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MERUERT SARSENOVA

Molecular and cellular landscape of endometriosis

Karolinska Institutet

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Institute of Clinical Medicine, University of Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

This PhD thesis is based on the following original research publications:

I. **Sarsenova, M**.*, Lawarde, A.*, Pathare, A. D. S., Saare, M., Modhukur, V., Soplepmann, P., Terasmaa, A., Käämbre, T., Gemzell-Danielsson, K., Lalitkumar, P. G. L., Salumets, A., & Peters, M. (2024). Endometriotic lesions exhibit distinct metabolic signature compared to paired eutopic endometrium at the single-cell level. Communications Biology, 7(1), 1026. doi:10.1038/s42003-024-06713-5.

II. **Sarsenova, M**., Boggavarapu NR., Kask K., Modhukur V., Samuel K., Karro H., Gemzell-Danielsson K., Lalitkumar PGL., Salumets A., Peters M., Lavogina D. (2024). Hypoxic conditions affect transcriptome of endometrial stromal cells in endometriosis and promote TGFBI axis. Frontiers in Endocrinology, 15. doi: 10.3389/fendo.2024.1465393.

III. **Sarsenova, M**., Stepanjuk, A., Saare, M., Kasvandik, S., Soplepmann, P., Mikeltadze, I., Götte, M., Salumets, A., & Peters, M. (2024). Carboxypeptidase Inhibitor LXN Expression in Endometrial Tissue Is Menstrual Cycle Phase-Dependent and Is Upregulated in Endometriotic Lesions. Genes, 15(8), 1086. doi:10.3390/genes15081086.

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* Joint first authorship

Author's personal contribution:

Study I: Participated in the design of study and in formulating and developing the study question; performed wet-lab experiments (tissue dissociation, library preparation, generated GEMs for 10x scRNA-sequencing); performed analysis and interpretation of bioinformatic results; visualized the graphical data; wrote the initial and final versions of the manuscript. Ankita Lawarde (PhD student in bioinformatics at UT) performed the bioinformatic analysis of raw scRNA-seq data using R packages.

Study II: Participated in the design of study and in formulating and developing the study hypothesis; performed wet-lab experiments (endometrial stromal cell isolation from tissues, endometrial stromal cell culturing, RNA-sequencing, qRT-PCR); performed analysis and interpretation of bioinformatic analysis results; analysis and interpretation of qRT-PCR, IHC data; visualized the graphical data; communicated results with supervisors and involved researchers to develop the study; wrote the initial and final versions of the manuscript.

Study III: Joined the ongoing project and performed wet-lab experiments (qRT-PCR of eutopic and ectopic endometrial tissue biopsies); performed analysis and interpretation of results; visualized the graphical data; communicated results with supervisors and involved researchers to develop the study; wrote the initial and final versions of manuscript.

ABBREVIATIONS

Acetyl-CoA	acetyl coenzyme A
Akt/PKB	protein kinase B
AMPK	AMP-activated protein kinase
ASRM	American Society for Reproductive Medicine
ATP	adenosine triphosphate
cDNA	complementary DNA
CK2	casein kinase 2
DEG	differentially expressed genes
DIE	deep infiltrating endometriosis
E2	estradiol
EcE	ectopic endometrium
EcESCs	ectopic endometrial stromal cells
ECM	extracellular matrix
ESR	gene encoding estrogen receptor
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-mesenchymal transition
ER (α, β)	estrogen receptor (α, β)
EuE	eutopic endometrium
EuESCs	eutopic endometrial stromal cells
FA	fatty acid
FC	fold change
FMT	fibroblast-to-myofibroblast transition
G1 phase	cell growth phase of cell cycle
G2/M phase	checkpoint and mitosis
HIF	hypoxia-inducible factor
IHC	immunohistochemistry
LXN	latexin
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
mRNA	messenger RNA
MTT assay	methylthiazolyldiphenyl-tetrazolium bromide cell viability assay
NE	normal endometrium (women without endometriosis or healthy controls)
NESCs	normal endometrial stromal cells from controls
OXPHOS	oxidative phosphorylation
P4	progesterone
PGR	gene encoding progesterone receptor
PK	protein kinase
PKAc	cAMP-dependent PK
PR	progesterone receptor
qRT-PCR	real-time quantitative reverse transcription PCR

ROCK	Rho-dependent PK
ROS	reactive oxygen species
S phase	DNA synthesis phase of cell cycle
scRNA	single-cell RNA
siLXN	small interfering RNA targeting LXN
TCA	tricarboxylic acid cycle
TF	transcription factor
TGFBI	transforming growth factor beta induced
TGF-β	transforming growth factor beta

1. INTRODUCTION

Endometriosis is a prevalent benign gynecological disease in women of reproductive age. It is estimated to affect globally around 190 million people, or approximately every tenth woman. Symptomatically endometriosis usually manifests with chronic pelvic pain, impaired fertility and reduced quality of life. These non-specific symptoms or asymptomatic course of disease make it difficult for women to suspect the presence of disease. Thereby, the diagnosis is often delayed on average for seven to nine years. In many cases, endometriosis is identified during medical examination and surgical intervention due to other pathology, or investigation of possible reasons for infertility. However, even after confirmation of endometriosis diagnosis women cannot be fully cured and their treatment is limited to the management of symptoms or surgical removal of endometriotic lesions (ectopic endometrium, EcE).

