-time PCR, immunocytochemistry and glucose uptake assay.	Insulin inhibits the expression of SLC2A1 on mRNA and protein level during decidualization resulting in a slight decrease in cellular glucose uptake potentially contributing to dysregulation of decidualization.
IV	To determine whether androgens modulate the effect of insulin on decidualizing ESCs.	Cell culture of human endometrial cells, RNA extraction, reverse transcription, Real-time PCR, flowcytometry, microscopy, wound healing assay, and spheroid invasion assay were used.	Insulin and androgens interact in regulating the expression of many decidualization markers and cellular morphology resulting in the enhancement of characteristics of decidualization. However, mainly the combined treatment with insulin and DHT inhibits stromal cell migration and trophoblast invasion potentially impairing decidual function.

3 RESEARCH AIMS

Overall aim

The overall aim of this project is to increase knowledge about the role of insulin alone or together with androgens in the complex process of decidualization of potential importance for decreased fertility and reproductive complications in conditions of hyperinsulinemia and hyperandrogenism.

Specific aims

Study I

To examine the effect of high insulin levels on the expression of FOXO1 target genes in decidualizing ESCs and on the morphological transformation of these cells.

Study II

To investigate the regulation of PROK1 by high insulin levels in decidualizing ESCs and whether this regulation affects ESC migration and trophoblast cell motility.

Study III

To examine the effect of high insulin levels on SLC2A1 expression and glucose uptake in decidualizing ESCs.

Study IV

To explore the possible interaction between insulin and androgens in the modulation of decidual marker expression, decidual cell size and ESC and trophoblast cell motility.

4 MATERIALS AND METHODS

4.1 SUBJECTS

In all four studies, cells from healthy women with regular menstrual cycles were used. For the first three papers endometrial biopsies were obtained from six subjects under paracervical local anesthesia on cycle day 5-9 using a soft plastic suction curette (Pipet Curet, CooperSurgical, USA). Nine subjects contributed to biopsies for the fourth study. Cycle day 5-9 was chosen in order to ensure a sufficient sample from the follicular phase of the menstrual cycle. A pelvic examination and vaginal ultrasound were performed before biopsy collection to ensure that no obvious pathologies were present. All participating women were 18-35 years old with a BMI between 19-28 meaning no obese women were included. Other exclusion criteria were smoking, hormonal treatments within 3 months prior to biopsy collection, chronic diseases, or medication use on a regular basis. All women signed a written informed consent.

4.2 CELL CULTURE (STUDY I, II, III AND IV)

After biopsy collection, the samples were immediately transported to the cell culture laboratory in Ham's F-10 Nut Mix medium (Thermo Fischer 118 Scientific, USA) supplemented with 0.2% penicillin streptomycin (Sigma-Aldrich, USA) and 20% of heat-inactivated fetal bovine serum (HI-FBS) (Sigma-Aldrich, USA) on ice and endometrial stromal cells were extracted using a protocol described by Arnold *et al* and Satyaswaroop *et al* with slight modifications (91,92). After washing, the biopsy was manually cut into 1 mm pieces and cleaned from red blood cells using a 100 μm pore size cell strainer (Falcon BD Biosciences, Belgium). Subsequently, single stromal cells were secured following sedimentation of the tissue at 140 g for 5 minutes at 4˚C and digestion with collagenase III (Worthington, USA) (1 mg/ml) supplemented with DNase I (40 μg/ml) (Sigma-Aldrich, USA) in phosphate-buffered saline (PBS) at 37˚C for 2-2.5 hours in a shaking thermostat. After centrifugation (120g, 10 min, 4˚C), we washed the cell suspensions with Ham's F-10 Nut mix prior to separating single stromal cells from glandular tubes using a 40 μm cell strainer (Falcon BD Biosciences, Belgium). The stromal cells were sedimented again (120g, 10 min, 4˚C), frozen in freezing mixture and stored in liquid nitrogen. We used sequential culturing and immunocytochemistry (cytokeratin as a marker for epithelial cells and CD10 for stromal cells) to confirm the purity of the ESCS.

For most experiments we used 6-well Costar plates (Sigma-Aldrich, USA) and seeded approximately 10^5 cells in each well in culture medium (DMEM/F12-Glutamax medium from Thermo Fischer Scientific, USA, supplemented with 10% HI-FBS from Sigma-Aldrich, USA, and 0.2% penicillin-streptomycin from Sigma-Aldrich, USA). The cells were checked daily and grown until 80-90% confluency. We induced *in vitro* decidualization using 1 μM MPA (Sigma-Aldrich, USA) and 0.5 mM N^6 2`-O-dibutyryladenosine cAMP (db-cAMP) (Sigma-Aldrich, USA) as decidualization agents. MPA was chosen instead of progesterone as it might give a more relevant decidual response because of its androgenic and glucocorticoid activity, which is a characteristic of *in vivo* decidualization. Furthermore, progesterone has a tendency to compartmentalize in culture (Jan Brosens 2017, personal communication, 20 March). The culture media during decidualization was phenol red free DMEM/F12 (Thermo Fischer Scientific, USA) supplemented with 2% charcoal-stripped fetal bovine serum (FBS) (Sigma-Aldrich, USA) and 0.2% penicillin-streptomycin (Sigma-Aldrich, USA). Phenol red free media was chosen because phenol red might mimic weak estrogens in the cell culture setting (93). The experiments with *in vitro* decidualization lasted 4-6 days, depending on the specific experiment, with the media always changed every 3 days to ensure sufficient nutrients for the cells.

In addition to decidualizing agents, various other substances were used to test the hypotheses we had.

- In study I, II and III, we decidualized the cells *in vitro* in the presence or absence of 5, 50 or 500 nM insulin for 6 days. In paper IV, only 100 nM insulin concentration was used to determine the effect of insulin on putative FOXO1-regulated decidualization markers.
- In paper IV, *in vitro* decidualization in the presence or absence of 1 μM DHT (Sigma-Aldrich, USA), 1 μM testosterone (Sigma-Aldrich, USA) or their combination with 100 nM insulin was performed for 6 days to determine the effect of insulin and androgens on decidualization.
- In papers I and II, the PI3K inhibitor Wortmannin was used to test the involvment of PI3K/Akt signal transduction cascade in the effect of insulin on decidualizing cells. For these experiments, the cells were predecidualized for 3 days before 1-hour treatment with only 500 nM Wortmannin and then 2-day treatment with 500 nM Wortmannin, 100 nM insulin and decidualizing agents.
- In papers I and III, 100 nM and 500 nM of the FOXO1 inhibitor AS1842856 (Merck Millipore, Germany, dissolved in DMSO) was used to determine the role of FOXO1 in

the effect of insulin on putative decidualization markers. The cells were treated with the FOXO1 inhibitor for 2 days in combination with decidualizing agents after pre-treatment with only the decidualizing agents for 3 days.

- In paper II, the involvement of hypoxia inducible factor 1 alfa (HIF1α) in the regulation of PROK1 by insulin was tested using echinomycin (Merck Millipore, Germany), a HIF1α inhibitor. The cells were predecidualized for 3 days before 1-hour treatment with only 5 nM echinomycin and then 2-day treatment with 5 nM echinomycin, 100nM insulin and decidualizing agents.

4.3 RNA EXTRACTION, REVERSE TRANSCRIPTION, REAL-TIME PCR (STUDY I, II, III AND IV)

Total RNA from cell pellets was purified with RNeasy® Mini Kit (Qiagen GmbH, Hilden, Germany) or Quick-RNA Miniprep Kit (Zymo Research, USA) following the protocol provided by the manufacturer. A DNase treatment step was included as a precaution. After measuring the extracted total RNA, 1 µg was subjected to reverse transcription using SuperScript VILO cDNA Synthesis Kit (Thermo Fischer Scientific, USA). Subsequently, the expression of all genes of interest was measured with Real-Time PCR (polymerase chain reaction). Ribosomal protein L13a (RPL13A) acted as the housekeeping gene. Same amount of cDNA was used for measuring both target and housekeeping genes. All measurements were performed in triplicate using the $\Delta\Delta C_T$ method and StepOnePlus™ Real-Time PCR System (Thermo Fischer Scientific).

Table 1 TaqMan assays used (Thermo Fischer Scientific, USA):

Gene	TaqMan assay	Study number
PRL	Hs00168730_m1	I, II, IV
IGFBP1	Hs00236877_m1	I, II, IV
INSR	Hs00961554_m1	I
PROK1	Hs00951617_m1	II
SLC2A1	Hs00892681_m1	III
RPL13A	Hs01926559_g1	I, II, III, IV

Table 2 Oligonucleotides used (Sigma-Aldrich, USA):

Gene	Sequence	Study number
CTGF	5` -GCA GGC TAG AGA AGC AGA GC- 3` 5` -TGG AGA TTT TGG GAG TAC GG- 3`	I
DCN	5` -TGG CAA CAA AAT CAG CAG AG- 3` 5` -GCC ATT GTC AAC AGC AGA GA- 3`	I
LEFTY2	5` -CCC TGG ACC TCA GGG ACT AT- 3` 5` -CAG TTC TTG GCC CCA CTT CAT- 3`	I
FOXO1	5` -AAG AGC GTG CCC TAC TTC AA - 3` 5` - TTC CTT CAT TCT GCA CAC GA- 3`	I
STAT3	5`-GAG CTG GCT GAC TGG AAG AG- 3 5`-TGT TGA CGG GTC TGA AGT TG- 3	I
RPL13A	5` -CAG GTC CTG GTG CTT GAT G - 3` 5` - GTT GAT GCC TTC ACA GCG TA- 3`	I, IV
TF	5` -CCA AAC CCG TCA ATC AAG TC- 3` 5` -CGT CTG CTT CAC ATC CTT CA- 3`	IV
TIMP3	5` -GGG GAA GAA GCT GGT AAA GG- 3` 5` -ATC TTG GTG AAG CCT CGG TA- 3`	IV

4.4 IMMUNOFLUORESCENCE (STUDY I)

Immunofluorescence staining and microscopy were used to study the effect of insulin on the subcellular location of FOXO1. This method is based on using antibodies labeled with fluorescent dyes enabling visualization of subcellular structures under a light microscope. The PI3K inhibitor wortmannin (Sigma-Aldrich, USA) was used to assess the involvement of the PI3K pathway. Briefly, the ESCs were pre-decidualized for 3 days, followed by 1-hour treatment with 500 nM wortmannin and then 3-hour treatment with 100 nM insulin. Next, the cells were fixed (3.7% paraformaldehyde in PBS), permeabilized (0.1% Triton X-100 in PBS), blocked (3% BSA and 0.1% Tween 20 in PBS) and incubated with anti-FOXO1 antibody (Cell Signaling Technology, USA; 1:200 dilution) and the appropriate secondary antibody (Alexa Fluor 488 anti-mouse). The slides were evaluated and photographed with a Zeiss AxioVert 40 CFL microscope (630x magnification) equipped with a Zeiss AxioCAM MRM digital camera and the AxioVision software.

