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Metabolomics of uterine decidualization in mammals

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Sophie Pohlert BSc M.Sc.

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Abstract

Decidualization is a vital process for successful eutherian mammal embryo implantation, as it is a hormonally initiated transformation of endometrial stromal cells into specialized secretory cells. Impaired decidualization is associated with infertility and pregnancy loss in multiple species. However, the mechanisms by which decidualization enables pregnancy remain insufficiently understood. Even though previous studies have characterized gene expression changes during decidualization, changes within the metabolome remain largely unexplored.

This master's thesis aims to investigate the metabolic changes occurring during decidualization in a conventional laboratory inbred mouse strain (C57BL/6) as well as a transgenic mouse line carrying a human allele associated with pregnancy-related changes linked to decidual disorders. Using a standardized in- vitro model, decidualization is induced in primary cell culture using 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP) and progesterone. Through GC-MS (Gas chromatography - Mass spectrometry) a total of 33 metabolites were detected, out of which 7 showed statistical significance, suggesting significant changes during decidualization in central metabolic pathways.

Increased fructose-1,6-bisphosphate and pyruvic acid levels in WT cells suggest enhanced glycolysis and a Warburg-like effect, with pyruvic acid accumulation and partial decoupling from the TCA cycle. Decreasing glutamic acid levels indicate its potential use to compensate for a potential downregulation of the TCA cycle activity. Downregulated levels of the polyamine putrescine might indicate efficient turnover for downstream polyamine synthesis as they are considered crucial for cell proliferation and differentiation. In the transgenic line, no increase in fructose-1,6-bisphosphate was detected, possibly affecting the fructose-1,6-bisphosphate-IL-27 axis. Parallel decreasing glutamic acid as well as 2-oxoglutaric acid levels suggest insufficient compensation via glutaminolysis and a potential decrease in TCA cycle activity. In general, our findings support the hypothesis of metabolic reprogramming during decidualization, particularly involving glycolysis and the TCA cycle.

Keywords: Decidualization, Mouse endometrial stroma fibroblasts, Metabolites, GC-MS,

Zusammenfassung

Die Dezidualisierung, als eine hormonell initiierte Umwandlung von endometrialen Stromazellen in spezialisierte sekretorische Zellen, ist ein entscheidender Prozess für die erfolgreiche Einnistung von Embryonen bei eutherischen Säugetieren Eine beeinträchtigte Dezidualisierung ist bei mehreren Arten mit Unfruchtbarkeit und Schwangerschaftsverlust verbunden. Mechanismen, durch die die Dezidualisierung eine Schwangerschaft ermöglicht, sind noch nicht ausreichend verstanden. Obwohl frühere Studien Veränderungen der Genexpression während der Dezidualisierung charakterisierten, sind Veränderungen innerhalb des Metaboloms weitgehend unerforscht. Das Ziel dieser Masterarbeit ist es, die metabolischen Veränderungen, die während der Dezidualisierung in einer konventionellen Labor-Inzuchtmauslinie (C57BL/6) sowie in einer transgenen Mauslinie auftreten, zu untersuchen. Die transgene Mauslinie trägt dabei ein menschliches Allel, welches mit schwangerschaftsbedingten Veränderungen im Zusammenhang mit Dezidualisierungsstörungen verbunden ist. Unter Verwendung eines standardisierten In-vitro-Modells wird die Dezidualisierung in primären Zellkulturen induziert. Durch GC-MS (Gaschromatographie – Massenspektrometrie) wurden insgesamt 33 Metaboliten gemessen, von denen bei 7 statistisch signifikante Veränderungen während der Dezidualisierung in zentralen Stoffwechselwegen zeigen. Erhöhte Fructose-1,6-Bisphosphat- und Pyruvatspiegel in WT-Zellen deuten auf eine verstärkte Glykolyse und einen Warburg-ähnlichen Effekt mit Pyruvatakkumulation und teilweiser Entkopplung vom TCA-Zyklus hin. Sinkende Glutaminsäurespiegel deuten darauf hin, dass Glutaminsäure möglicherweise zum Ausgleich einer möglichen Senkung der TCA-Zyklusaktivität eingesetzt werden kann. Herunterregulierte Mengen des Polyamins Putrescin könnten auf einen effizienten Umsatz für die nachgeschaltete Polyaminsynthese hinweisen, da sie als entscheidend für die Zellproliferation und -differenzierung gelten. In der transgenen Linie wurde kein Anstieg von Fructose-1,6-Bisphosphat festgestellt, was möglicherweise Auswirkungen auf die Fructose-1,6-bisphosphat-IL-27-Achse hat. Gleichzeitig sinkende Glutaminsäure- und 2-Oxoglutarsäure-Spiegel deuten auf eine unzureichende Kompensation durch Glutaminolyse und einen möglichen Rückgang der TCA-Zyklusaktivität hin. Die Ergebnisse stützen die Hypothese einer metabolischen Umprogrammierung während der Dezidualisierung, insbesondere durch die Beteiligung der Glykolyse und des TCA-Zyklus.

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1 Introduction

Decidualization is a hormonally regulated transformation of endometrial stromal cells into specialized secretory cells that is crucial for successful eutherian mammal embryo implantation as well as for pregnancy support. Impaired decidualization is associated with infertility and pregnancy loss in multiple species (Gellersen & Brosens, 2014; Zhou et al., 2022). Yet the mechanisms by which decidualization enables pregnancy remain insufficiently understood. While gene expression changes during this transformation have been well characterized at least in humans and mice, recent models suggest that the role that single cell types play in the tissue may be better captured by the metabolome.

Therefore, this master's thesis aims to investigate the metabolic changes occurring during decidualization in the house mouse (*Mus musculus*). In addition to the conventional laboratory inbred mouse strain C57BL/6 (henceforth "wild-type"), we also make use of a transgenic mouse line. The transgenic line carries a human allele linked to pregnancy changes associated with decidual disorder. The line was generated on the background of C57BL/6, and thus the two lines differ only in a single nucleotide (with the exception of possible random mutations). Using a standardized in vitro model, decidualization is induced in primary cell culture using cyclic AMP and progesterone. The metabolic profiles acquired by GC-MS (Gas chromatography - Mass spectrometry) will help to identify key pathways involved in decidualization, such as glycolysis and Tricarboxylic Acid (TCA) cycle, as well as their change in the deviating line. The overall goal is to better understand the metabolic changes of mouse endometrial stromal cells during decidualization. Before explaining the experimental work, I will in the following provide some biological context of the study.

1.1 Uterine biology and the menstrual/estrus cycle in mammals

The uterus, as well as the oviducts, cervix, and vagina, are the essential organs that are entailed under the term *female reproductive tract* (FRT) in mammals (Spencer et al., 2005). The uterus is a muscular, hollow organ with the primary functions to house and nourish the foeti during pregnancy (Lüllmann-Rauch & Asan, 2019).

The mammalian uterine wall consists of three layers: the perimetrium, the myometrium consisting of smooth muscle, and the outermost, lumen-facing lining of the corpus called the endometrium, which is of interest here (Lüllmann-Rauch & Asan, 2019). Under the hormonal control of the ovarian cycle, the endometrium changes to prepare for potential pregnancies. This nonpregnant cycle is variably long in different eutherian species (Basanta & Pavličev, 2025). In all, the uterine phases include the estrogen-dominated proliferative phase before ovulation, and the secretory phase that follows ovulation and is the phase in which implantation could occur. In particular, the secretory phase may involve various degrees of endometrial tissue changes depending on the species. In humans and many primates, for example, it involves spontaneous decidualization (i.e., regardless of the presence of an embryo), and it presumably for that reason ends with the shedding of the tissue, the menstruation (Finn, 1987). Spontaneous decidualization is rare across eutherians (Emera et al., 2012) and is absent in mice, which decidualize only when pregnant. It has been shown that menstruation can be artificially induced in mice by

triggering decidualization through scratching the surface of the endometrium. Following the withdrawal of progesterone, such decidualized tissue is shed also in mouse (Xu et al., 2007). Although triggers for normal decidualization are different in mice and humans, they both show striking similarities in hormonal needs as well as in cellular response (Wang & Dey, 2006). Causal for the phases of the endometrium are the phases of the ovarian cycle, with their dynamics of hormone release. Both in mice and in humans, successful implantation is dependent on a preovulatory increase in estrogen secretion to stimulate epithelial cell proliferation and postovulatory progesterone production by the corpus luteum, which induces the differentiation process, decidualization (Finn & Martin, 1974; Gellersen & Brosens, 2014).

1.2 Decidualization: mechanisms and function

In most eutherian species, as in stem Eutherian, decidualization is closely associated with embryo implantation and occurs only in pregnancy (Wagner et al. 2014). The onset of pregnancy is initiated by attachment and implantation of the embryo in the endometrium (Teklenburg et al., 2010). This phase is characterized by a complex, bidirectional communication between the maternal endometrium and the trophectoderm of the embryo (Teklenburg et al., 2010).

Implantation success relies on the competence of the blastocyst as well as a receptive uterine endometrium. Decidualization, i.e., the stromal remodelling focused on in the present study, is a key maternal factor (Gellersen & Brosens, 2014; Muter et al., 2023). In this process, the endometrial stroma transforms into a differentiated tissue called decidua (Lüllmann-Rauch & Asan, 2019). This occurs under the influence of progesterone and cAMP (De Clercq et al., 2017; Lüllmann-Rauch & Asan, 2019; Stadtmauer & Wagner, 2022)

Steroid hormones estrogen and progesterone hold key roles in regulating early uterine events crucial for successful pregnancy. They induce a series of structural and functional changes in the endometrial tissue, ensuring its readiness for implantation. Estrogen is produced by ovarian granulosa cells and signals via estrogen receptor alpha (ESR1) to trigger the endometrial proliferation (Carp, 2021). Progesterone, produced by the corpus luteum, the tissue of the ovulated follicle, drives the decidualization of endometrial stromal cells (De Clercq et al., 2017; Lüllmann-Rauch & Asan, 2019).

Another driver for decidualization is the activation of the cyclic AMP (cAMP)/protein kinase A (PKA) pathway stimulated through prostaglandin E2 (PGE2) (Stadtmauer & Wagner, 2022).

The most apparent sign of decidual transformation is that the endometrial stromal fibroblasts change into the larger and round decidual stromal cells. At the same time, the fibroblasts undergo a substantial transcriptional and secretory change, providing growth factors and cytokines which promote the development of the embryo (Brosens et al., 2002).

Upon the activation of signalling pathways, endometrial stromal cells undergo drastic transcriptional reprogramming, such as the activation of pathways like WNT4 signalling,

which is considered to drive the differentiation process (Franco et al., 2011). In contrast, pathways associated with stress, such as Jun N-terminal kinase (JNK) and p38, are being inhibited (Gellersen & Brosens, 2014).

The decidua, as a tissue in which endometrial stromal cells are the main component, also bears many important differences from nonpregnant endometrium. Enabled through the key transcription factor FOXO 1 (Forkhead box transcription factor of the "O" subclass), CCAAT/Enhancer-Binding Protein β (C/EBPβ), as well as progesterone receptor (PGR), decidual cells are able to produce prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1) which are considered to be important canonical markers of decidual differentiation (Gellersen & Brosens, 2014).

Additionally, the change from proliferative to a secretory state in endometrial glands entails functional changes such as the accumulation of glycogen and lipids, increased ER/Golgi activity, and secretion of uterine fluid. The glandular epithelium also plays a major role in paracrine signalling that initiates and supports decidualization. Signals produced by the glandular epithelium are activin/inhibin dimers (TGF-β family members), interleukin-11 (IL-11), leukaemia inhibitory factor (LIF), and heparin-binding epidermal growth factor (HB-EGF). Decidualization also coincides with the remodelling of the spiral arteries, as well as an immunoregulatory role, reflected in the gathering of special types of lymphocytes, the uterine natural killer cells (uNK) (Gellersen & Brosens, 2014).