4.5 ENZYME-LINKED IMMUNOSORBENT ASSAY (STUDY II)

Cells were *in vitro* decidualized in the presence and absence of different concentrations of insulin as described previously under "Culture conditions". Conditioned culture media was collected and secreted PROK1 was analyzed with enzyme-linked immunosorbent assay (ELISA) (Abcam, Cambridge, UK) in duplicates, according to instructions provided by the manufacturer. ELISA is a method that enables quantification of peptides and proteins by using target-specific antibodies linked to reporter enzymes, which produce a measurable signal when incubated with an appropriate substrate.

4.6 FLOWCYTOMETRY (STUDY IV)

The cells were harvested (AutoMACS Rinsing solution by Miltenyi Biotec, USA) and pelleted (400g for 7 minutes) before resuspension in Cytofix/Cytoperm (BD Biosciences, USA) and transfer to 96-well plate on ice for 20 minutes. This was followed by double centrifugation (450g for 6 min) with washing of the cells in between (Perm/Wash buffer by BD Biosciences, USA). To avoid non-specific antibody binding by Fc receptors, found on certain immune cells causing background fluorescence, the cells resuspended in the Perm/Wash buffer were treated with Fc block (BD Biosciences, USA). Incubation with APC-conjugated connexin 43 (CX43) or isotype control antibody (R&D Systems, USA) was then followed by washing and resuspension in Fixation buffer (Biolegend, USA). The fluorescent signal of the cells was finally measured with a NovoCyte flow cytometer (ACEA Biosciences, Inc., USA) and the data were analyzed with the FlowJo software (TreeStar, USA).

4.7 TRANSWELL MIGRATION ASSAY, WOUND HEALING ASSAY, SPHEROID INVASION ASSAY (STUDY II AND IV)

4.7.1 Wound healing assay (study II and IV)

In order to evaluate the effect of PROK1 on the proliferation and migration of undifferentiated ESCs, a wound-healing assay was used (Study II). The cells were seeded, cultured in DMEM/F12-Glutamax (Thermo Fischer Scientific) supplemented with 10% HI-FBS and 0.2% penicillin–streptomycin until confluency, scratched with a 200 µl pipette tip,

washed and cultured in DMEM/F12 media supplemented with 2% charcoal-stripped FBS and 0.2% penicillin–streptomycin with or without 50 ng/ml PROK1. The cells were then observed with time-lapse imaging using ZEN2 Software in a TIRF microscope incubation chamber, at constant 37°C and under 5% CO_2, for 24 hours with images recorded every 15 minutes. Photographs were analyzed using ImageJ software (NIH, Bethesda, Maryland, USA).

In study IV, the cells were decidualized in the presence or absence of 100 nM insulin, 1 μM DHT or their combination for 6 days prior to scratching with a 200 μl pipette tip, removing the media, washing and adding phenol red-free DMEM/F12 media supplemented with 2% charcoal-stripped FBS and 0.2% penicillin–streptomycin. The closure of the wound was then observed for 24 hours by IncuCyte S3 Live-Cell Analysis System (Sartorius, USA) with a 4x objective. We used ImageJ software to analyze the images.

4.7.2 Cell viability analysis (study II)

To ensure that the differences in cellular migration in the wound healing assay did not depend on changes in cell viability, we used the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay (Thermo Fisher Scientific, USA). This assay employs the ability of viable cells to transform yellow MTT reagent into purple formazan crystals. 10^4 ESCs were seeded in a 96-well plate in 200 μl media consisting of DMEM/F12-Glutamax, 10% HI-FBS and 0.2% penicillin-streptomycin. Twenty-four hours later, the cells were washed and 100 μl media with or without 50 ng/ml PROK1 (Sigma-Aldrich) was added. The media consisted of phenol red-free DMEM/F12, 2% charcoal-stripped FBS and 0.2% penicillin-streptomycin. After 24 hours, the cells were labeled with 10 μl MTT reagent followed by 100 μl 10% sodium dodecyl sulphate solution in tris-buffered saline (TBS), which solubilized the purple formazan crystals produced of MTT by viable cells. The viability was subsequently quantified colorimetrically by measuring absorbance in a plate-reader with a 595 nm filter.

4.7.3 Transwell migration assay (study II)

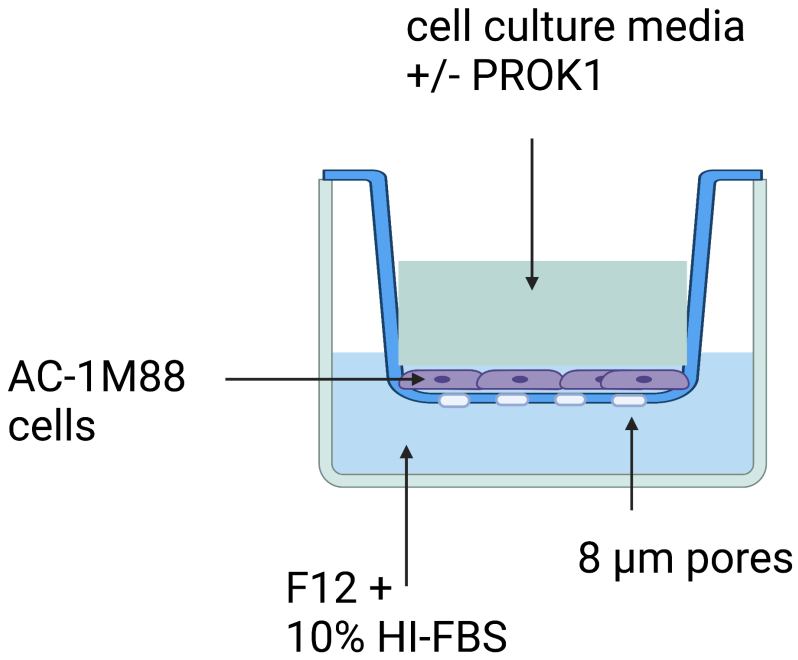

Figure 7 Transwell migration assay setting. © Ivika Jakson. Created with BioRender.com.

We used AC-1M88 choriocarcinoma–trophoblast hybrid cells (DSMZ, Braunschweig, Germany) that are produced by the fusion of choriocarcinoma cell line JEG-3 and term trophoblast cells. This cell line is suitable to study the adhesion and migration of cells (76). To study how PROK1 affects the migration of these cells, we plated cells to cell culture inserts with 8 µm pores (Merck Millipore) covered with cell culture media with or without PROK1 (Figure 7). The cell culture inserts were placed in 24-well plates with F12 media supplemented with 10% HI-FBS in each well for 48 hours. Staining with 0.5% crystal violet and removing the cells on the upper side of the membrane allowed us to visualize the cells that had migrated through the porous membrane. For analysis, the results were photographed with 40× magnification on a Nikon Eclipse TS 100 inverted microscope using a Leica DFC420 C digital camera, images converted to gray scale images with Adobe Photoshop and quantified using ImageJ software.

4.7.4 Spheroid invasion assay (study II and IV)

We used HTR-8/SVneo cell line (a gift of Mona Nystad at the University of Tromsø, Norway) consisting of immortalized first trimester villous explants suitable for studying

adhesion, migration, invasion and proliferation (94). Spheroids were formed by suspending $3*10^5$ cells in a mixture consisting of 9.11 ml RPMI 1640 medium and 890 µl 2.8% methylcellulose solution (R&D Systems, Minneapolis, Minnesota, USA). Spontaneous formation of spheroids followed within 24 hours. Spheroids are thought to better mimic *in vivo* conditions compared to single cells (51).

In study II, after 24 hours the spheroids were embedded in 100 µl of 6 mg/ml growth factor reduced Basement Membrane Extract (R&D Systems), diluted with serum free RPMI 1640 media in the presence or absence of 50 ng/ml PROK1 and centrifuged for 5 min at 300g at 4°C (Figure 8). Complete media was added on the top of the solidified gel and incubated for 5 days at 37°C in 5% CO_2 with daily microphotographs taken with 40× magnification with a Leica DFC420 C digital camera on a Nikon Eclipse TS 100 inverted microscope. The images were analyzed with ImageJ software.

In Study IV, the spheroid and stromal/decidual cell co-culture experiments were performed in 24-well Costar plates (Sigma-Aldrich, USA) (Figure 8). Primary endometrial stromal cells were seeded at a density of 10^5/well and cultured until 90% confluency. Cells were either decidualized in the presence or absence of 100 nM insulin, 1 µM DHT or their combination as described above for 6 days or left untreated. After 6 days, media was changed to 700 µl phenol-red free DMEM/F12 supplemented with 2% charcoal-stripped FBS and 0.2% penicillin-streptomycin. One HTR-8/SVneo spheroid was carefully transferred to each well onto the confluent stromal/decidual cells using 1 ml pipette tip previously cut with sterile blade in order to widen it. The invasion of HTR-8/SVneo spheroids was followed by an IncuCyte S3 Live-Cell Analysis System (Sartorius, USA) using a 4x objective (whole well, phase contrast imaging). The invasion areas of spheroids were measured using ImageJ.