The word decidua originates from the Latin word for dying, falling off, or detaching, decidere (Gellersen & Brosens, 2014), which hints at the event that occurs in the absence of a conceptus in humans, menstruation. Menstruation is initiated by the withdrawal of the steroid hormone progesterone at the end of the luteal phase in humans, upon which the decidualized cell layer of endometrium sheds (Emera et al., 2012). In the presence of a conceptus, human trophoblast cells produce human chorionic gonadotropin (hCG), which hinders luteolysis, thereby maintaining the production of progesterone up to the point where the placenta is able to take over (Carp, 2021). While few eutherians undergo endometrial shedding in the nonpregnant cycle, most species do decidualize during pregnancy (Emera et al., 2012; Wagner et al., 2014).

As mentioned, in rodents and most other eutherian species, a blastocyst´s presence is required to initiate decidualization (Emera et al., 2012; Finn & Martin, 1974). Although decidualization triggers thus differ between the two species, the intensification of transformation in humans after embryo implantation suggests a common underlying mechanism (Gellersen & Brosens, 2014). In mice, the decidua begins to develop at day 4,5 of pregnancy, which is the time of blastocyst attachment. During the following 3 days, decidualized fibroblasts at the site of implantation start to proliferate and transform into binucleated or polyploid cells (Ansell et al., 1974). The then-formed decidua delivers nutrients to the blastocyst before the later-formed placenta takes over (Zygmunt et al., 2003).

1.3 The role of decidualization in pregnancy and reproductive success

Studying decidualization is important for several reasons. Evolutionarily, it is a key novelty of the eutherian lineage, enabling the invasive placentation (Wagner et al., 2014). The mechanisms by which this enabling occurs are multiple and probably involve domestication of the innate immune reaction to injury of the embryo (Chavan et al., 2021). Physiologically in most eutherians, decidualization is likely also at the heart of a very critical decision in early pregnancy. It has been shown that decidualizing stromal cells can impose quality selection in humans, i.e., distinguish between individual preimplantation embryos and decide whether a preimplantation embryo is allowed to implant or is rejected (Teklenburg et al., 2010). In other words, the decidualized endometrium is critical for embryo implantation and sustaining early pregnancy. Failure of this process is considered a major cause of infertility and recognized as a key contributor to early pregnancy loss, particularly in the context of recurrent pregnancy loss (RPL) in humans (Gellersen & Brosens, 2014). 75% of pregnancy failures in humans are associated with defects during the peri-implantation period (Cha et al., 2012).

The reasons for miscarriages are often attributed to chromosomal abnormalities of the blastocyst; however, in the case of RPL, the definite cause remains largely unknown. Studies have found that in some of the women, human endometrial stromal cells (HESCs) showed prolonged or even disordered proinflammatory responses during decidualization. Moreover, it has been shown that HESCs from RPL patients display reduced decidual gene expression, increased oxidative stress sensitivity, and impaired embryo sensing. This dysregulation may impair the embryo-endometrial dialogue, leading to implantation of poor-quality embryos pathological implantation sites, and can ultimately end in fetal loss (Gellersen & Brosens, 2014).

While most work on decidualization has been performed in humans, its evolution has been less studied. Even decidualization in mice is decisively less well understood. Of course, studying this complex mechanism in humans is inherently limited by biological, and logistical, as well as ethical constraints, which is why rodent models play a crucial part in understanding the underlying mechanisms involved during decidualization. In this study, we will be working with mice, both a wild-type line and a transgenic line carrying the human rs3820282 allele linked to decidual disorders. As early key reproductive processes, including the progesterone-driven decidualization, are evolutionarily conserved between mice and humans, mice serve as an ideal model organism (Pavličev et al., 2024).

1.4 Metabolomics in reproductive biology

Metabolites are intermediates and endproducts of biochemical reactions and hold an important role in fundamental cell maintenance, as well as mediation between the various regulatory processes that act within cells, forming a network that connects cellular self-maintenance, with processes of growth, differentiation, and major organismal function. Metabolic concentration is dependent upon the amount and characteristics of enzymes involved, which are controlled through regulatory mechanisms such as gene expression and protein interactions. The functional state of cells is therefore reflected in their

metabolic profile, and has an influence on their phenotype in response to environmental and/or genetic changes (Villas-Bôas et al, 2005).

Recent research has emphasized the role of metabolic complementation as a key mechanism in the evolution of tissue organization. The described concept explains how one cell type can support another by sharing metabolic resources, thereby allowing the supported cell to enhance its specialized function (Pavličev et al. 2024).

It is likely, that decidual stromal cells might act similarly in the context of early pregnancy. Not only providing structural and signalling support, but also directly contributing metabolic substrates to the embryo, such as glucose, lactic acid, amino acids, or antioxidants. By analysing the metabolic profile of decidual cells, we can begin to uncover what they produce and release, as well as potentially provide to the embryo.

While studies such as Stadtmauer & Wagner (2022) as well as Stadtmauer et al. (2025) have already provided deep transcriptomic insights into the gene expression changes of decidualization, the metabolomic perspective remains largely unexplored, in particular in species other than humans.

Studies by Jia et al. (2024) as well as Citrinovitz et al. (2022) were already able to show that various metabolites play a crucial role in the transformation of endometrial stromal cells into endometrial decidual cells in humans. Jia et al. (2024) found, using single-cell RNA sequencing, that there is an upregulation in amino acids and sphingolipid metabolism in humans. Corresponding metabolic reprogramming of amino acids and lipid metabolism can also be seen between the non-pregnant uterus and the pregnant uterus of pigs, cattle, and mice (Jia et al., 2024).

A study by Citrinovitz et al. (2022), compared in vitro decidualized and non-decidualized primary HESCs, using mRNA expression levels of key enzymes involved in the glucose- as well as fatty acid metabolism. They showed that there is major metabolic reprogramming involved during decidualization, particularly in the glucose- and fatty acid metabolism (Citrinovitz et al., 2022).

1.4.1 GC-MS

Chromatography is a physical-chemical separation process to divide mixtures between two immiscible phases. One phase is stationary, while the other is mobile and moves through the stationary phase, carrying the analytes. In gas chromatography (GC), the mobile phase is a gas (e.g., nitrogen, helium, or argon) that flows through a column. The separation is based on how strongly the analytes interact with the stationary phase (Kolb, 2012). The following step, the mass spectrometry (MS) then enables the analysis of the mass-to-charge ratio and the estimation of the abundance of components by analysing the gas-phase ions (Glish & Vachet, 2003).

1.5 The model organism mouse, for studying decidualization

Studying the complex processes of endometrial remodeling of decidualization in humans is inherently limited by ethical, logistical, and biological constraints. Therefore, rodent models, especially mice, which have efficient reproduction and a well-defined genetic

profile (Liu et al., 2020), have become an essential tool for dissecting the molecular and cellular mechanisms underlying endometrial function and early pregnancy. Unlike human tissue, which is limited by donor variability as well as clinical factors such as endometriosis or cycle phase, murine endometrial tissue provides a reliable base for studying decidualization under controlled hormonal and cellular conditions (De Clercq et al., 2017).

One of the key advantages of using laboratory mice lies in the conservation of fundamental reproductive processes across mammals. Many aspects of hormone-regulated endometrial transformation, particularly the progesterone-driven decidualization in stromal cells, are evolutionarily conserved between mice and humans. For instance, Wnt4, a gene central to endometrial biology and decidualization, is regulated by estrogen and progesterone in both species (Pavličev et al., 2024). Thanks to the genetic manipulability of mice and also the establishment of protocols for in vitro decidualization through supplementing the culture medium with the hormone progesterone and second messenger cAMP (De Clercq et al., 2017), it is possible to study decidualization not only in vivo but also in vitro. Even though in vivo studies of decidualization have shown the complex signalling involved, detailed mechanistic research, like the interaction between epithelial and stromal signalling, remains limited. Nevertheless, in vitro models are a valuable tool to study key mechanisms through the active manipulation of specific pathways. Primary endometrial stromal cells derived from mice with specific genetic modifications enable us to study very specific characteristics (De Clercq et al., 2017), for example, in our case, the metabolic profile of decidualizing stromal cells.

1.5.1 A Wnt4 regulatory variant knock-in mouse model (rs3820282)

Through the process of CRISPR/Cas9 knock-ins in mice, the effects of specific human alleles can be better studied, as linkage disequilibrium does not obscure the effect of specific alleles. So by introducing rs3820282 into a mouse genome, it is possible to avoid the confounding effects of neighboring SNPs, normally present in human populations (Pavličev et al., 2024).

The transgenic mouse model in this study, carrying the human rs3820282 allele, has provided direct insights into Wnt4's role in the endometrial receptivity and stromal gene expression, mirroring effects relevant to human reproductive traits and disorders such as endometriosis and implantation failure. These transgenic mice show an estrogen-dependent upregulation of Wnt4 in stromal cells, which is linked to increased uterine *invadability* and metabolic reprogramming during the implantation window (Pavličev et al., 2024).

2 Material and methods

This master's thesis aims to investigate the metabolic changes that occur during decidualization in endometrial stromal cells in wild-type mice (*Mus musculus*) and comparing this normal process to the one in the transgenic mouse model carrying a human rs3820282 allele associated with decidual disorders (Pavličev et al. 2024). The following chapter provides an overview of the methodology as well as materials used in the analysis, ensuring the reproducibility of the findings.

2.1 Animal husbandry

The animals for this study (transgenic as well as wild-type) were kept under a light-dark cycle of 12 hours (lights on at 0700) in single-sex groups of two to four animals per unit. The bedding consisted of wood chips, and food (standard mouse chow) and water were available *ad libitum*. The laboratory conditions ensured a relatively constant temperature of 22 ± 2C°and relative humidity of 50 ± 5%. The individuals used were euthanized by cervical dislocation, immediately before the harvest of the uteri.

The transgenic mouse line used was originally described by Pavličev et al. (2024). In these mice, the mouse allele at the genomic location corresponding to the human rs3820282 SNP was replaced with the human allele via CRISPR/Cas9-mediated genome editing. The mice used were from a C57BL/6 genetic background, the protocol is described by Pavličev et al. (2024). Successful targeting was confirmed by PCR and Sanger sequencing (Pavličev et al., 2024).

2.2 Primary cell isolation

For the isolation of mouse endometrial stromal fibroblasts (mESF) and mouse endometrial epithelial cells (mEEC), we followed the standardized protocol by De Clercq et al. (2017). Only cycling mice at the beginning of the estrus phase of the cycle were selected for uterine dissection. Shortly before the isolation, mice were checked for their cycle phase via a vaginal swab, which was then stained using Giemsa stain. The appearance of predominantly cornified epithelial cells indicated the estrus phase (Figure 1). After euthanasia, the abdominal skin and muscle layers were cut to locate the reproductive organs and harvest the uterus. The mEEC and mESF cells were then enzymatically isolated using pancreatin and trypsin (De Clercq et al., 2017). Uteri of a total of six wild-type and 11 transgenic mice were used for this study. The mESF were cultured in DMEM/F12 (1:1) (with phenol red, L-glutamine, and HEPES) medium containing 10% FBS (Foetal Bovine Serum), 0.5 µg/ml amphotericin B, and 100 µg/ml gentamicin, and the MEEC in DMEM medium containing 25% MCDB-105 medium, 10% FBS, 0.9 µg/ml amphotericin B, 100 µg/ml gentamicin, and 5 µg/ml insulin. The cultures were washed with PBS and the growth medium was replaced approximately every 3 days.

2.3 Immunocytochemistry

To verify the identity of mouse endometrial stromal fibroblasts (mESF), immunocytochemical staining was performed. To this end, the mESF were grown to near confluence on the chamber slides (Thermo Fisher Scientific Inc. Lab-Tek II Chamber Slide w/Cover) and stained with antibody against a cell-family marker protein. We used a non-conjugated anti-Vimentin primary antibody (Table 1), against a marker for mesenchymal cells. We subsequently incubated the cells with the a Fluoropore-conjugated secondary antibody (Table 1) to visualize the protein expression. DAPI was used to visualize cell nuclei. Images were acquired using fluorescence microscopy (EVOS™ M7000 Imaging System; ThermoFischer).

Table 1: Overview of used primary and secondary antibodies.

Primary Antibody				
Antibody	**Target (Gene/Protein)**	**Company**	**Marker/Function**	**Dilution**
ab92547	Vimentin (EPR3776)	Abcam	Mesenchymal marker	1:200
14371-1-AP	Wnt4	Proteintech	Wnt signaling pathway	1:200

Secondary Antibody				
Antibody	**Company**	**Specifity**	**Cojugate**	**Dilution**
ab150077	Abcam	Anti-rabbit	Alexa fluor 488	1:1000

2.4 In vitro decidualization

In vitro decidualization was performed according to the protocol by De Clercq et al. (2017), using 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP) and progesterone as well as preconditioned MEEC medium, to ensure potential secreted signals from the epithelial cells. Cells were incubated in the decidualization medium for 5 days at 37 °C, 6.5 % CO_2, and 5.0 % O_2 atmosphere. During this time, the cultures were only washed with PBS once. Successful decidualization was confirmed by immunocytochemistry using an anti-Wnt4 (Table 1). Decidualized cells show binucleation, and their growth is significantly decreased. On day five, the confluence was annotated, and the cultures were washed with 0.9 % NaCl and then immediately frozen using liquid N2 and stored at -70 °C until the metabolite extraction.

2.5 Metabolomics techniques

Metabolite extraction and derivatization for the GC-MS analysis, as well as the assessment of the metabolome, were performed in collaboration with the Vienna Metabolomics Center (VIME, https://metabolomics.univie.ac.at/). For the metabolite extraction, the cells in each well, previously stored at -70 °C, were incubated for 15 min with 80 % MeOH on ice. After the cells were scraped off using a cell scraper and transferred to a safe-lock tube. 10 µl of an isotopically-labeled internal standard mixture was added to each sample. The samples were centrifuged for 10 min at 21 000 rpm at 4 °C. 80 % of the supernatant was

then transferred to new safe-lock tubes, and 10 % of all supernatants were used to generate a pool sample to create a metabolite concentration baseline for the GC analysis. After all the samples were dried using a SpeedVac, derivatization was carried out under the guidance of Martin Brenner from the Vienna Metabolomics Center. The derivatization process was initiated by incubating the sample with the addition of 20 µl of methoxyamine hydrochloride solution (40 mg dissolved in 1ml pyridine) on a thermal shaker (90 min, 30 °C and 700 rpm). Next, 80 µl of n-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) were added to the mixture and again incubated for 30 minutes at 37 °C and 700 rpm. Lastly, the samples were centrifuged for 10 minutes at room temperature at 21 000 x g, and 70 µl of the supernatant were then moved into glass vials containing inserts. The vials were then sealed with crimp caps. GC-MS was used for the metabolomic analysis of the cells.

2.6 Metabolome data processing and statistical analysis

All samples for the GC-MS measurements were randomized before processing. Altogether, 48 separate samples were measured, belonging to 4 categories: non-decidualized and decidualized wild-type strain, and non-decidualized and decidualized transgenic strain. Each of the samples consisted of approximately 1.5 to 2 million cultured cells from multiple individuals of a given genotype. The focus was on comparing metabolite abundance of non-decidualized and decidualized stromal cells of wild-type mice and transgenic mice. We used the software Metaboanalyst v6.0 (https://www.metaboanalyst.ca/) and R.2.2 (https://www.r-project.org/) for the analysis of the GC-MS results.

Metabolome assessment was carried out at the Vienna Metabolomics Center. Data processing, multivariate statistical analysis, and interpretation were performed in collaboration with Univ.-Prof. Dr. Wolfram Weckwerth, using standardized protocols and an-inhouse toolbox COVAIN (Sun & Weckwerth, 2012).

3 Results

As previously described, the cycle phase was determined through distinct differences cellular composition of Giemsa-stained vaginal smears. The presence of cornified epithelial cells indicated estrus phase (Figure 1). After primary cell isolation, mouse endometrial stromal fibroblasts (mESF) and mouse endometrial epithelial cells (mEEC) were cultured separately. The two cell types were distinguishable through their difference in morphology (Figure 2).

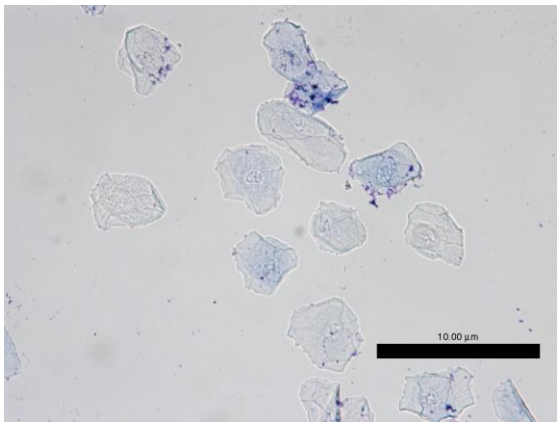

Figure 1: Cornified vaginal epithelial cells indicating the estrus phase of the cycle.

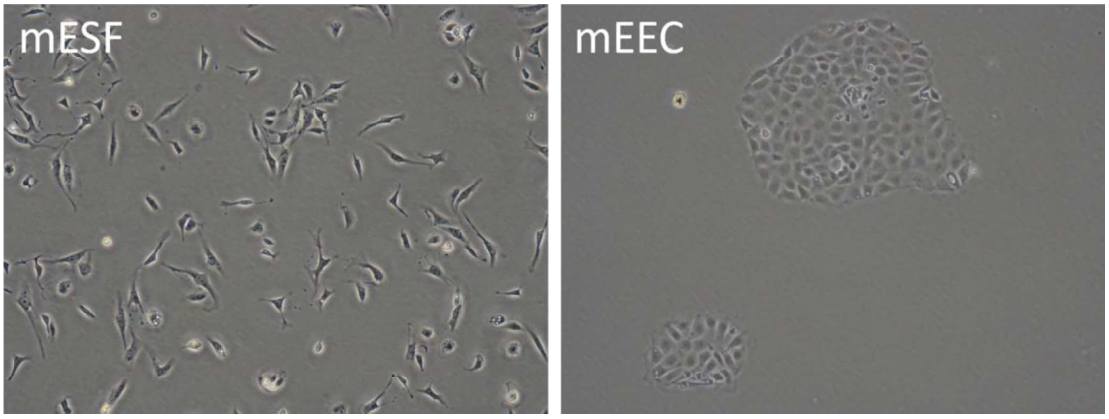

Figure 2: Picture of the morphological characteristics of mouse endometrial stromal fibroblasts (mESF, left) and mouse endometrial epithelial cells (mEEC, right) (Light microscopy, magnification 200X)

3.1 Immunocytochemistry

Through immunofluorescence staining of marker genes, identification of mouse endometrial stromal fibroblasts (mESF) was possible (Figure 3). Following the treatment for decidualization with progesterone and 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP) over a duration of five days, decidualization was ensured through the confirmation of binucleated cells via light microscopy (Figure 4) as well as immunohistochemical staining with a Wnt4 antibody (Figure 5).

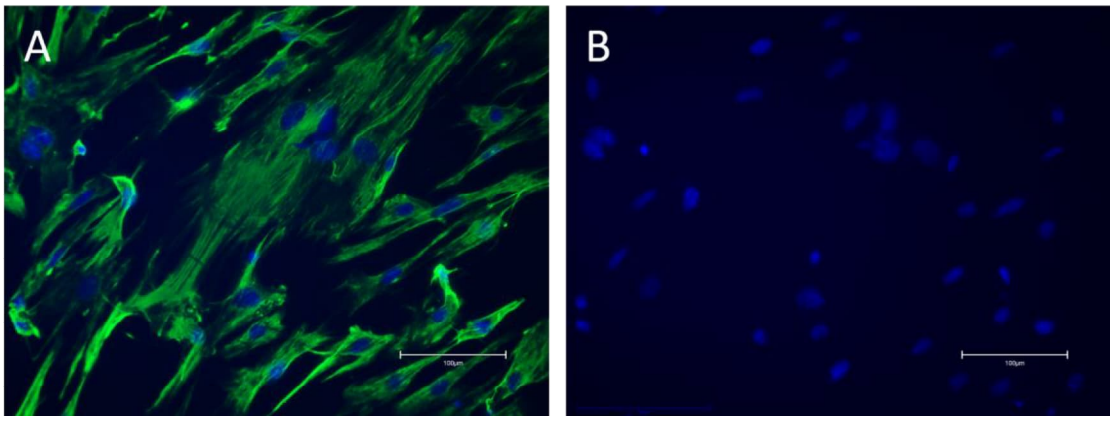

Figure 3: Immunofluorescence staining of mouse endometrial stromal fibroblasts (mESF) (A, B) using an anti-vimentin antibody (green) to visualize the cytoskeleton (Table 1). Nuclei were counterstained with DAPI (blue). Images show merged DAPI/GFP channels (A) and DAPI-only channels (B). Scale bar as indicated.

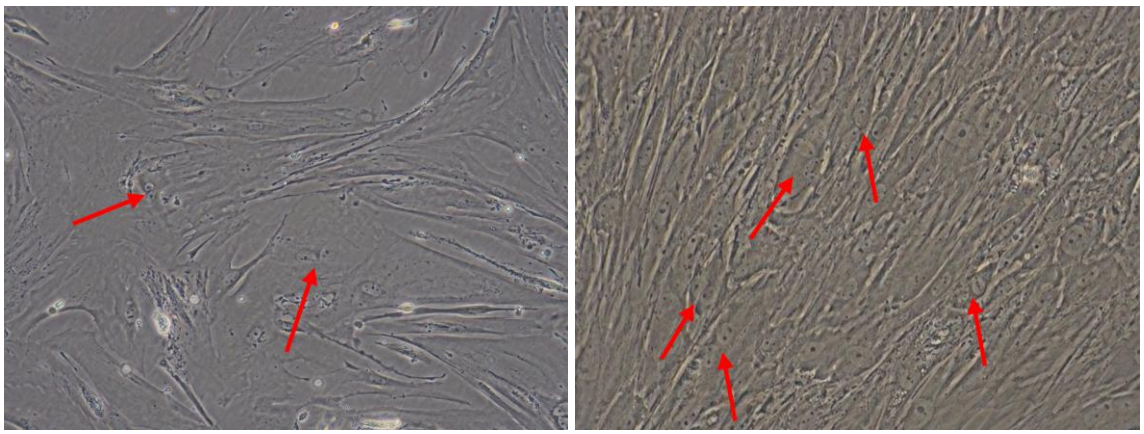

Figure 4: Light microscopy of decidualizing mouse endometrial stromal fibroblasts (mESF). Decidualization is indicated by binucleated cells (arrows). Representative images show mESF following decidualization; arrows pointing to cells containing two nuclei.