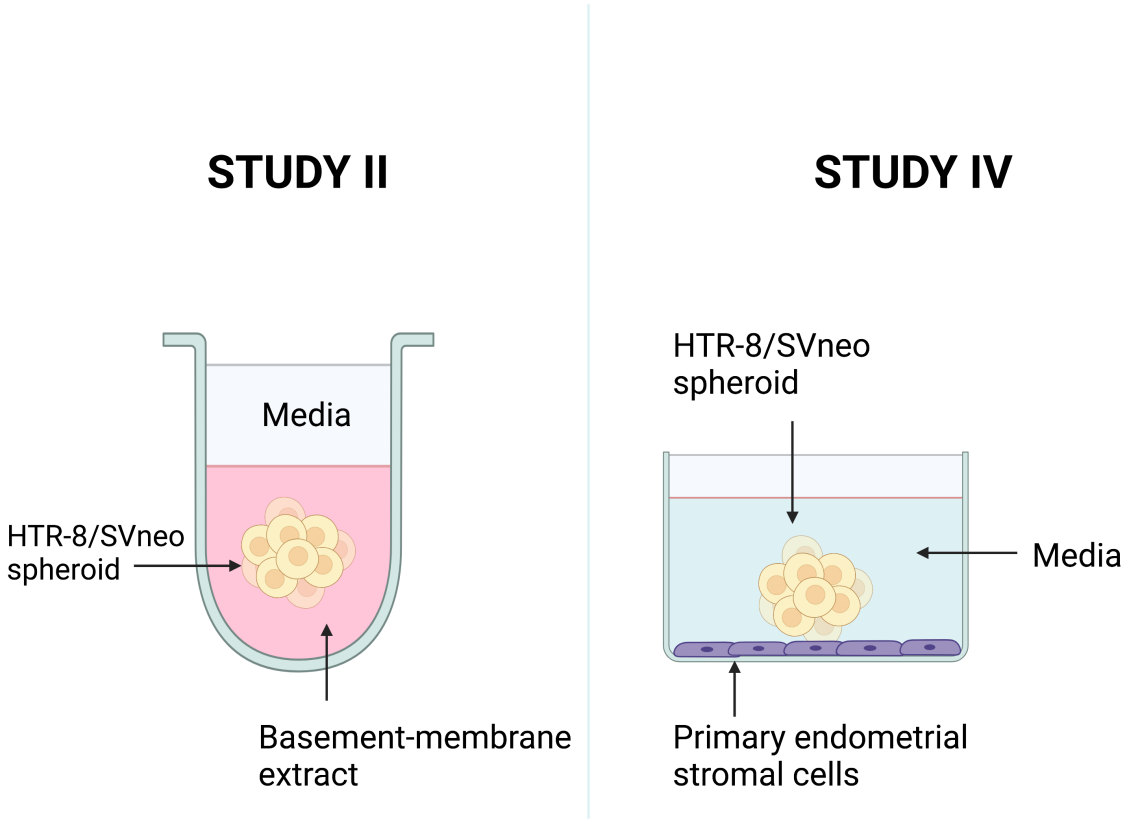

Figure 8 Spheroid invasion assay settings. © Ivika Jakson. Created with BioRender.com.

4.8 IMMUNOCYTOCHEMISTRY (STUDY III)

ESCs were decidualized with MPA and db-cAMP in the presence or absence of 500 nM insulin and fixed with 4% paraformaldehyde. Next, the cells were permeabilized (0.1% Triton-X for 15 minutes), washed with PBS and TBS, blocked with Background Sniper (Histolab Products AB, Sweden) for 10 minutes, washed and incubated with the diluted primary SLC2A1 antibody for 1 hour at room temperature. The following antibodies were used: ab32551 - rabbit polyclonal antibody to SLC2A1 (Abcam, Cambridge, UK), dilution 1:200, and LS-C87465 – mouse polyclonal antibody to SLC2A1 (LifeSpan BioSciences, Inc, USA.), dilution 1:400. Primary antibody specificity control (using two antibodies against same protein but different epitopes), secondary antibody control and labeling controls were included. The subsequent steps included incubation with MACH3 Mouse or Rabbit Probe (depending on the primary antibody, Histolab Products AB, Sweden), MACH3 Mouse or Rabbit M-Polymer HRP and Betazoid DAB Chromogen (Histolab Products AB, Sweden). The samples were washed with TBS between using different reagents. We finished off the staining process with counter staining with concentrated hematoxylin, dehydrating and mounting with Pertex.

Because of the innately irregular nature of the decidualization process, we chose the four most decidualized areas on each slide at a magnification of 100x and photographed them using image analysis system (Leica Imaging System Ltd., Cambridge, UK). Three independent evaluators (M.P., E.D.M., D.U.) completed a blinded evaluation of the staining intensity on a 5 point-scale: 0 (no staining), 1 (very weak staining), 2 (weak staining), 3 (moderate staining) and 4 (strong staining). They also assessed the percentage of cells with each intensity. Afterwards, a score ranging from 0 to 400 was calculated using the following equation: 4 x percentage of strongly staining cells + 3 x percentage of moderately staining cells + 2 x percentage of weakly staining cells + 1 x percentage of very weakly staining cells. The highest score for each condition was used in statistical evaluation.

4.9 GLUCOSE UPTAKE ASSAY (STUDY III)

ESCs were cultured and treated for 6 days using decidualizing agents in the presence and absence of different concentrations of insulin as described under Culture conditions. Prior to determining glucose uptake, cells were starved for four hours in an insulin- and serum-free media containing only decidualizing agents. Radioactive tritiated 2-deoxy-D-glucose ([3H]-2DG) was used to quantify cellular glucose uptake. Starving the cells from insulin and serum was followed by incubation with [3H]-2DG for 15 minutes, washing and solubilizing with sodium dodecyl sulfate (SDS). After overnight freezing, radioactivity of the samples was measured in a liquid scintillation counter. To adjust the glucose uptake to the amount of protein, we quantified the protein amount in each treatment well with the Micro BCA™ protein assay kit (Thermo Fisher Scientific, USA) and calculated the relative glucose uptake. All samples were run at least in duplicate.

4.10 STATISTICAL ANALYSIS

Data analysis was performed with GraphPad Prism 7.0 and 9.0. Normality of data was tested with either Shapiro-Wilk or Kolgomorov-Smirnov test. Data was log-transformed in case of skewness (Study IV). One-way ANOVA, Friedman test and Wilcoxon test were used to compare differences between treatment groups, depending on the normality of data, followed by multiple comparisons when appropriate. In case the numerical value of the control group was 1, as in study III, 95% confidence interval was calculated, and differences considered significant if the confidence interval did not include 1. When comparing two normally

distributed groups, paired and unpaired t-tests were used. In study IV, two-way ANOVA followed by simple main effects tests were used to evaluate interactions between androgens and insulin. A p-value of <0.05 was considered significant.

5 RESULTS

STUDY I

In study I, we showed that db-cAMP alone and in combination with MPA, but not MPA alone, induce decidualization *in vitro* after 6 days. Decidualization was confirmed by the increased gene expression of several decidualization markers. IGFBP1, connective tissue growth factor (CTGF), INSR, DCN and left-right determination factor 2 (LEFTY2) are all FOXO1 targets, as indicated by their transcriptional suppression after FOXO1 inhibition (Table 3). However, the decidualization marker PRL does not seem to be a FOXO1 target gene, as FOXO1 inhibition did not change PRL expression (fold change 1.1, not significant).

Table 3 Fold change in gene expression of decidualization markers in the presence of a FOXO1 inhibitor (AS1842856).

Gene	Fold change in gene expression in response to FOXO1 inhibition compared to decidualized cells (arbitrarily chosen 1)
IGFBP1	↓ 0.13 *
CTGF	↓↓ 0.2 **
INSR	↓↓↓ 0.2 ***
DCN	↓↓ 0.43 **
LEFTY2	↓↓↓↓ 0.005 ****

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. Significance levels represent the statistical significance between control and FOXO1 inhibitory treatments.

Furthermore, we found that increasing doses of insulin inhibit the expression of IGFBP1, CTGF, INSR and DCN (Figure 9), whereas insulin has no significant effect on LEFTY2 and in contrast upregulates the expression of PRL (data not shown).

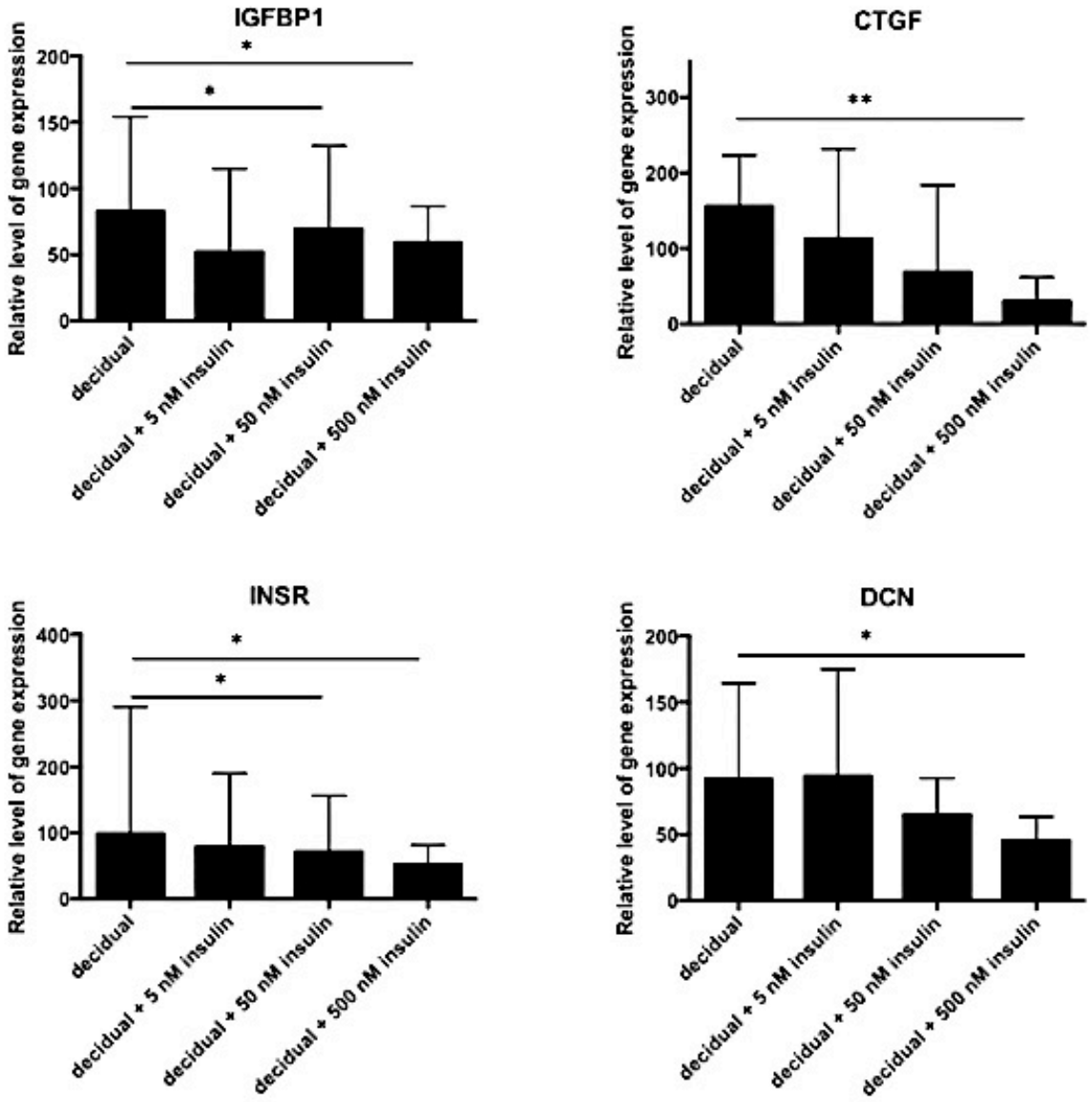

Figure 9 Relative gene expression levels of IGFBP1, CTGF, INSR and DCN in response to 5, 50 and 500nM insulin in *in vitro* decidualized ESCs.

- = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

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Insulin slightly inhibits the transcription of FOXO1 gene and induces nuclear export of FOXO1 protein. The regulation of FOXO1 by insulin is at least partly mediated by the PI3K pathway. However, treating decidualizing ESCs with insulin did not significantly affect their size suggesting that insulin does not inhibit the morphological transformation of ESC during decidualization although several markers were suppressed (Table 4).

Table 4 The effect of decidualization and insulin treatment on cell size of decidualizing ESCs

Groups compared	Direction of change in cell size and statistical significance of the difference
Stromal vs decidual	↑, $p < 0.05$
Stromal vs decidual + insulin	↑, $p < 0.05$
Decidual vs decidual + insulin	Not significant

STUDY II

In study II, we found that db-cAMP alone and in combination with MPA, but not MPA alone, induce upregulation of PROK1 during *in vitro* decidualization after 6 days. High concentrations of insulin drastically enhance the upregulation of PROK1 expression (Figure 10). Furthermore, we could demonstrate that the enhancement by insulin is mediated by HIF1α, and that the PI3K pathway is involved in the regulation of PROK1 by insulin.

PROK1 has no effect on ESC viability but inhibits ESC migration as indicated by reduced wound healing after 24 hours. PROK1 also inhibits the invasion of HTR-8/SVneo (trophoblast cell line) spheroids (Table 5).

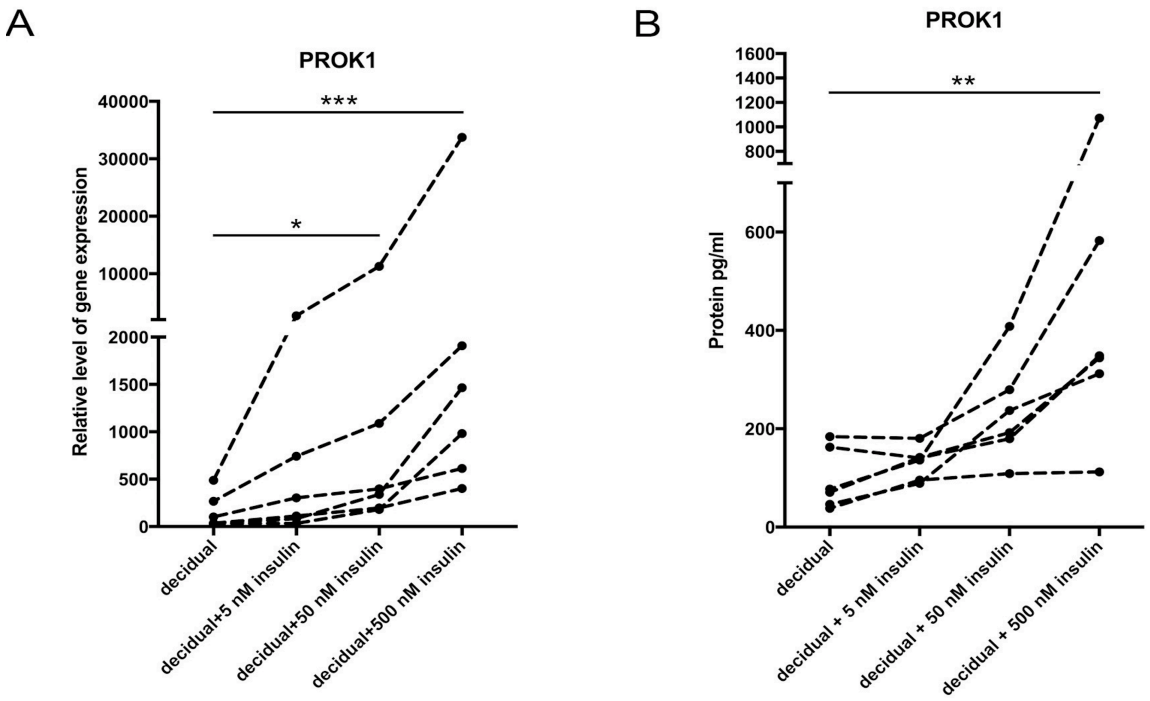

Figure 10 Relative gene expression (A) and protein (B) levels of PROK1 in response to 5, 50 and 500nM insulin in *in vitro* decidualized ESCs.

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$

Study II. Reprinted from Journal of Cellular Molecular Medicine; copyright 2018: Open access. CC-BY license.

Table 5 The effect of PROK1 treatment on ESC migration and trophoblast cell motility

Measure	Control (mean ± SD)	50 ng/ml PROK1 (mean ± SD)	Statistical significance between treatment groups
Closure of the wound in ESC wound healing assay after 1 day (%)	73 ± 15	68 ± 15	$p < 0.05$
Total area of migrated AC-1M88 cells after 1 day (pixel^2)	421 140 ± 38 991	255 889 ± 30 175	$p < 0.01$
Total area of invaded HTR-8/SVneo cells after 3 days (pixel^2)	766 875 ± 23 824	631 421 ± 96 732	$p < 0.01$

STUDY III

In study III, we demonstrated that high concentrations of insulin suppress the gene and protein expression of SLC2A1 (glucose transporter 1) in decidualizing ESCs (Figure 11 and 12). Furthermore, we could show that SLC2A1 is a FOXO1 target, as indicated by transcriptional suppression after FOXO1 inhibition. The suppression of SLC2A1 by insulin was followed by a slight decrease in glucose uptake of decidualizing ESC.

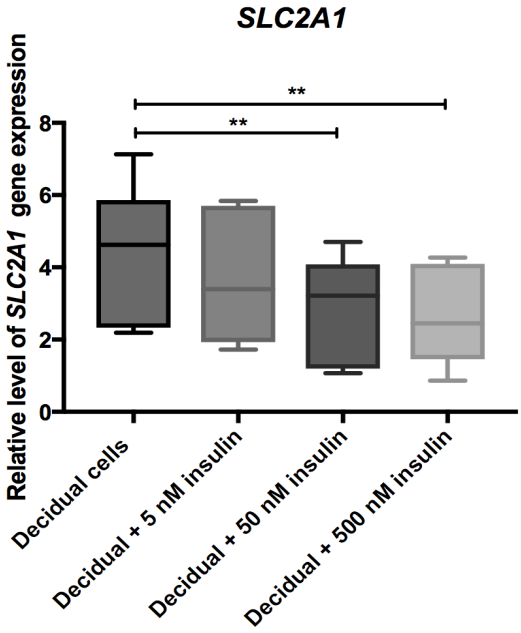

Figure 11 The relative expression of SLC2A1 mRNA in response to insulin treatment in decidualizing ESC. The box plots show minimum, first quartile, median, third quartile and maximum. ** = $p < 0.01$.
Study III. Reprinted from Reproductive Biology and Endocrinology; copyright 2020: Open access. CC-BY license.

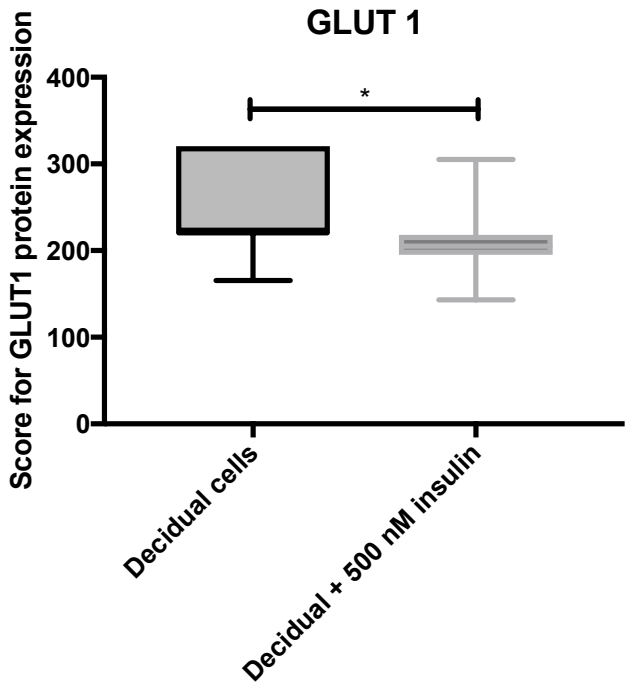

Figure 12 The relative expression of SLC2A1 protein in response to insulin treatment in decidualizing ESC. The box plots show minimum, first quartile, median, third quartile and maximum. * = $p < 0.05$.
Study III. Reprinted from Reproductive Biology and Endocrinology; copyright 2020: Open access. CC-BY license.

STUDY IV

In study IV, we showed that insulin interacts with DHT in enhancing the gene expression of PRL and TF messenger RNA (mRNA) and protein expression of CX-43. Both insulin and DHT alone enhance the expression of TIMP3 without significant interaction (Table 6). Furthermore, testosterone alone increases the production of PRL, IGFBP1, TF and TIMP3, however without interaction with insulin.