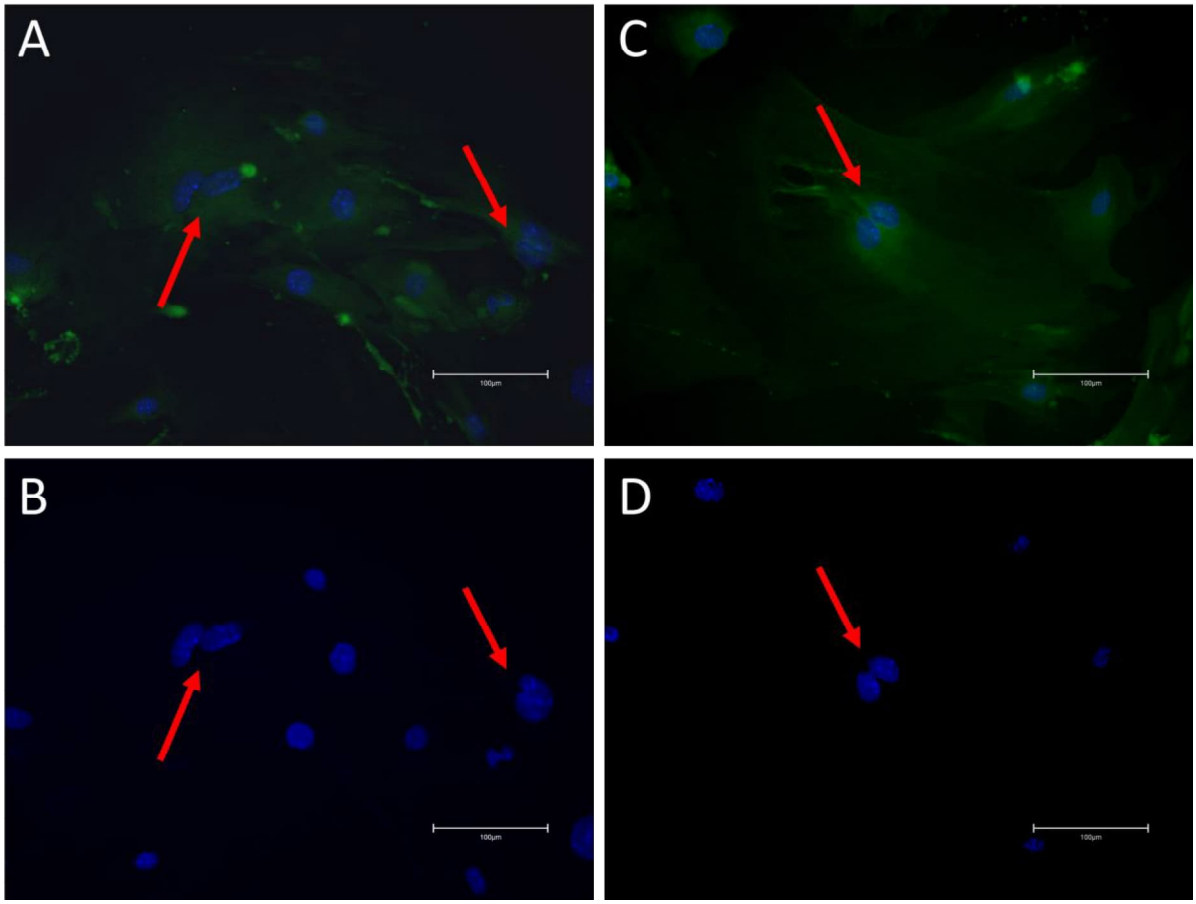

Figure 5: Immunohistochemical staining of decidualized mESF of WT mice with Wnt4 antibody. Wnt4 immunoreactivity was detected using a GFP-conjugated secondary antibody (green) (Table 1). Nuclei were counterstained with DAPI (blue). Decidualization is indicated by binucleation (see arrows). Images show merged DAPI/GFP channels (A, C) and DAPI only channels (B, D). Scale bar as indicated.

3.2 GC-MS results

A total of 30 WT samples and 18 K1 samples were acquired and tested for different metabolite concentrations using GC-MS. Decisions on which metabolites to test were based on literature research, while covering the core measured set of metabolites.

In total, 7 outlier samples were identified as judged by a heatmap illustrating consistently deviating metabolite concentration values across the metabolites, as compared to other decidualized and non-decidualized WT and K1 samples. As there were two outlier samples accumulated in the first round of GC-MS measurements, we decided to exclude the whole first round, consisting of 5 samples, from further analysis. In addition, two more samples were identified as outliers and therefore excluded from the study. This left us with 30 WT and 11 K1 samples for further analysis.

A total 33 metabolites were detected through GS-MS. In our analysis, we focus on the TCA cycle, glycolysis, glutaminolysis, polyamine pathway, and serine and glycine biosynthesis, therefore some detected metabolites outside of these pathways were not further analysed.

3.2.1 Overall results

Overall analysis of the samples using the principal component analysis reveals that the decidualized and non-decidualized categories of samples can be distinguished in terms of their metabolic profile, despite expected overlap. Moreover, it can be seen that the decidualization in both lines, WT and transgenic, moves in the same overall direction in the metabolomic “space”. Interestingly, the deviation of the transgenic line from the WT also appears to follow the direction of decidualization (Figure 6).

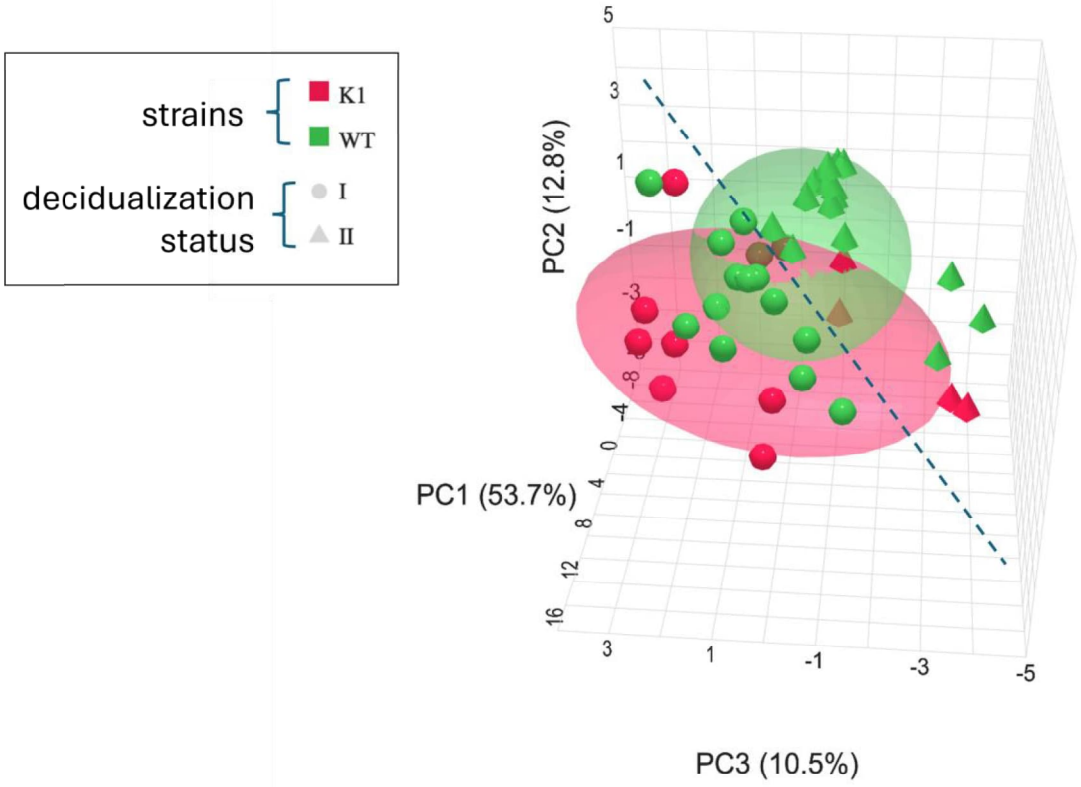

Figure 6: 3D PCA (principal component analysis) showing the mouse strains K1 (red) and WT (green) and also their decidualization status, non-decidualized (circle) and decidualized (triangle).

To identify significant differences between the genotypes (WT, K1) across both treatments, as well as to identify the main effect of the decidualization across both mouse lines, a two-way ANOVA was performed. In addition to direct effects of the genotype, we also asked whether the changes in metabolite profiles, associated with decidualization, differ between the two mouse lines, i.e., whether the mutation in Wnt4 changes the metabolic trajectory of decidualization (Table 2). As the two-way ANOVA does not directly test whether WT vs. K1 differ within the decidualized group only, post hoc simple effects (pairwise) comparisons were applied.

Table 2: Two-way ANOVA results. In red are the effects significant at the level $p \leq 0.05$. Factor 1 tests whether there is an overall difference between the mouse lines (WT vs. K1) regardless of decidualization status, and factor 2 tests whether there is an overall difference between decidualized and non-decidualized mouse endometrial stromal fibroblasts (mESF) independent of the mouse lines. The interaction then tests whether the effect of decidualization depends on the mouse line. The Raw p is the unadjusted p-value directly produced by the ANOVA for main effect or interaction, the adjusted.p is the p-value after applying the Benjamini-Hochberg false discovery rate correction method to control for multiple testing.

Metabolites	Factor1(F.val)	Factor1(raw.p)	Factor1(adj.p)	Factor2(F.val)	Factor2(raw.p)	Factor2(adj.p)	Interaction(F.val)	Interaction(raw.p)	Interaction(adj.p)
Glycine	26.504	8.92e-06	0.00029436	41.027	1.78e-07	1.749e-06	6.856	0.013	0.053625
Proline	18.087	0.000138	0.002277	22.623	2.99e-05	0.00019734	0.078	0.782	0.83352
Putrescine	13.601	0.000722	0.007942	1.473	0.232	0.40295	10.648	0.002	0.0132
Pyruvic acid	10.177	0.003	0.02475	0.415	0.523	0.68538	21.98	3.68e-05	0.0006072
DHAP	9.24	0.004	0.0264	0.265	0.61	0.71642	12.502	0.001	0.00825
Ornithine	6.365	0.016	0.088	9.136	0.005	0.0165	2.139	0.152	0.3135
Malic acid	5.54	0.024	0.095333	9.336	0.004	0.014667	6.976	0.012	0.053625
myo-Inositol	5.552	0.024	0.095333	0.021	0.885	0.885	0.08	0.778	0.83352
F-1;6-P	5.405	0.026	0.095333	53.732	1.03e-08	3.399e-07	8.359	0.006	0.033
Lactic acid	5.16	0.029	0.0957	0.382	0.54	0.68538	0.272	0.605	0.83352
Taurine	4.573	0.039	0.117	20.049	6.99e-05	0.00038445	0.237	0.63	0.83352
Fumaric acid	2.145	0.152	0.39854	3.68	0.063	0.1386	2.709	0.108	0.25457
2-Oxoglutaric a	2.088	0.157	0.39854	10.305	0.003	0.012375	25.518	1.2e-05	0.000396
Galactose	1.783	0.19	0.44786	0.302	0.586	0.71622	0.019	0.892	0.892
Hypotaurine	1.434	0.239	0.5258	0.891	0.351	0.50361	0.215	0.646	0.83352
Aspartic acid	1.306	0.26	0.53625	0.941	0.338	0.50361	0.145	0.705	0.83352
Threonine	1.062	0.31	0.60176	15.594	0.000339	0.0015981	2.425	0.128	0.2816
Serine	0.857	0.36	0.66	40.963	1.81e-07	1.749e-06	3.552	0.067	0.201
Fructose	0.544	0.465	0.759	0.138	0.713	0.73528	0.036	0.851	0.87759
Valine	0.503	0.483	0.759	1.351	0.252	0.4158	1.577	0.217	0.42124
Isoleucine	0.502	0.483	0.759	6.219	0.017	0.04675	2.83	0.101	0.25457
Threitol	0.384	0.539	0.79554	0.192	0.664	0.71642	1.218	0.277	0.50716
Leucine	0.347	0.559	0.79554	5.08	0.03	0.070714	2.958	0.094	0.25457
Phenylalanine	0.294	0.591	0.79554	3.356	0.075	0.15469	0.816	0.372	0.6138
Glucose	0.244	0.625	0.79554	1.875	0.179	0.34747	3.913	0.055	0.1815
Glutamic acid	0.212	0.648	0.79554	40.306	2.12e-07	1.749e-06	17.894	0.000147	0.001617
Maltose	0.206	0.652	0.79554	0.225	0.638	0.71642	0.169	0.684	0.83352
Tyrosine	0.179	0.675	0.79554	1.189	0.283	0.44471	0.077	0.783	0.83352
Lysine	0.12	0.731	0.83183	1.592	0.215	0.39417	0.595	0.445	0.69929
Succinic acid	0.073	0.789	0.85161	0.406	0.528	0.68538	0.251	0.619	0.83352
Methionine	0.065	0.8	0.85161	0.181	0.673	0.71642	1.143	0.292	0.50716
Citric acid	0.031	0.861	0.88791	5.124	0.03	0.070714	4.739	0.036	0.132
Alanine	0.000553	0.981	0.981	8.235	0.007	0.021	0.134	0.716	0.83352

3.2.2 Metabolic changes during decidualization in wild-type-derived endometrial stromal fibroblasts

Within the conventional laboratory mouse line (C57BL/6, "WT"), the process of decidualization is accompanied in the endometrial stromal cells with widespread differences in metabolites, with 30% of measured metabolites changing their abundance (Table 2). We assessed the normal distribution of residuals using Q-Q plots and the Shapiro-Wilk test. As no deviation from normality was detected, differences between decidualized and non-decidualized WT samples were subsequently analysed using Welch's two-sample t-test. When residuals deviated significantly from normality (Shapiro-Wilk test $p<0.05$), the non-parametric Wilcoxon rank-sum (Mann-Whitney U) test was applied.