Insulin and DHT show an interaction effect resulting in enhanced decidual cell size (Table 6). Moreover, insulin and DHT in combination decrease the migration of decidualizing ESC and decrease the invasion of trophoblast cell line (HTR-8/SVneo) spheroids (Table 6).

Table 6. Various parameters in *in vitro* decidualized endometrial stromal cells in response to insulin, DHT or insulin+DHT

Measure	Decidual	Insulin	DHT	Insulin+DHT	Interaction and Main effect	Post-hoc effects
PRL	306 (39-3,386)	823 (56-1,986)	326 (44-2,917)	1,164 (71-2,228)	$1^{(*)}$	$a^{(*)}$, $b^{(**)}$, $d^{(**)}$
TF	2.22 (0.62-39.04)	5.82 (1.37-248.00)	3.01 (0.92-71.60)	4.60 (1.35-220.50)	$1^{(*)}$	$a^{(***)}$, $b^{(**)}$, $c^{(**)}$
TIMP3	2.41 (1.33-9.63)	16.74 (2.38-69.46)	4.08 (2.13-19.62)	36.47 (3.39-121.5)	$A^{(**)}$, $B^{(*)}$	
CX43	$1.8x10^5$ ($1.4x10^5$-$3.2x10^5$)	$2.5x10^5$ ($1.7x10^5$-$3.8x10^5$)	$1.7x10^5$ ($1.2x10^5$-$3.0x10^5$)	$2.5x10^5$ ($1.7x10^5$-$4.0x10^5$)	$1^{(*)}$	$a^{(**)}$, $b^{(**)}$
Pulse width	113.0 (89.0-149.0)	121.0 (101.0-144.0)	114.0 (87.0-149.0)	121.0 (107.0-152.0)	$1^{(p=0.058)}$	$b^{(**)}$, $d^{(**)}$
Cell size	2,468 (1,817-2,833)	3,184 (2,492-3,388)	2,257 (1,794-2,586)	3,456 (2,719-3,652)	$1^{(*)}$	$b^{(*)}$, $d^{(**)}$
Closure of the wound (%)	57.56 (45.12-74.96)	45.72 (42.63-63.35)	57.09 (52.96-70.21)	39.29 (27.17-41.92)	$1^{(p=0.102)}$	$b^{(**)}$, $d^{(*)}$
Spheroid invasion 12 h	3.29 (2.15-5.10)	2.68 (2.13-3.66)	3.14 (2.36-5.23)	1.87 (1.38-3.24)	$1^{(p=0.111)}$	$b^{(*)}$, $d^{(*)}$

Data are median and ranges (min-max). Values of PRL, TF and TIMP3 are relative gene expression levels compared with those in stromal cells. Values of CX43 protein levels and pulse width are AU (arbitrary unit) of mean fluorescence intensity. The units of cell size are pixel^2. Values of spheroid invasion are fold changes compared with the area of the spheroids at 0h in each condition. 1 = Interaction between insulin and DHT, A = Main effect of insulin, B = Main effect of DHT. Post-hoc test: a = insulin vs. decidual, b = insulin+DHT vs. DHT, c = DHT vs. decidual, d = insulin+DHT vs. insulin. *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$.

6 DISCUSSION

6.1 THE EFFECT OF INSULIN ON DECIDUALIZATION MARKERS AND FOXO1 TARGET GENES

One of the most often used methods to evaluate decidualization is to quantify changes in cellular mRNA or protein expression of decidual markers. Decidualizing ESCs express drastically different mRNA and protein expression patterns compared to non-decidualized ESCs (95). PRL and IGFBP1 are two of the most often used decidualization markers that are thought to positively correlate to the extent of decidualization. These two markers are, however, regulated in different directions by insulin, meaning that more information is needed for relevant interpretation. Thousands of genes are differentially expressed during decidualization and could potentially be used as decidualization markers (43). The best markers, however, show very low expression in non-decidualized ESCs and marked increase in decidual dells, such as PRL and IGFBP1.

6.1.1 PRL

PRL is a well-established decidualization marker used to assess the onset and extent of decidualization, either alone or in combination with other markers (96,97). The exact role of PRL produced by the decidua is unknown, but it has been proposed to act in an autocrine manner inhibiting the synthesis of other decidualization markers and regulating implantation, angiogenesis, trophoblast cell growth and local immune reactions (75,98). In accordance with earlier studies, we showed in studies I and IV that insulin enhances the production of PRL in decidualizing ESCs (Table 7) (78). However, it has been demonstrated that the knockdown of IRS2, insulin-like growth factor 1 receptor (IGF1R) and INSR does not significantly alter the expression of PRL, as opposed to several other decidualization markers (66). This might point towards insulin not having a direct effect on PRL synthesis.

Previous studies have shown both increased and decreased production of PRL upon FOXO1 silencing, whereas our results do not support a regulatory role of FOXO1 since there was no significant change in PRL levels by FOXO1 inhibition (Table 7) (36,99). The varying results are a good reflection on the complex nature of PRL regulation in the decidua involving co-operation of several transcription factors (97). The use of different decidualizing agents and methods for FOXO1 inhibition may also explain some of the discrepancies between studies.

6.1.2 IGFBP1

IGFBP1 is another well-established marker used to evaluate decidual transformation. It has been proposed to modulate feto-maternal interaction during pregnancy, and high levels might limit the invasion of cytotrophoblasts into the maternal decidua (100). It is known that insulin suppresses the expression of IGFBP1 in many tissues and that is true even for the endometrium, as shown in study I (Table 7) (79,80). We could also confirm IGFBP1 as a FOXO1 target (36). In study IV, the effect of insulin on IGFBP1 regulation was not completely consistent because in 2 of the 9 patient samples, there was instead an upregulation of IGFBP1 by insulin. The reason for that discrepancy could not be explained by patient characteristics.

Based on the literature and the studies presented in this thesis, the use of PRL and IGFBP1 only is unsuitable for evaluation of the effect of insulin on decidualization. Insulin is a known potent regulator of IGFBP1 production, and thereby might not be the best indicator of overall decidual progression. Additionally, IGFBP1 and PRL are regulated in opposite directions by insulin making it difficult to draw any conclusions on overall decidual transformation.

6.1.3 INSR, CTGF, DCN, LEFTY2

CTGF, INSR, DCN and LEFTY2 are all genes known to be upregulated during decidualization according to the literature (Table 7) (48,99,101–103). In study I, we established CTGF, INSR and DCN as FOXO1-targets and confirmed the FOXO1-regulation of LEFTY2. In our experiments, CTGF expression was not increased during decidualization. However, previous studies have also shown only a small increase in CTGF synthesis in the decidua compared to ESCs (101). We demonstrated in the first paper how insulin causes nuclear export of FOXO1 thereby decreasing the transcription of FOXO1-upregulated target genes resulting in the suppression of many decidualization markers by insulin (Figure 13). One might hypothesize about the possible function of the individual proteins and what effect the downregulation by insulin might have on aspects of decidualization, but drawing functional conclusions from studies determining mRNA expression is not correct. The exact function of most decidual proteins is unclear. In study I, the goal was to describe potential FOXO1-regulated genes, not genes with a specific putative function. The studied factors have been proposed to have a role in regulating events during implantation and placenta formation (CTGF and DCN) and energy homeostasis (SLCA1 and INSR) (48,77,101). LEFTY2 has been shown to inhibit decidualization in an autocrine manner, similarly to PRL (104).

However, the functional importance of the decrease in the studied FOXO1 target genes by insulin is not clear.

6.1.4 SLC2A1

SLC2A1 is not a classical marker of decidualization because despite the significant increase in mRNA expression during decidualization, it is highly expressed in both ESCs and decidual cells. Decidual transformation is characterized by an increase in energy uptake and storage. Increased energy demand is being met by a rise in glucose transporter levels, mainly SLC2A1, and increased glucose uptake (65). The expression of SLC2A1 seems to be crucial for decidualization because knockout studies have demonstrated extensive downregulation of decidualization markers upon loss of SLC2A1 (64). Insulin has been shown to regulate SLC2A1 expression in other tissues (68,69).

Previous studies by our group have investigated the effect of lifestyle intervention and weight reduction on the endometrium of women with PCOS (70). After the intervention, there was an increased endometrial expression of SLC2A1 together with decreased circulating insulin levels. We hypothesized that high levels of insulin would suppress the expression of SLC2A1 leading to decreased glucose uptake during decidualization. In support, we showed a decrease in SLC2A1 mRNA and protein expression upon insulin treatment (Table 7). Because insulin treatment does not lead to loss of SLC2A1, the changes in transcriptory profile of the decidualizing cells are not as pronounced as in knockout studies (64).

Our results are in line with Neff *et al* who described IRS2 as a central molecule conveying the effects of insulin on decidualization (66). They treated decidualizing ESCs with insulin resulting in decreased INSR expression. In experiments using small interfering RNA (siRNA) and knockout models they showed how knockdown of INSR results in decreased IRS2 expression and knockdown of IRS2 suppressed SLC2A1 expression via PI3K/AKT and MAPK pathways and decreased glucose uptake. Our study could show a direct link between increasing insulin levels and suppressed SLC2A1 expression. We suggest that FOXO1 mediates the effect of insulin on SLC2A1 levels together with other proteins, such as IRS2, since we established SLC2A1 as a FOXO1 target gene. We propose that a decline in SLC2A1 levels is a symptom of the dysregulating effect insulin has on decidualization, but not the cause of it.

Table 7 The effect of decidualization and insulin treatment on selected decidual markers and FOXO1 targets (FOXO1 targets verified in our studies marked with *)

Marker	Effect of decidualization in our experiments (in the literature)	Effect of insulin compared to decidualized cells in our experiments (in the literature)
PRL	↑(↑,(99)	↑(↑,(78)
IGFBP1 *	↑(↑,(99)	↓→(↓,(79,80)
INSR *	↑(↑,(103)	↓(↓(66)
CTGF *	→(↑,(101)	↓(no data)
DCN *	↑(↑,(48,99)	↓(no data)
LEFTY2	↑(↑,(102)	→(no data)
SLC2A1 *	↑(↑,(62)	↓(no data)
FOXO1	↑(↑(99)	↓(no data)
PROK1	↑(↑,(105)	↑(no data)
TF	↑(↑,50)	↑(no data)
TIMP3	↑(↑,108)	↑(no data)
CX-43	→(↑,109)	↑(no data)

↑ - increased gene expression, ↓ - decreased gene expression, → - no change in gene expression.