The results in Figure 7, show the influence of decidualization on metabolite abundance (y-axis) between decidualized (II) and non-decidualized (I) (x-axis) mouse endometrial stromal fibroblasts (mESF) in WT. We found a significant difference in the abundance of amino acid glycine between decidualized and non-decidualized WT stromal fibroblasts ($t=5.267$, $p= 1.547e-05$, Figure 7A). Glycine levels thereby decreased significantly with decidualization. The same direction of change was also observed in glutamic acid ($t=3.056$, $p= 0.005$, Figure 7F) and in diamine putrescine ($t=2.736$, $p= 0.012$, Figure 7G). In contrast, the fructose-1,6-bishosphate, a metabolic intermediate of glycolysis, increased

significantly during decidualization (W=0, p=3.358e-06; Figure 8B). Importantly, we also saw an increase of pyruvic acid, the main product of glycolysis (W=49, p=0.008, Figure 7E). Dihydroxy-aceton-phosphate (DHAP) (Figure 7C), a three-carbon sugar phosphate intermediate in glycolysis, as well as lactic acid values (Figure 7D) indicated no significant changes between decidualized and non-decidualized WT mESFs (t=1.189, p=0.246 and W= 91, p= 0.389, respectively) (Figures 7C, 7D).

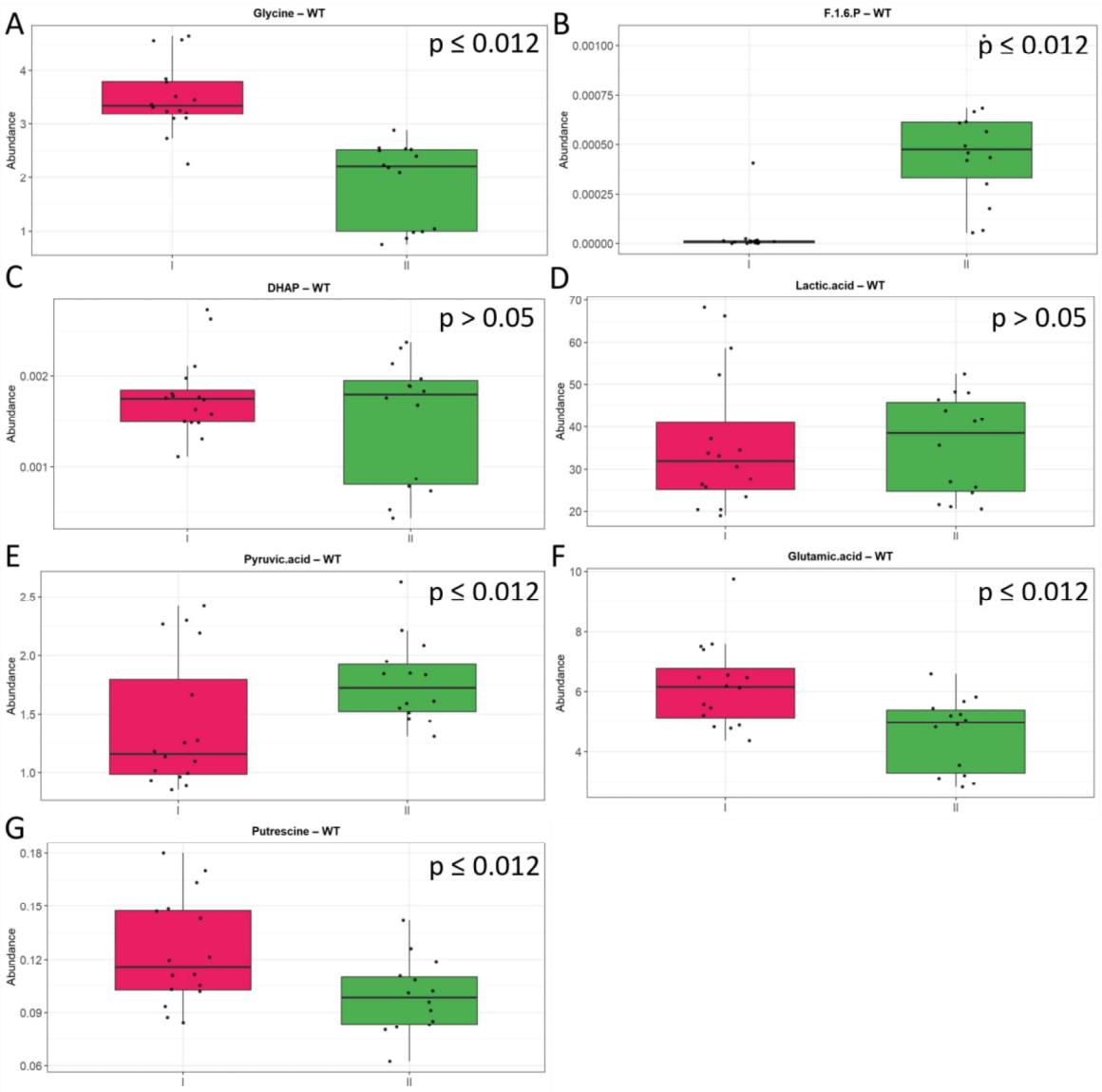

Figure 7: Abundance of seven metabolites illustrating the effect of decidualization on metabolite levels in wild-type (WT) mouse endometrial stromal fibroblasts (mESF). Metabolite levels were compared between non-decidualized (I) and decidualized (II) mESF. Boxplots show median and interquartile range; dots represent individual samples; p-values as indicated. Panels show: (A) Glycine, (B) Fructose-1,6-bisphospahte, (C) Dihydroxyacetone phosphate (DHAP), (D) Lactic acid, (E) Pyruvic acid, (F) Glutamic acid, (G) Putrescine.

3.2.3 Deviation in decidualization-related metabolic changes in K1 transgenic mouse ESF

Figure 8 shows the abundance of three metabolites (A: fructose-1,6-bisphosphate, B: dihydroxy-aceton-phosphate (DHAP), and C: 2-oxoglutaric acid) in decidualized and non-decidualized mESF of both genotypes (WT versus K1). In these metabolites, the changes caused by decidualization significantly affected metabolite abundance only in one genotype. For example, applying two-way ANOVA showed a significant interaction of genotype and decidualization status for fructose-1,6-bishosphate levels (raw.p=0.006; adj.p=0.033). Post hoc simple-effects analysis with Holm correction showed that decidualization significantly enhanced fructose-1,6-bishosphate levels in WT mice ($p<0.0001$), whereas no statistically significant effect was observed in K1 mice ($p=0.2410$) (Figure 8A). A contrary observation was made concerning DHAP and 2-oxoglutaric acid where the changes were only significant within the transgenic line, not in the WT. The results revealed a significant interaction between genotype and decidualization status for DHAP levels (raw.p=0.001; adj.p=0.00825) and post hoc simple-effects analysis with Holm correction showed that decidualization had no significant effect in WT mice ($p=0.19$), whereas it significantly increased DHAP levels in K1 mice ($p=0.0021$), (Figure 8B). This observation could also be made for 2-oxoglutaric acid where there appears to be a significant interaction between genotype and decidualization for 2-oxoglutaric acid levels (raw.p=1.2e-05; adj.p=0.000396). Post hoc simple-effects analysis with Holm correction showed that decidualization significantly decreased 2-oxoglutaric acid levels in K1 mice ($p<0.0001$) but not in the WT mice ($p=0.8187$) (Figure 8C).

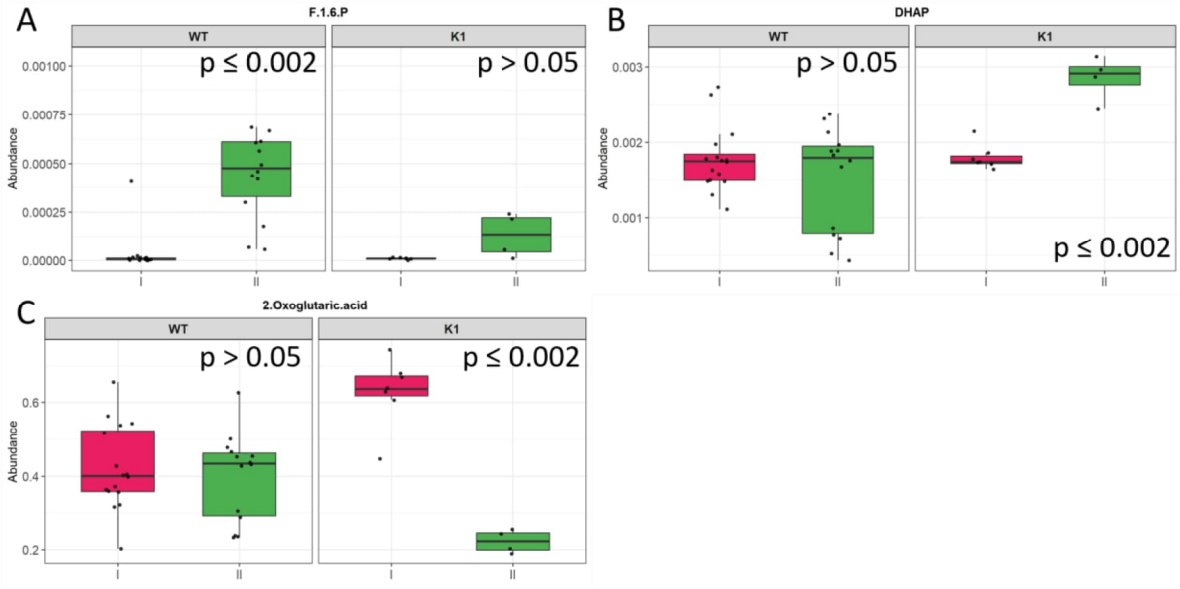

Figure 8: Three metabolites exhibiting changes in the same direction upon decidualization in wild-type (WT) and transgenic line (K1) mouse endometrial fibroblasts (mESF), although the magnitude of these changes differs between the genotypes. Metabolite abundance was compared between non-decidualized (I) and decidualized (II) mESF. Boxplots show median and interquartile range; dots represent individual samples; p-values are indicated. Panels show: (A) Fructose-1,6-bisphosphate, (B) Dihydroxyacetone phosphate (DHAP), (C) 2-Oxoglutaric acid.

The metabolites pyruvic acid and putrescine show a change in opposite directions in WT and K1 upon decidualization, indicating genotype-specific regulations. Pyruvic acid exhibits a significant interaction between genotype and decidualization status mESF (raw.p=3.68e-05; adj.p=0.0132). Additional post hoc simple-effects analysis with Holm correction showed that decidualization significantly decreased pyruvic acid levels in K1 mice (p<0.0006) and significantly increased in the WT mice (p=0.0060) (Figure 9A). A significant interaction is also found between the effect of a genotype and decidualization for putrescine levels (raw.p=0.002; adj.p=0.0132). Post hoc simple-effects analysis with Holm correction showed that decidualization significantly reduced putrescine levels in WT (p=0.0106) but significantly enhanced putrescine abundance in K1 mice (p<0.0335) (Figure 9B).

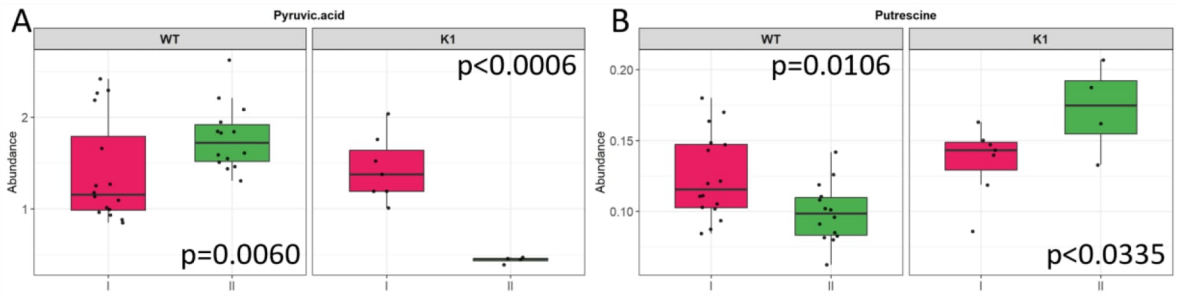

Figure 9: Abundance of two metabolites displaying deviating changes upon decidualization between wild-type (WT) and transgenic line (K1) mouse endometrial stromal fibroblasts (mESF). Metabolite levels were compared between non-decidualized (I) and decidualized (I) mESF. Boxplots show median and interquartile range; dots represent individual samples; p-values are indicated. Panels show: (A) Pyruvic acid, (B) Putrescine.

Overall, 7 out of the 33 detected central metabolites showed a significant response to decidualization in either WT and/or K1 mouse endometrial stromal fibroblasts (mESF). Among these seven metabolites, three (fructose-1,6-bisphosphate, DHAP, and pyruvic acid) are part of the glycolytic pathway; two (glycine and glutamic acid) belong to the amino acid pathway; putrescine is associated with the polyamine pathway and 2-oxoglutaric acid (i.e., alpha-ketoglutaric acid) is a component of the TCA cycle. Therefore, in this study, a focus was put on these pathways (Figure 10).

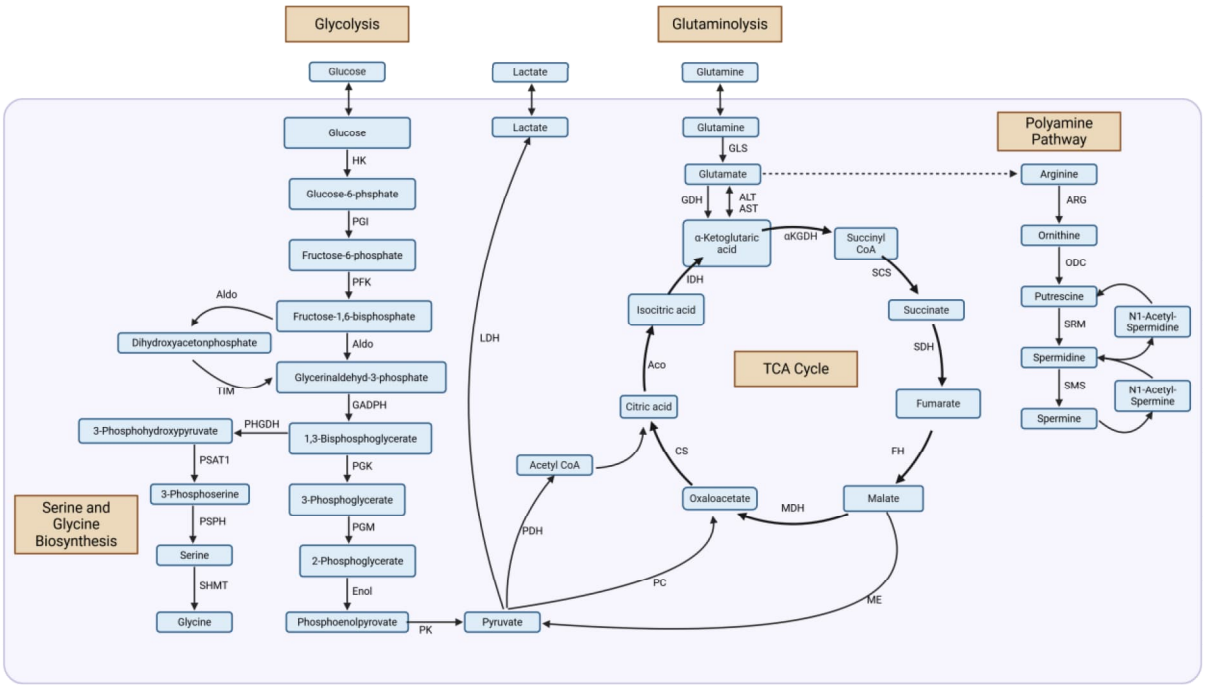

Figure 10: Metabolic pathways and interactions of analysed metabolites. Enzymes are shown in abbreviated form; full names are provided in Table 3. (BioRender, 2026; Citrinovitz et al., 2022; Löffler, 2007; Wallace et al., 2003; Zhao et al., 2008; Zuo et al., 2015).

Table 3: List of enzyme abbreviations and their corresponding full names as used in Figure 10.

Enzymes			
Abbreviation	**Full name**	**Abbreviation**	**Full name**
HK	Hexokinase	GLS	Glutaminase
PGI	Phosphoglucose isomerase	GDH	Glutamate dehydrogenase
PFK-1	Phosphofructokinase-1	ALT	Alanine aminotransferase
Aldo	Aldolase	AST	Aspartate aminotransferase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	αKGDH	α-Ketoglutarate-dehydrogenase
PGK	Phosphoglycerate kinase	SCS	Succinyl-CoA synthetase
PGM	Phosphoglycerate mutase	SDH	Succinate dehydrogenase
Enol	Enolase	FH	Fumarase
PK	Pyruvatkinase	MDH	Malate dehydrogenase
LDH	Lactate dehydrogenase	CS	Citrate synthase
PDH	Pyruvate dehydrogenase	Aco	Aconitase
PC	Pyruvate carboxylase	IDH	Isocitrate dehydrogenase
TIM	Triose phosphate isomerase	ARG	Arginase
PHGDH	Phosphoglycerate-dehydrogenase	ODC	Ornithine decarboxylase
PSAT1	Phosphoserine aminotransferase 1	SRM	Spermidine synthase
PSPH	Phosphoserine phosphatase	SMS	Spermine synthase
SHMT	Serine hydroxymethyltransferase	ME	Malic enzyme

4 Discussion

This study investigated metabolic changes that accompany the decidualization process in mouse endometrial stromal cells. In addition, we compared "wild-type" mice (C57BL6 line of *Mus musculus*) to a transgenic mouse line carrying a human minor allele at the locus rs3820282, associated with decidual disorders (Pavličev et al. 2024). This allele has been shown to cause a premature decidualization in nonpregnant mice post-ovulation.

Metabolic changes during the in vitro decidualization of mouse endometrial stromal cells

First, we found significant alterations in the metabolite profile of mouse endometrial stromal fibroblasts (ESFs) during decidualization. Notably, several significantly altered metabolites were identified as key intermediates of central metabolic pathways, particularly glycolysis and TCA cycle, but potentially also other pathways.

A significant difference in metabolite abundances was observed for fructose-1,6-biphosphate (F-1,6-P), pyruvic acid, glycine, 2-oxoglutaric acid, glutamic acid, and putrescine. Surprisingly, a number of intermediates of glycolysis and the TCA cycle, in contrast, showed no differences (e.g., lactic acid, malic acid, glucose) between decidualized and non-decidualized stromal cells.

Fructose-1,6-biphosphate (F-1,6-P) is a crucial glycolytic intermediate, into which most of the glucose is turned after entering the cell. The rising abundance of F-1,6-P during decidualization in the mouse had been previously linked to the upregulation of phosphofructokinase 1 (PFK1), an enzyme that catalyzes the production of F-1,6P, and/or a downregulation of fructose-bisphosphatase 1 (FBP1), an enzyme that catalyzes the opposite reaction in gluconeogenesis (Zhou et al., 2022). As the decidualization is a process occurring in preparation for pregnancy support, the above observation could be indicative of an increase in the glucose uptake and thus an increased rate of glycolysis by the cell, suggesting the upregulation of PFK1. Indeed, due to a good positive correlation between levels of F-1,6-P and the rate of glycolysis, F-1,6-P is used as a readout of the activity of glycolysis in the liver cancer cells (Pérez-Chávez et al., 2024).

In the next step of glycolysis, the 6-carbon sugar F-1,6-P is cleaved into two 3-carbon molecules: glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP), of which we only measured the DHAP. DHAP abundance did not differ significantly between decidualized and non-decidualized mESF. We did, however, detect an increase in the final product of glycolysis, pyruvic acid.

Zuo et al. (2015) proposed a so-called *Warburg effect* during the decidualization of mouse ESCs. This effect has also been suggested in human ESCs (Citrinovitz et al., 2022). The Warburg effect, often observed in tumor cells, is a form of metabolic reprogramming (Potter et al., 2016). It is a consequence of the increased rate of glycolysis, which causes the accumulation of pyruvic acid, as is observed in our cells. The conversion of pyruvic acid by the enzyme pyruvate dehydrogenase (PDH) to create acetyl-CoA, an important metabolite in the TCA cycle, cannot occur at the same rate. The surplus pyruvic acid is thus converted into lactic acid by the enzyme lactate dehydrogenase A (LDHA) when PDH is saturated (Vayakkattil et al., 2025). This processing of accumulated pyruvic acid is

also necessary to regenerate NADH from NAD+ to be used in further glycolysis. This conversion towards lactic acid is variably associated with downregulation of TCA cycle. Historically, the conversion of pyruvic acid to lactic acid had been thought to occur only in the absence of oxygen, in which case lactic acid represents an alternative to pyruvic acid. However, it has been shown later that lactic acid production is common even in the presence of sufficient oxygen (Kaplon et al., 2013; Potter et al., 2016), both in cancer cells and other proliferative cells that subsequently also reduce the TCA cycle, but also in other contexts where the surplus lactic acid is often exchanged among cells. The Warburg metabolism (lactic acid production in the presence of oxygen) is thus associated with increased glucose uptake and enhanced activity of glycolytic enzymes. It is believed that the Warburg effect represents an adaptive strategy that supports biosynthesis, redox homeostasis, and cell proliferation (Potter et al., 2016). In the case of decidualization, it is important to note here that cells differentiate and stop proliferating.

A possible counterindication to the proposed Warburg metabolism is the absence of increased lactic acid levels in decidualized mESF. Under the Warburg effect, lactic acid may be expected to increase in decidualized cells. We did not observe an increase in our experiment, consistent also with findings in human decidualized cells (Citrinovitz et al., 2022). However, this is but a weak argument against Warburg metabolism, because lactic acid may be rapidly exported via monocarboxylate transporters (MCT4), and used by other cells, making it extracellular and therefore not detectable in our measurements (Citrinovitz et al., 2022; Zuo et al., 2015). Indeed, Zuo et al. (2015) have found that MCT4 is induced in decidualized mouse stroma cells. They also found that the lactate transporter MCT1 and the proliferation marker Ki-67 are sited in yet undifferentiated cells, suggesting active import of lactic acid in proliferating cells (Zuo et al., 2015). In support of Warburg metabolism, Citrinovitz et al. (2022) also observed an upregulation of lactate dehydrogenase (LDH) in human decidualization, clearly indicating increased lactic acid production. Different than the above studies in mouse and human decidualization, we detected an increase in pyruvic acid values in decidualized WT cells. However, this observation likely does not speak against Warburg metabolism, considering that it results from increased rates of glycolysis and pyruvic acid production.