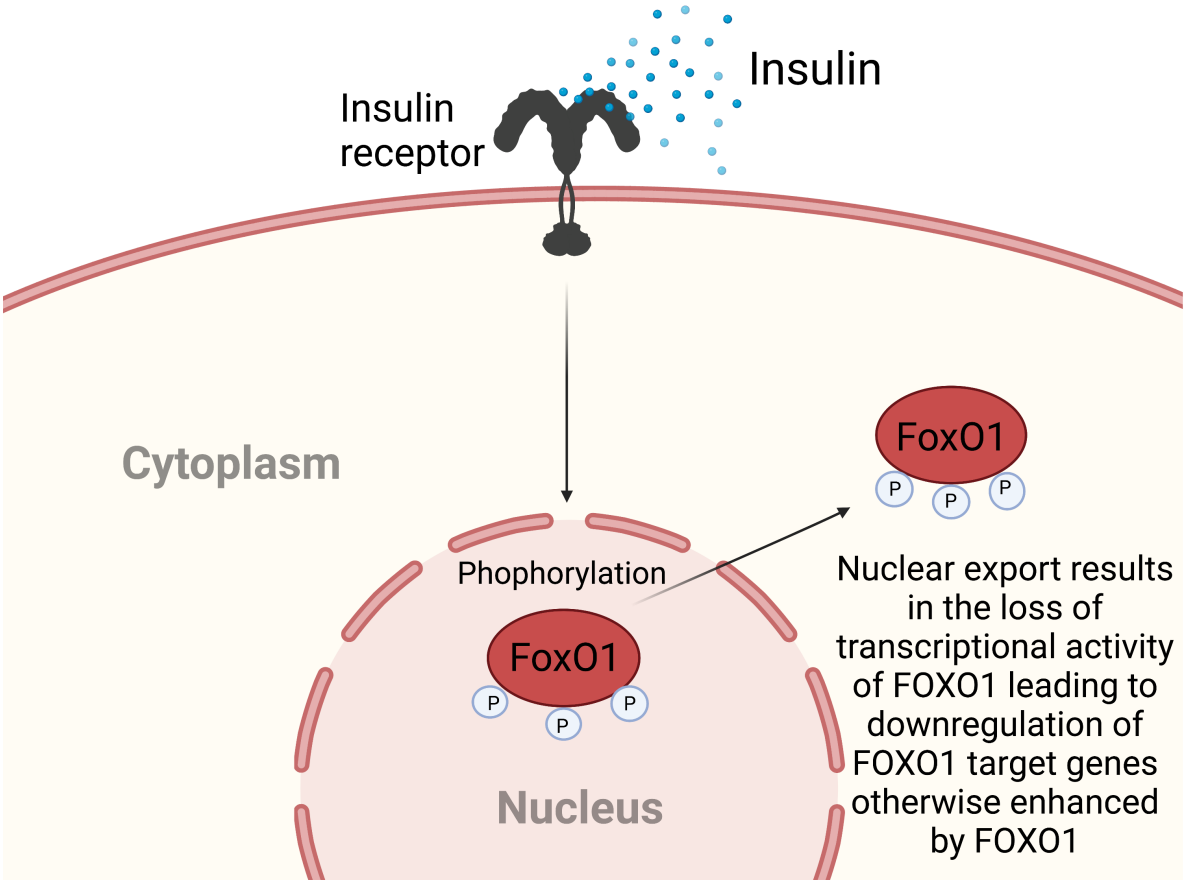

Figure 13 Insulin regulation of FOXO1. © Ivika Jakson. Created with BioRender.com.

6.1.5 PROK1

PROK1 is an angiogenic factor secreted by many tissues. It is expressed in the epithelial, stromal and endothelial cells in the endometrium and its synthesis is upregulated by decidualization in ESCs (106). It shows fluctuating expression levels during the menstrual cycle and pregnancy and is proposed to act as a regulator of endometrial receptivity (33). PROK1 is not a FOXO1 target and it is highly expressed in the first trimester in both placenta and maternal tissues (106–108). While insulin downregulates many FOXO1 target genes, PROK1 is upregulated by insulin, as shown in study II (Table 7). We demonstrated that the upregulation is mediated by the HIF1α via the PI3K pathway. Increased PROK1 levels have been noted in endometrial biopsies of patients suffering from recurrent pregnancy loss and other complications (33,109). The exact mechanisms that trigger the fluctuating levels at relevant time points during pregnancy remain to be elucidated, but insulin seems to have a strong upregulating effect on its expression.

6.1.6 TF, TIMP3, CX43

The expression of TF, TIMP3 and CX 43 is upregulated by decidualization and they are thought to regulate several decidual cell functions, including hemostasis and trophoblast invasion (47,49). CX43 is a gap junction protein proposed to coordinate the intercellular communication necessary for decidualization (110). In study IV, we evaluated the effect of high insulin levels on several decidualization markers, describing increased expression of TF and TIMP3 mRNA and CX43 protein (Table 7). We did not elucidate the potential mechanisms behind the upregulation by insulin, however previous studies have described an inhibiting effect of FOXO1 on TIMP3 synthesis (111). Because insulin decreases the effect of FOXO1 on transcription by inducing nuclear export, it is probable that the upregulation of TIMP3 by insulin is at least partly mediated by the loss of the inhibitory effect of FOXO1 on TIMP3 production.

6.1.7 Conclusion

In summary, insulin is a potent regulator of decidual mRNA and protein synthesis. By affecting the function of various transcription factors and other regulatory proteins, it exerts a unique effect on different targets. Both inhibition and enhancement of a certain factor are possible results of insulin regulation, depending on the regulatory proteins and pathways involved. We focused on FOXO1 regulation, describing the inhibition of many genes by loss of FOXO1 transcriptional activity. Nuclear (transcriptionally active) FOXO1 inhibits the expression of some genes (TIMP3), as shown by Buzzio *et al*, at the same time enhancing the expression of others (IGFBP1, INSR, DCN) (111). Therefore, loss of FOXO1 transcriptional activity by insulin may lead to opposing results, depending on the gene of interest. Furthermore, we described the upregulation of PROK1 mediated by transcription factor HIF1α, presenting another example of a regulatory mechanism of insulin during decidualization. Hence, the sum effect of insulin on the expression of decidual markers depends on regulatory proteins involved in their regulation. It is difficult to conclude what overall effect insulin has on decidualization based on changes in decidual gene expression patterns only.

6.2 THE EFFECT OF INSULIN ON THE FUNCTIONAL ASPECTS OF DECIDUALIZING ENDOMETRIAL STROMAL CELLS

6.2.1 Migration and invasion of ESCs and trophoblast cells

Motility of cells on both the maternal and embryonal side is essential for successful implantation, placenta formation and pregnancy. High insulin levels have been proposed to be one of the possible causes leading to impaired decidual trophoblast invasion in patients with PCOS (112). Therefore, studying cellular motility is of great importance to understand early pregnancy events. Implantation and decidualization cannot be studied in an *in vivo* environment for obvious ethical and technical reasons. Hence, *in vitro* model systems have been developed to try to mimic the *in vivo* environment and study the mechanisms involved.

In study II, we showed that insulin drastically upregulates the expression of PROK1 in decidualizing ESCs. In an attempt to study the functional role of this upregulation, we found that PROK1 induced a decrease in migration of both ESCs and trophoblast cells and decreased invasion of trophoblast spheroids (Figure 14). This can be taken as indirect evidence of the ability of insulin to inhibit both migration and invasion of the mentioned cells via upregulation of PROK1.

In study IV, we showed that decidualization did not seem to affect the migratory potential of ESCs or the invasive capacity of trophoblast spheroids. The absent effect of decidualization as such on migration is in agreement with results by Gellersen *et al*, whereas Godbole *et al* reported that decidualization promotes EVT invasion (46,52). This discrepancy can probably be explained by the use of different cell lines and invasion assays.

In the same study (IV), we investigated the effect of insulin and androgen treatment alone or in combination on the migration of decidualizing ESCs. Insulin alone did not significantly affect the migration of decidualizing ESCs (Figure 14). Thereby we can conclude that insulin-induced PROK1 can inhibit the migration of non-decidualized ESCs, as reported in study II, whereas insulin does not seem to affect the migration of decidualized cells. Insulin treatment did, however, inhibit the invasion of trophoblast spheroids into decidualized ESCs, which is in line with the results from study II.

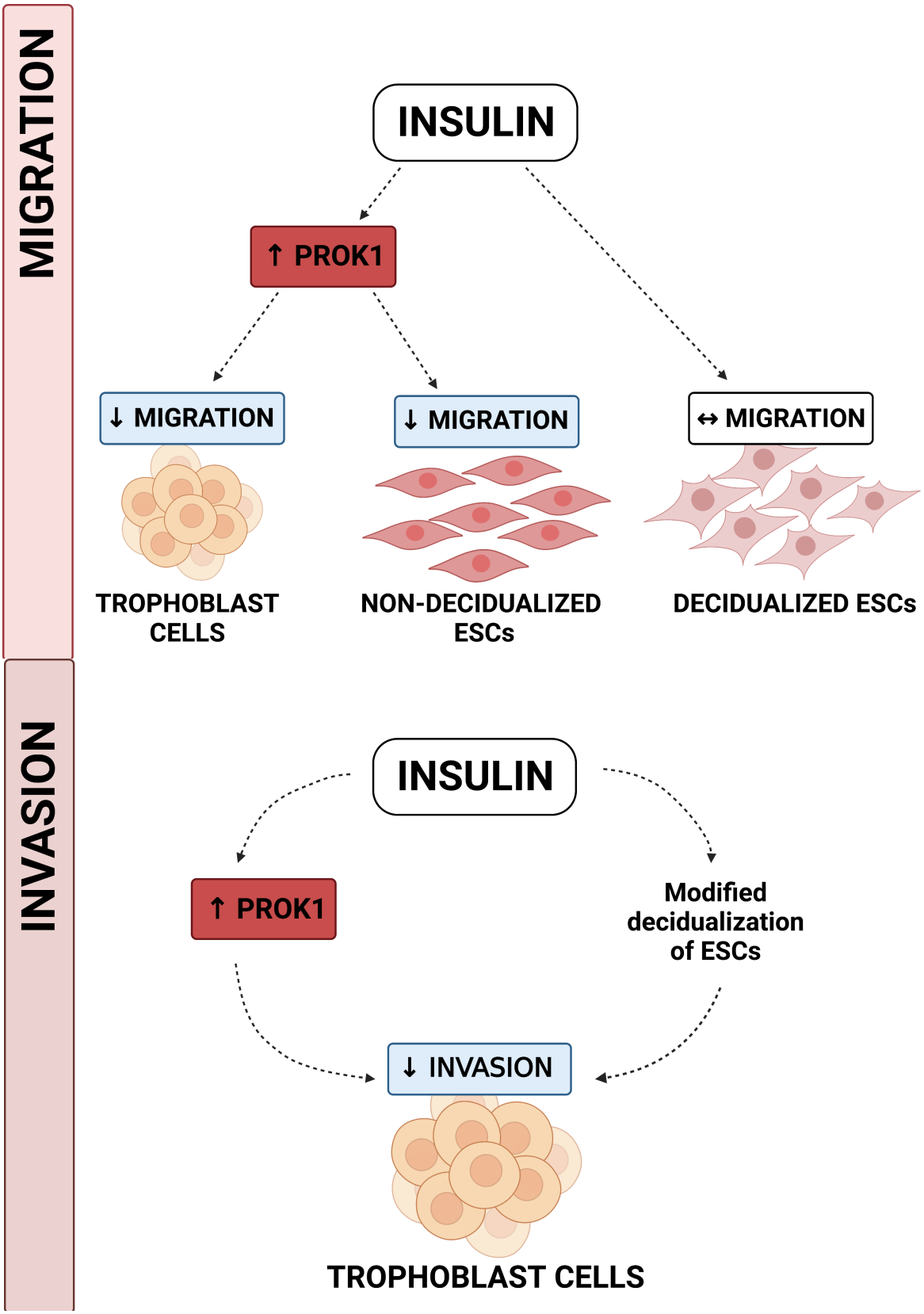

Figure 14 The effect of insulin on the migration and invasion of ESCs and trophoblast cells. © Ivika Jakson. Created with BioRender.com.

In summary, we could show that insulin has the ability to inhibit cellular motility of both non-decidualized ESCs and trophoblast cells. The effect on trophoblast invasion was mediated by modified decidualization of ESCs. To the best of our knowledge there is no previous data about the effect of insulin on the motility of these cell types. Earlier results regarding the effect of decidualization on ESC motility and EVT invasion have been contradictory, depending on the type of assay and cells used (cell lines, primary cells). Defective decidualization has become a topic of interest in recent years because of its possible role in the pathogenesis of PE. Decreased invasion of trophoblast cells into the spiral arteries and the subsequent insufficient vascular remodeling is proposed as an important aspect of the pathogenesis of PE, intrauterine growth-restriction and miscarriage (113). Thus, although data on the effect of decidualization on ESC and EVT motility *in vitro* is contradictory, inhibited invasion of EVT has been implicated in the pathogenesis of several obstetrical complications. It is clear that a coordinated motility of EVT and ESCs is necessary for a successful pregnancy. Hence, sustained hyperinsulinemia might disturb the balance required for implantation and placenta formation.

6.2.2 Morphological transformation

The decidual transformation consists of several changes in cellular properties. Morphologically fibroblast-like elongated ESCs remodel into large polygonal cells with a round nucleus, many nucleoli, glycogen and lipid depots and dilated rough endoplasmic reticulum and Golgi systems (Figure 3) (104). These structural differences make it possible to evaluate the progress of decidual transformation by measuring the size and internal complexity of the cells by flowcytometry or, alternatively, assess the changes using microscopy. The assumption is that the increase in cell size and granularity correlates to the extent of decidualization. A study by Wu *et al* from 1995 described a correlation between individual decidual cell size and prolactin production (29). However, not all data supports the correlation of PRL expression with morphological decidual transformation. Neff *et al* studied the effect of IRS2 knockdown on various decidual cell properties and described how IRS2 knockdown resulted in cells maintaining their fibroblastic shape characteristic to non-decidualized cells at the same time exhibiting PRL levels comparable to those of decidualized cells (66). This raises the possibility that morphological and secretory decidual transformation might not necessarily move in the same direction in all conditions.

We estimated the effect of insulin on cell size and granularity during decidualization in studies I and IV. In study I, although a significant increase in both cell size and granularity could be detected when comparing non-decidualized and decidualized cells, the addition of

insulin did not affect these parameters. In study IV, we were not able to show a change in cell size with flow cytometry or light microscopy by insulin, in agreement with results from study I. Decidualizing the cells in the presence of insulin resulted, however, in increased SSC-A describing the internal complexity of the cells. There is no previous data about the effect of insulin on the morphology of decidualizing endometrial cells. Because our initial hypothesis was that high levels of insulin inhibit decidual transformation, we expected insulin treatment to make the cells retain their fibroblast-like form and result in smaller cells. However, this was not the case. Our results indicate that morphological transformation characteristic of decidualization and development of a decidua-specific transcriptory and secretory profile are not necessarily interdependent.

6.2.3 Glucose uptake

An increase in glucose uptake seems to characterize decidualization, therefore quantifying and comparing glucose uptake might provide a further piece of evidence about the effect of insulin on decidualization.

SLC2A1 has been shown to be the most abundant glucose transporter isoform in the endometrium and therefore we expected changes in SLC2A1 expression to correspond to changes in glucose uptake. In study III, we demonstrated an increase in SLC2A1 mRNA levels and glucose uptake during *in vitro* decidualization. This has previously been shown in ESCs of a mouse model and recently even in human cells (65,66). Furthermore, we demonstrated a dose-dependent decline in SLC2A1 mRNA expression in response to decidualization in the presence of increasing insulin concentrations. SLC2A1 protein levels were also suppressed by insulin, although less than the mRNA levels. However, the resulting decline in glucose uptake was discrete and significant only for treatment with the lowest dose of insulin. This can partly be explained by the subtle changes in SLC2A1 levels. Furthermore, insulin treatment does not lead to a complete loss of SLC2A1, as in knockout studies (62). Another explanation could be that SLC2A1 is not the only glucose transporter in the endometrium. At least seven more are also present. Glucose uptake of decidualizing ESCs is therefore not a function of SLC2A1 exclusively, but a combined outcome of all glucose transporters in the endometrium (64,70). How insulin regulates the other seven glucose transporters was not the topic of study III, but the involvement of these could also be of importance for our findings.

We can also conclude that the slight changes in glucose uptake induced by insulin do not result in the inhibition of morphological transformation characteristic to decidualization, as

shown in studies I and IV, where insulin treatment did not result in changes in cell size. Adequate levels of glucose are certainly necessary for decidualization, but the optimal concentrations are not known. Since glucose has a crucial role in many metabolic processes, evolutionarily conserved defense mechanisms against fluctuating glucose levels are highly likely to be present making the process of decidualization possibly less susceptible to energy deficit.

6.3 THE COMBINED EFFECT OF ANDROGENS AND INSULIN ON DECIDUALIZATION

Clinical conditions of hyperinsulinemia in women are often associated with hyperandrogenism. This is true for PCOS, but also in obesity without PCOS, which most likely is due to dysregulation of the reproductive axis by obesity, as well as insulin-induced suppression of SHBG, which increases free androgen levels (114,115). Both hyperinsulinemia and hyperandrogenism are related to impaired fertility and pregnancy complications, and the combination of both could potentiate adverse effects (115,116). The association between these two conditions makes it relevant to also study the combined effect of high levels of both insulin and androgens on decidualization.

Both insulin and DHT have been shown to enhance the expression of PRL during decidualization, while DHT also enhances IGFBP1, in contrast to insulin (78,79,88,89). In study IV, we examined how the combination of insulin and androgens affects the expression of various decidual markers and cellular properties. We studied the effect of both DHT and testosterone on decidualization markers. The two androgens regulated the studied decidual markers in the same direction, but testosterone did not exhibit a clear interaction effect, therefore we only examined the effect of DHT on the function of the cells. It was previously reported that DHT alone enhances the expansion of trophoblast spheroids, modeling the later phase of implantation involving adhesion and invasion, whereas it has no effect on trophoblast cell invasion when measured in a co-culture assay consisting of trophoblast cells and endometrial stromal cell line (45,117).

We found that insulin and DHT interacted in the regulation of PRL, TF and CX43 (Table 8). Insulin enhanced the expression of these markers both in the presence and absence of DHT. In case of PRL, the combined treatment resulted in higher expression levels than for either

Table 8 The effect of insulin and DHT treatment alone and in combination on selected decidual cell parameters (interaction effect between insulin and DHT marked with *)

Parameter	**The effect of insulin alone**	**The effect of DHT alone**	**Combined effect** (compared to insulin alone /compared to DHT alone)
PRL gene expression (*)	↑	→	↑/↑
IGFBP1 gene expression	→	→	-
TF gene expression (*)	↑	↑	→/↑
TIMP3 gene expression	↑	↑	-
CX43 protein expression (*)	↑	→	→/↑
FSC-A (*)	→	→	↑/→
SSC-A (*)	↑	→	→/↑
Pulse width (*)	→	→	↑/↑
Cell size (*)	→	→	↑/↑
Closure of the wound (%)	→	→	↓/↓
Spheroid invasion 12h	→	→	↓/↓
Spheroid invasion 16h	↓	→	-

↑ increased gene expression; ↓ decreased gene expression; → no change in gene expression; - not calculated

treatment alone. No interaction was found in the regulation of IGFBP1 and TIMP3, although both insulin and DHT separately enhanced the expression of TIMP3. Insulin did not significantly downregulate the expression of IGFBP1 because in two of the nine samples insulin did not suppress IGFBP1 production (Table 8). No interaction effect between insulin and testosterone could be observed for the studied decidualization markers, although testosterone and insulin individually affected the expression of PRL, TF and TIMP3 in the same direction as DHT.

When it comes to cell morphology, insulin and DHT showed an interaction in the regulation of all parameters studied. Cell size and pulse width (reflecting cell diameter) were both increased by combined treatment with insulin and DHT more than by treatment with any of the treatments alone. Insulin increased SSC-A (reflecting granularity of the cells) both in the presence and absence of DHT, whereas the effect of DHT on FSC-A (reflecting cell volume) was dependent on the presence of insulin. Thus, insulin and DHT interacted in the regulation of all studied cell morphology parameters and the combined treatment with insulin and DHT resulted in larger cells indicating enhanced morphological transformation characteristic to decidualization.