In short, our findings indicate a substantial upregulation of glycolytic flux during decidualization. This is in alignment with previous metabolic studies reporting drastic metabolic changes during decidualization in humans as well as mice (Citrinovitz et al., 2022; Zhou et al., 2022).

To understand the changes downstream of glycolysis, we focused on TCA-related metabolites. As mentioned, an increased production of lactic acid can but need not be associated with decreased activity of the TCA cycle. We found no decidualization-related changes in the intermediate metabolites of the TCA cycle: malic acid, succinic acid, or 2-oxoglutaric acid (i.e., alpha-ketoglutarate). There is thus no direct evidence for a reduction in the TCA cycle activity due to decidualization in the WT line. We did, however, find decreased levels of glutamic acid upon decidualization. Glutamic acid is one of the alternative sources for 2-oxoglutaric acid. During glutaminolysis, glutamine is converted to glutamic acid via the enzyme glutaminase, and the glutamic acid is then converted to 2-oxoglutaric acid via glutamate dehydrogenase (GLUD) (Tang et al., 2023). This alternative mechanism may compensate for reduced pyruvic acid input, maintaining the

TCA cycle. A decrease in the level of glutamic acid may indicate that this pathway is active and that this compensation successfully maintains the constant levels of the intermediates of the TCA cycle. Alternatively, decreased glutamic acid levels may suggest downregulation, the TCA cycle not requiring compensatory input. Either way, the TCA cycle does not appear restricted in WT based on present data.

In contrast, previous studies showed that in human decidualization, the Warburg metabolism is associated with the downregulation of the TCA cycle. Citrinovitz et al. (2022) reported that the mRNA expression level of the enzyme isocitrate dehydrogenase 2 (IDH2), which catalyzes the rate-limiting step of the TCA cycle, is significantly downregulated in decidualized compared to non-decidualized cells. In contrast, mRNA levels of later-acting enzymes, such as succinate dehydrogenase A (SDHA) and fumarate hydratase (FH), were upregulated in decidualized cells, suggesting enhanced activity in the latter parts of the TCA cycle. This observation led them to propose that intermediate metabolites, other than from the glucose or fatty acids, might enter the TCA cycle (Citrinovitz et al., 2022). Glutaminolysis might play such a role in decidualization. To test this hypothesis Tang et al. (2023) examined mRNA expression levels of enzymes involved with the glutaminolysis, such as glutaminase 1 (GLS1), Glu dehydrogenase 1 (GLUD1), glutamic-oxaloacetic transaminase 2 (GOT2), glutamic-pyruvic transaminase 2 (GPT2), as well as succinate dehydrogenase (SDHB). Indeed, an overall upregulation in decidualized cells was found compared to non-decidualized cells in humans (Tang et al., 2023). This supports our interpretation of reduced levels of glutamic acid as an indication of a compensatory mechanism to maintain TCA cycle activity.

Another pathway of interest is the polyamine pathway as polyamines are considered to play an important role in decidualization and implantation (Zhao et al., 2008). Putrescine was the only metabolite detected through GC-MS within the polyamine pathway that showed a significant decrease in decidualized mouse endometrial stromal fibroblast (mESF) in WT. Putrescine is derived from arginine via conversion to ornithine by the enzyme arginase, followed by decarboxylation through ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine synthesis (Zhao et al., 2008; Wallace et al., 2003). It has been shown that polyamine synthesis is enhanced at the location of implantation and that in the case of blockage of ODC, pregnancy rates dropped drastically in mice. These finding led to the conclusion that polyamine synthesis is crucial for implantation (Zhao et al., 2008). A study by Liu et al. (2017) looking at metabolic changes during decidualization in the mouse uterus of 8-day pregnant mice, did find increased putrescine and spermidine levels in decidualized tissue. These findings align with the found effect of polyamines, regulating gene expression, partly through chromatin remodelling and promotion of cellular differentiation and proliferation. In contrast to the tissue-level increase reported by Liu et al. (2017). However, the decreased putrescine abundance observed in decidualized WT mESF in our study, may also reflect efficient metabolic turnover of putrescine for downstream polyamine synthesis during the differentiation induced through decidualization. Rather than reduced pathway activity, there is a difference in the distinct biological context of tissue-level composition and signalling in vivo versus stromal-cell-intrinsic metabolism in vitro. That said, our results likely reflect the general consensus that polyamines are essential for cell proliferation and

differentiation, and the polyamine pathway is influenced by decidualization (Wallace et al, 2003).

The decidualization-associated metabolic trajectory in the transgenic line deviates from WT

Interestingly, we found deviating results in the transgenic mice. The general expectation for both mouse lines was an overall increased energy demand during decidualization, as decidualization itself is a highly energy-intensive process, and previous studies both in humans and mice have shown this trend (Zhou et al., 2022).

Contrary to what the observations in WT, there was no significant increase in fructose-1,6-biphosphate abundance in the K1 line during decidualization. It has been argued that reduced accumulation of F-1,6-P is associated with impaired activation of the fructose-1,6-biphosphate -IL27 signalling axis (Zhou et al., 2022). This axis is considered a case of an *immuno-metabolic coupling*, where the accumulated glycolytic metabolite fructose-1,6-biphosphate (due to an upregulation of the upstream enzyme PFK1 and downregulation of its catabolic enzyme FBP1), acts as a signal inducing the expression of Interleukin-27 (IL-27). It does so by binding to pyruvate kinase 2 (PKM2), the enzyme of which cytosolic form catalyses pyruvic acid production. This enzyme, however, can also enter the nucleus, activate ERK and downstream c-FOS, and induce IL-27 expression (Zhou et al., 2022). IL-27 is an immunoregulatory cytokine that regulates immune cells through signal transducer and activator of transcription (STAT) signalling pathways, activating both pro-inflammatory and anti-inflammatory effects depending on the biological context (Xu et al., 2024). In the context of decidualization, IL-27 is considered to contribute to the stabilization and quality of decidualization as it induces the production of specialized macrophages (COX-2^{+}M2-like macrophages), which are considered to support decidualization, invasion of the trophoblast, development of the placenta and the establishment of immune tolerance towards the embryo (Zhou et al., 2022).

Even though both WT and K1 stromal cells were decidualized under identical in vitro conditions, we know from previous research that the K1 line enters decidualization from a pre-primed stromal state, as these stromal cells already show an upregulation of WNT4 and progesterone-regulated genes (Pavličev et al., 2024). While WT mESF show a clear increase in F-1,6-B levels during decidualization, this increase was less pronounced in K1 cells. As F-1,6-P reflects glycolytic activity, this could imply that the K1 stromal cells might undergo a different or attenuated metabolic transition during decidualization, potentially due to their pre-primed state (Pérez-Chávez et al., 2024).

However, the K1 decidualization does show an increase of Dihydroxyacetone phosphate (DHAP), one of the two glycolytic metabolites directly downstream of F-1,6-P, a change that was not detected in WT cells. Accumulation is associated in the literature with pathological conditions, such as fibrosis or diabetes, and it is not clear at present whether it may also occur in association with an upregulated rate of glycolysis, as is the case in WT. Interestingly, the pyruvic acid level is significantly decreased in the decidualized mESFs of the transgenic line, in contrast to WT mESFs, where the pyruvic acid increased upon decidualization.

Other results in the transgenic line compared to the WT also differ. While the glutamic acid levels decrease during decidualization in the transgenic line as they do in WT, in this case, the levels of 2-oxoglutaric acid also significantly decrease with decidualization in the transgenic line. This is generally observed when cells are starved for carbon sources; in this case, this would be reflected in decreased pyruvic acid input. If our logic is correct, together with low levels of pyruvic acid, this suggests a lack of 2-oxoglutaric acid, which is not readily compensated by glutaminolysis and results in reduced activity of the TCA cycle in K1.

The stable 2-oxoglutaric acid levels in WT could thus possibly indicate no major alteration in TCA activity. A possible explanation for the significantly decreased 2-oxoglutaric acid levels in K1 could be an insufficient production of 2-oxoglutaric acid due to restrictions within the cycle, as well as insufficient compensation by glutaminolysis, while downstream pathways still consume 2-oxoglutaric acid, which could result in reduced levels due to lower production but “normal” turnover.

Another difference between the transgenic line and the WT is the deviating results of putrescine abundance. Even though putrescine abundance was decreased in WT mESF, putrescine was significantly increased in decidualized K1 mESF. As of now, there is not enough evidence that allows for well-reasoned explanation on this effect, which is why further investigation into the mRNA gene expression of enzymes such as arginase, ornithine decarboxylase, spermidine synthase, and spermine synthase could potentially offer further insights. Nonetheless, as already said, the polyamine synthesis seems to play a vital part in decidualization and implantation and therefore provides sufficient justification for further investigation (Zhao et al., 2008).

One of the limiting factors of this study is that metabolomic measurements alone do not provide insight into whether a specific metabolic pathway is upregulated or suppressed, as accumulation can result from either decreased catalysis (inactivity downstream) or increased production (activity upstream), and thus do not alone indicate a specific change in pathway flux. Therefore, an additional layer of analysis is necessary for an accurate testing of the hypothesis. Combining liquid chromatography with transcriptomic analysis of specific pathways will help identify metabolic changes correlating to certain regulatory mechanisms. Furthermore, increasing the sample size would strengthen the robustness of the results and allow a more accurate representation of observed phenomena.

Pavličev et al. (2024) have previously shown that knock-in of the allele in this transgenic line leads to a higher expression of WNT4 in the endometrium, positively impacting pregnancy length and implantation success. The present study further shows that this genetic modification is accompanied by metabolic alterations that significantly differ from those observed in the wild-type.

In conclusion, we observed changes in the abundance of several metabolites during decidualization, which aligns with findings in previous studies that found metabolic reprogramming during decidualization, especially in glycolysis and the TCA cycle (Citrinovitz et al., 2022; Zhou et al., 2022). Our results also align with previously stated observations of a Warburg-like effect in decidualized stromal cells (Citrinovitz et al., 2022). But to conclude whether our results indicate the activation or inactivation of

metabolic pathways, transcriptomic analysis of the same cells will be inevitable in the future.