Regarding the effect on cellular motility, insulin and DHT showed a possible interaction in the regulation of decidualizing ESC migration and invasion of trophoblast spheroids. The combined treatment with DHT and insulin suppressed the migration of decidualizing ESCs and invasion of trophoblast spheroids more than insulin or DHT alone. Previous studies have reported DHT not having a significant effect on trophoblast cell invasion, which is in line with our results showing a significant effect of DHT only in the presence of insulin (45).

Our experiments unveiled an interaction effect of insulin and DHT on most parameters studied. The combined treatment with DHT and insulin resulted in the largest increase in PRL expression and cell size, and strongest inhibition of decidual cell migration and trophoblast spheroid invasion. Furthermore, our group has previously published how DHT potentiates the upregulating effect of insulin on PROK1 production (118). This suggests that whatever effect insulin alone has on decidualization, adding DHT might significantly modify these effects. The decidualization markers studied in paper IV often showed enhanced upregulation upon concomitant treatment with insulin and DHT whereas cellular motility was suppressed. Therefore, the presence of hyperandrogenemia and hyperinsulinemia simultaneously *in vivo* might modulate the dysregulating effect that high insulin levels have on decidualization.

6.4 THE OVERALL EFFECT OF INSULIN ON DECIDUALIZATION

Insulin is a peptide hormone with a wide range of cellular functions, including regulation of glucose, lipid and protein metabolism (1). Energy balance and reproduction are two well-integrated systems making it an essential study topic (119). Insulin seems to be an important regulator of reproduction. The exact molecular mechanisms, however, are often difficult to study in humans, because hyperinsulinemia and insulin resistance usually co-exist with other metabolic abnormalities (8).

Because of the many functions of decidual cells, it is challenging to clearly define inhibited and enhanced decidualization. Decidual transformation consists of changes in cellular morphology, secretome, trophoblast invasion, responses to oxidative stress and immunotolerance (120). Studying all known aspects of decidualization is problematic. Insulin signaling is necessary for decidualization, as determined by knockout studies, however, determining the effect of high insulin levels is complicated because decidual markers are regulated in different directions. Some are upregulated (PRL, PROK1, TF, CX-43, TIMP3), whereas others are downregulated (IGFBP1, CTGF, INSR, DCN, SLC2A1). Because the exact function of most decidual proteins is unknown, it is not possible to explain the effect of insulin-induced changes based on that. The most correct term for describing the effect of insulin on decidualization would be ‘imbalance’ or ‘dysregulation’, not ‘inhibition’ or ‘enhancement’.

Morphological changes accompany the development of a decidua-specific secretome. Therefore, we measured the effect of insulin on cellular morphology. The assumption was that the bigger and more complex the cell, the more decidualized. We showed that despite the significant effect on many decidual markers, the morphological transformation was not affected by insulin alone. The effects on cellular morphology and secretory properties are accompanied by inhibition of the migratory potential of both ESCs and trophoblast cells and the invasion of trophoblast cells. Inhibition of EVT invasion has been shown to be important in the development of PE and other pregnancy complications, which would imply a negative effect of high insulin levels on the function of decidual cells (113). In addition to the effects insulin alone exerts on decidualization, it also seems to interact with androgens potentially exaggerating the effects of hyperandrogenemia.

The results of this thesis present a complex effect of high insulin levels on decidualization. They draw attention to the importance of not relying on one characteristic or test, such as expression of PRL and IGFBP1, when evaluating an intricate biological process. A recent

study on the effect of prednisolone on decidualization supports this notion with prednisolone having no effect on the classical decidualization markers PRL and IGFBP1 while suppressing the expression of decidual cytokines and modulating decidual proteins and interactions between the trophoblasts and decidua (121). Different aspects of the decidual transformation can be regulated independently, which is also supported by data showing that the knockdown of IRS2, an important mediator of the decidual transformation, prominently inhibits the morphological transformation of ESCs into decidual cells without affecting PRL production. This data altogether describes a multilayered regulation of decidualization by insulin.

To conclude, the data included in this thesis propose that high insulin levels have a dysregulating effect on decidualization with different aspects affected in contrasting directions. Such complex regulation seems appropriate for a hormone with extensive effect on cellular metabolism. This dysregulation might play a role in the pathogenesis of reproductive disorders associated with hyperinsulinemia.

6.5 STRENGTHS AND LIMITATIONS

The studies included in the thesis fill a significant gap of knowledge about the effect of insulin on the endometrium and decidualization. When the work for this thesis began in 2013, information about insulin and endometrial function was very scarce. We had the possibility to obtain fresh endometrial biopsies and thus work with primary ESCs instead of a cell line. Although cell lines try to mimic the cellular properties of primary cells, they can never completely replace them.

There are, however, some limitations that need to be discussed. The insulin concentrations we used in our experiments were higher than physiologic plasma concentrations. *In vitro* model systems try to mimic *in vivo* conditions, however the tissue levels of insulin are unknown in the endometrium. It has been reported that tissue concentrations of insulin can be up to 25-100 times higher than plasma concentrations (122,123). Culture conditions used in our experiments are based on available literature (124–126). We have used several concentrations of insulin whenever possible to see whether there is an apparent dose-dependent effect on the parameter studied. Besides high insulin levels, our model system also included glucose levels of 17.5 mmol/l in the cell culture media, which is significantly higher than blood glucose levels. However, even in this case, levels of exact tissue concentrations *in vivo* are unknown.

In Study IV, we examined the possible interaction of androgens and insulin. Whereas in gene expression experiments we included treatments with both testosterone and DHT, in all other experiments we only studied the effect of DHT. Because testosterone and DHT did not show marked differences in the gene regulation patterns, we chose DHT for following experiments. Firstly, because of its higher affinity to AR (127). Secondly, because the effect of testosterone is also mediated by its conversion to both estrogen and DHT (88).

Another aspect that needs to be addressed is the aspect of temporal and spatial regulation of migration and invasion during implantation and placentation *in vivo*, which cannot be recaptured in an *in vitro* model. This calls for caution in the interpretation of *in vitro* model results. Because the effect of high insulin levels on decidualization seems to affect the motility of cells on both maternal and fetal side of the maternofetal interface, it seems probable that consistently high insulin levels during implantation and placentation might disturb coordinated events during early pregnancy. However, to further elucidate on the exact implications of this dysregulation, more knowledge is needed about implantation and placentation under normal conditions.

While we used primary ESCs in all studies, in experiments including trophoblast cells we chose appropriate cell lines instead. Many aspects make the use of cell lines a more practical alternative to primary cells. Lower price, unlimited supply of cells with consistent properties and less ethical concerns are just some positive traits. At the same time, cell lines can never completely mimic all aspects of primary cells and have often shown to be contaminated with other cell types and microorganisms (51). Illustrating possible downsides of cell lines are studies comparing them to primary cells. For example, primary EVT cells secrete progesterone, whereas HTR-8/SVneo cells do not (128). Furthermore, conditioned media from primary EVT cells enhances PRL production in non-decidualized ESCs and enhances the migration, adhesion and proliferation of decidualized ESCs compared to conditioned media from HTR-8/SVneo cells (54). However, we chose cell lines that were most appropriate for our experiments and therefore believe that the data we acquired is highly relevant.

Studies I-III included samples from six different women and Study IV from nine women. Whereas the limited number of subjects might be seen as a potential limitation if the results were non-significant, we report significant differences induced by our *in vitro* treatments. Furthermore, experiments assessing basic molecular mechanisms are often done using commercial cell lines where the material comes from only one patient. Therefore, we deem our patient number to be suitable for this type of study.

In conclusion, despite certain methodological limitations, we believe the results presented in this thesis present valid novel information to the field of reproductive biology.

7 CONCLUSIONS

Study I

High insulin levels suppress the expression of FOXO1 target genes IGFBP1, CTGF, INSR, and DCN by inducing nuclear export of FOXO1 via PI3K pathway in decidualizing ESCs. Nuclear export of FOXO1 does not inhibit the morphological transformation of the cells.

Study II

High insulin levels upregulate the mRNA and protein expression of PROK1 via HIF1α and PI3K pathway. The increased PROK1 levels inhibit the migration of ESCs and invasion and migration of trophoblast cell lines.

Study III

The downregulation of SLC2A1 mRNA and protein expression by high insulin levels is accompanied by slight inhibition of glucose uptake in decidualizing ESCs. The downregulation of SLC2A1 by insulin is partly mediated by FOXO1.

Study IV

The combination of insulin and androgens enhanced the synthesis of decidual PRL, TF, CX-43 and TIMP3, lead to increased size and suppressed migration of decidualizing ESCs, and decreased invasion of trophoblast spheroids. Furthermore, androgens and insulin interacted in the regulation of PRL, TF, CX-43 and decidual cell size.

8 POINTS OF PERSPECTIVE

While *in vitro* models studying the biology of a single cell type are important for obtaining information about basic molecular mechanisms, it is crucial to strive for multicellular models and thereafter *in vivo* validation of results. In the endometrium, the two most abundant cell types are ESCs and endometrial epithelial cells. It has been shown that endometrial stromal and epithelial cells significantly affect each other's function meaning that it would be valuable to study the effect of insulin on both cell types during decidualization (91,129,130). Several new 3D models have been proposed, including those where ESCs interact with organoids consisting of endometrial epithelial cells embedded in various matrices (131). Others have proposed an approach incorporating endometrium-on-a-chip microfluidics in order to achieve a multicellular environment similar to the endometrium (132). Incorporating multicellular models in some form is a clear future direction that should be undertaken.

While multicellular endometrial models would provide a more layered picture of the effect of insulin on the endometrium, *in vivo* studies are also required. Studying decidualization by analyzing biopsies from the secretory phase of non-pregnant women is not optimal because the decidual transformation is still at its early stages at that point. However, it might be relevant if the biopsies obtained from specific patient groups, such as obese women or women with PCOS who also exhibit hyperinsulinemia, were further cultured *in vitro* in the hope of detecting molecular patterns suspected from *in vitro* studies and saved in the cellular "memory" as in studies on recurrent pregnancy loss, endometriosis and PE, where cells retained specific pathological molecular characteristics even *ex vivo* (33,44,131,133).

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