5 References

Ansell, J. D., Barlow, P. W., & Mclaren, A. (1974). Binucleate and polyploid cells in the decidua of the mouse. Development, 31(1), 223–227. https://doi.org/10.1242/dev.31.1.223

Basanta, S., & Pavlicev, M. (2025). The Shifting Role and Regulation of the Corpus Luteum in Vertebrate Reproduction: A Synthetic Review. The Quarterly Review of Biology, 100(3), 189–231. https://doi.org/10.1086/737357

BioRender (2026). Circular Pathway (Layout). https://app.biorender.com/biorender-templates/details/t-61fb36061edaa300a3b70b13-circular-pathway-layout

Brosens, J. J., Pijnenborg, R., & Brosens, I. A. (2002). The myometrial junctional zone spiral arteries in normal and abnormal pregnancies: A review of the literature. American Journal of Obstetrics and Gynecology, 187(5), 1416–1423. https://doi.org/10.1067/mob.2002.127305

Carp, H. J. A. (2021). Progestogens in luteal support. Hormone Molecular Biology and Clinical Investigation, 42(2), 143–148. https://doi.org/10.1515/hmbci-2019-0067

Cha, J., Sun, X., & Dey, S. K. (2012). Mechanisms of implantation: Strategies for successful pregnancy. Nature Medicine, 18(12), 1754–1767. https://doi.org/10.1038/nm.3012

Chavan, A. R., Griffith, O. W., Stadtmauer, D. J., Maziarz, J., Pavlicev, M., Fishman, R., Koren, L., Romero, R., & Wagner, G. P. (2021). Evolution of Embryo Implantation Was Enabled by the Origin of Decidual Stromal Cells in Eutherian Mammals. Molecular Biology and Evolution, 38(3), 1060–1074. https://doi.org/10.1093/molbev/msaa274

Citrinovitz, A. C. M., Hauke, J., Jauckus, J., Langhans, C.-D., Schwarz, K., Zorn, M., Strowitzki, T., Okun, J. G., & Germeyer, A. (2022). Glucose and fatty acids catabolism during in vitro decidualization of human endometrial stromal cells. Journal of Assisted Reproduction and Genetics, 39(12), 2689–2697. https://doi.org/10.1007/s10815-022-02637-3

De Clercq, K., Hennes, A., & Vriens, J. (2017). Isolation of Mouse Endometrial Epithelial and Stromal Cells for In Vitro Decidualization. Journal of Visualized Experiments : JoVE, (121), 55168. https://doi.org/10.3791/55168

Emera, D., Romero, R., & Wagner, G. (2012). The evolution of menstruation: A new model for genetic assimilation. BioEssays, 34(1), 26–35. https://doi.org/10.1002/bies.201100099

Finn, C. A. (1987). Why Do Women and Some Other Primates Menstruate? Perspectives in Biology and Medicine, 30(4), 566–574.

FINN, C. A., & MARTIN, L. (1974). THE CONTROL OF IMPLANTATION. Journal of Reproduction and Fertility, 39(1), 195–206. https://doi.org/10.1530/jrf.0.0390195

Franco, H. L., Dai, D., Lee, K. Y., Rubel, C. A., Roop, D., Boerboom, D., Jeong, J.-W., Lydon, J. P., Bagchi, I. C., Bagchi, M. K., & DeMayo, F. J. (2011). WNT4 is a key regulator of normal postnatal uterine development and progesterone signaling during embryo implantation and decidualization in the mouse. The FASEB Journal, 25(4), 1176–1187. https://doi.org/10.1096/fj.10-175349

Gellersen, B., & Brosens, J. J. (2014). Cyclic Decidualization of the Human Endometrium in Reproductive Health and Failure. Endocrine Reviews, 35(6), 851–905. https://doi.org/10.1210/er.2014-1045

Glish, G. L., & Vachet, R. W. (2003). The basics of mass spectrometry in the twenty-first century. Nature Reviews Drug Discovery, 2(2), 140–150. https://doi.org/10.1038/nrd1011

Jia, Z., Wei, Y., Zhang, Y., Song, K., & Yuan, J. (2024). Metabolic reprogramming and heterogeneity during the decidualization process of endometrial stromal cells. Cell Communication and Signaling, 22(1), 385. https://doi.org/10.1186/s12964-024-01763-y

Kaplon, J., Zheng, L., Meissl, K., Chaneton, B., Selivanov, V. A., Mackay, G., van der Burg, S. H., Verdegaal, E. M. E., Cascante, M., Shlomi, T., Gottlieb, E., & Peeper, D. S. (2013). A key role for mitochondrial gatekeeper pyruvate dehydrogenase in oncogene-induced senescence. Nature, 498(7452), 109–112. https://doi.org/10.1038/nature12154

Kolb, B. (2012). Gaschromatographie in Bildern: Eine Einführung. John Wiley & Sons.

Liu, J.-L., Zhang, W.-Q., Zhao, M., & Huang, M.-Y. (2017). Integration of Transcriptomic and Metabolomic Data Reveals Enhanced Steroid Hormone Biosynthesis in Mouse Uterus During Decidualization. PROTEOMICS, 17(19), 1700059. https://doi.org/10.1002/pmic.201700059

Liu, T., Shi, F., Ying, Y., Chen, Q., Tang, Z., & Lin, H. (2020). Mouse model of menstruation: An indispensable tool to investigate the mechanisms of menstruation and gynaecological diseases (Review). Molecular Medicine Reports, 22(6), 4463–4474. https://doi.org/10.3892/mmr.2020.11567

Löffler, G. (2016). Löffler Stoffwechsel [Poster]. Heidelberg: Springer Medizin Verlag.

Lüllmann-Rauch, R., & Asan, E. (2019). Taschenlehrbuch Histologie (6th ed., pp. 607–614). Thieme.

Muter, J., Lynch, V. J., McCoy, R. C., & Brosens, J. J. (2023). Human embryo implantation. Development, 150(10), dev201507. https://doi.org/10.1242/dev.201507

Pavlicev, M., DiFrisco, J., Love, A. C., & Wagner, G. P. (2024). Metabolic complementation between cells drives the evolution of tissues and organs. Biology Letters, 20(11), 20240490. https://doi.org/10.1098/rsbl.2024.0490

Pavličev, M., McDonough-Goldstein, C. E., Zupan, A. M., Muglia, L., Hu, Y.-C., Kong, F., Monangi, N., Dagdas, G., Zupančič, N., Maziarz, J., Sinner, D., Zhang, G., Wagner, G., & Muglia, L. (2024). A common allele increases endometrial Wnt4 expression, with antagonistic implications for pregnancy, reproductive cancers, and endometriosis. Nature Communications, 15(1), 1152. https://doi.org/10.1038/s41467-024-45338-4

Pérez-Chávez, I., Koberstein, J. N., Pueyo, J. M., Gilglioni, E. H., Vertommen, D., Baeyens, N., Ezeriņa, D., Gurzov, E. N., & Messens, J. (2024). Tracking fructose 1,6-bisphosphate dynamics in liver cancer cells using a fluorescent biosensor. iScience, 27(12). https://doi.org/10.1016/j.isci.2024.111336

Potter, M., Newport, E., & Morten, K. J. (2016). The Warburg effect: 80 years on. Biochemical Society Transactions, 44(5), 1499–1505. https://doi.org/10.1042/BST20160094

Spencer, T. E., Hayashi, K., Hu, J., & Carpenter, K. D. (2005). Comparative Developmental Biology of the Mammalian Uterus. In Current Topics in Developmental Biology (Vol. 68, pp. 85–122). Academic Press. https://doi.org/10.1016/S0070-2153(05)68004-0

Stadtmauer, D. J., Basanta, S., Maziarz, J. D., Cole, A. G., Dagdas, G., Smith, G. R., van Breukelen, F., Pavličev, M., & Wagner, G. P. (2025). Cell type and cell signalling

innovations underlying mammalian pregnancy. Nature Ecology & Evolution, 9(8), 1469–1486. https://doi.org/10.1038/s41559-025-02748-x

Stadtmauer, D. J., & Wagner, G. P. (2022). Single-cell analysis of prostaglandin E2-induced human decidual cell in vitro differentiation: A minimal ancestral deciduogenic signal. Biology of Reproduction, 106(1), 155–172. https://doi.org/10.1093/biolre/ioab183

Sun, X., & Weckwerth, W. (2012). COVAIN: A toolbox for uni- and multivariate statistics, time-series and correlation network analysis and inverse estimation of the differential Jacobian from metabolomics covariance data. Metabolomics, 8(1), 81–93. https://doi.org/10.1007/s11306-012-0399-3

Tang, L., Xu, X.-H., Xu, S., Liu, Z., He, Q., Li, W., Sun, J., Shuai, W., Mao, J., Zhao, J.-Y., & Jin, L. (2023). Dysregulated Gln-Glu-α-ketoglutarate axis impairs maternal decidualization and increases the risk of recurrent spontaneous miscarriage. Cell Reports Medicine, 4(5). https://doi.org/10.1016/j.xcrm.2023.101026

Teklenburg, G., Salker, M., Molokhia, M., Lavery, S., Trew, G., Aojanepong, T., Mardon, H. J., Lokugamage, A. U., Rai, R., Landles, C., Roelen, B. A. J., Quenby, S., Kuijk, E. W., Kavelaars, A., Heijnen, C. J., Regan, L., Brosens, J. J., & Macklon, N. S. (2010). Natural Selection of Human Embryos: Decidualizing Endometrial Stromal Cells Serve as Sensors of Embryo Quality upon Implantation. PLOS ONE, 5(4), e10258. https://doi.org/10.1371/journal.pone.0010258

Vayakkattil, A. B., Pazhanchery, A. S., Andrews, S., & Vayakkattil, U. (2025). The Warburg Effect Redefined: A Kinetic and Regulatory Perspective. Cureus, 17(9). https://doi.org/10.7759/cureus.93331

Villas-Bôas, S. G., Mas, S., Åkesson, M., Smedsgaard, J., & Nielsen, J. (2005). Mass spectrometry in metabolome analysis. Mass Spectrometry Reviews, 24(5), 613–646. https://doi.org/10.1002/mas.20032

Wagner, G. P., Kin, K., Muglia, L., & Pavličev, M. (2014). Evolution of mammalian pregnancy and the origin of the decidual stromal cell. International Journal of Developmental Biology, 58(2–4), 117–126. https://doi.org/10.1387/ijdb.130335gw

WALLACE, H. M., FRASER, A. V., & HUGHES, A. (2003). A perspective of polyamine metabolism. Biochemical Journal, 376(1), 1–14. https://doi.org/10.1042/bj20031327

Wang, H., & Dey, S. K. (2006). Roadmap to embryo implantation: Clues from mouse models. Nature Reviews Genetics, 7(3), 185–199. https://doi.org/10.1038/nrg1808

Xu, W.-D., Wang, D.-C., Zhao, M., & Huang, A.-F. (2024). An updated advancement of bifunctional IL-27 in inflammatory autoimmune diseases. Frontiers in Immunology, 15. https://doi.org/10.3389/fimmu.2024.1366377

Xu, X. B., He, B., & Wang, J. D. (2007). Menstrual-like changes in mice are provoked through the pharmacologic withdrawal of progesterone using mifepristone following induction of decidualization. Human Reproduction, 22(12), 3184–3191. https://doi.org/10.1093/humrep/dem312

Zhao, Y.-C., Chi, Y.-J., Yu, Y.-S., Liu, J.-L., Su, R.-W., Ma, X.-H., Shan, C.-H., & Yang, Z.-M. (2008). Polyamines Are Essential in Embryo Implantation: Expression and Function of Polyamine-Related Genes in Mouse Uterus during Peri-Implantation Period. Endocrinology, 149(5), 2325–2332. https://doi.org/10.1210/en.2007-1420

Zhou, W.-J., Yang, H.-L., Mei, J., Chang, K.-K., Lu, H., Lai, Z.-Z., Shi, J.-W., Wang, X.-H., Wu, K., Zhang, T., Wang, J., Sun, J.-S., Ye, J.-F., Li, D.-J., Zhao, J.-Y., Jin, L.-P., & Li, M.-Q. (2022). Fructose-1,6-bisphosphate prevents pregnancy loss by inducing decidual COX-2+ macrophage differentiation. Science Advances, 8(8), eabj2488. https://doi.org/10.1126/sciadv.abj2488

Zuo, R.-J., Gu, X.-W., Qi, Q.-R., Wang, T.-S., Zhao, X.-Y., Liu, J.-L., & Yang, Z.-M. (2015). Warburg-like Glycolysis and Lactate Shuttle in Mouse Decidua during Early Pregnancy *. Journal of Biological Chemistry, 290(35), 21280–21291. https://doi.org/10.1074/jbc.M115.656629

Zygmunt, M., Herr, F., Münstedt, K., Lang, U., & Liang, O. D. (2003). Angiogenesis and vasculogenesis in pregnancy. European Journal of Obstetrics & Gynecology and Reproductive Biology, The Regulation of Fetal Growth, Fetal Oxygen Supply and Intrauterine Development, 110, S10–S18. https://doi.org/10.1016/S0301-2115(03)00168-4