Endometriotic lesions are presented as extrauterine ectopic growth of endometrial-like tissues. They are mostly found in the peritoneum but can also form ovarian endometrioid cysts or spread locally or distally to other organs and tissues, affecting their function. The origin of this multifaceted disease remains unclear, and the theory of retrograde menstruation is the most widely accepted. According to this theory, fragments of shed endometrial tissues spread outside the uterus and implant on surrounding tissues and organs, adapting to the unfavorable microenvironment. Endometriotic lesions exhibit a molecular signature distinct from eutopic endometrium (EuE), which allows their survival and growth in ectopic sites. Research on the pathogenesis of endometriosis unraveled a key role of hormone imbalance and hypoxia on the regulation of processes, like cell proliferation, migration, metabolism and angiogenesis.

With the development of advanced powerful techniques, such as high-throughput transcriptomics, proteomics and metabolomics, we can perform a comprehensive analysis of tissues. Moreover, the identified molecular signatures can be linked to the single cell populations. By utilizing these techniques to study endometriosis pathogenesis, we can explore RNA, protein and metabolite profiles of single cell populations within endometriotic lesions. This knowledge will facilitate further understanding of disease pathobiology with potential applications in diagnostics and treatment of endometriosis.

2. REVIEW OF THE LITERATURE

2.1 Endometriosis

2.1.1 Eutopic endometrium: hormone regulation and menstrual cycle

Human endometrium is a complex tissue with the main function of facilitating embryo implantation and maintaining pregnancy. Endometrium comprises two parts: stratum basalis and stratum functionalis, containing stromal, epithelial and vascular compartments. Stroma is largely composed of stromal fibroblasts, epithelial, perivascular and endothelial cells. During reproductive period, morphological changes of healthy endometrium happen throughout the menstrual cycle (Figure 1) and are finely controlled by steroid hormones: estradiol (E2) primed in the proliferative phase and progesterone (P4) dominating secretory phase (Critchley et al., 2020). These hormones are mostly produced by ovaries in response to follicle-stimulating hormone and luteinizing hormone secretion, following the progression of ovarian cycle. During proliferative phase, E2 binds to estrogen receptors α and/or β (ERα or ERβ) acting as transcription factors (TFs) in nuclei of endometrial stromal and epithelial cells, inducing expression of genes related to endometrial growth. P4 binds to its receptor (PR) expressed in nuclei throughout the menstrual cycle, and inhibits proliferation while inducing cell differentiation, a process known as decidualization. During decidualization, stromal fibroblasts undergo mesenchymal-to-epithelial transition and become rounded, ready for implantation of embryo. In the absence of pregnancy, P4 withdrawal in the late secretory phase leads to initiation of inflammation, influx of immune cells and tissue breakdown. During this phase, cells comprising the functional layer of endometrium desquamate and shed (menstruation), followed by tissue repair, where stratum basalis containing stem cell niche serves as the source for stratum functionalis. The described processes may be, however, perturbed in different pathologies.

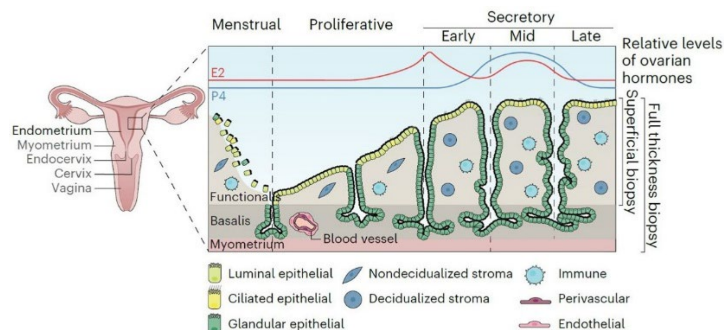

Figure 1. Schematic illustration of the human uterus and the cellular composition of the endometrium as it undergoes morphological changes across the menstrual cycle. Reproduced with permission from Marečková et al., 2024, Nature Genetics, licensed under the Creative Commons Attribution 4.0 International License.

2.1.2 Endometriosis prevalence and characteristics

Endometriosis is a common hormone-dependent and chronic inflammatory disorder, impacting about 190 million women worldwide and affecting 10% of women of reproductive age (Saunders & Horne, 2021; Zondervan et al., 2020). It clinically manifests as chronic pelvic pain, dysmenorrhea, and subfertility, affecting the quality of life, but may also be asymptomatic. Endometriosis is characterized by the presence of endometrial-like cells growing outside the uterus, known as endometriotic lesions, with approximately 80% located in the peritoneum (Figure 2, lower part) (Saunders & Horne, 2021). EcE affects the structure and function of organs and tissues. For example, in ovaries EcE forms an endometrioid cyst with accumulated blood (called endometrioma), while in the peritoneum lesion growth is accompanied by inflammation and formation of adhesions. Adhesions affect spatial localization and function of involved tissues and organs (e.g., intestine, bladder) and contribute to pain and infertility. Endometriotic lesions may also exhibit invasive growth, penetrating organs or tissues, called deep infiltrating endometriosis (DIE). In rare cases, lesions are found in organs distant to uterus, like lungs, causing accumulation of blood in the pleural cavity. The widely used system of endometriosis classification by American Society for Reproductive Medicine (ASRM) evaluates lesion phenotype and location, presence and extent of adhesions and involvement of organs and tissues, categorizing endometriosis into four stages, from minimal to severe ("Revised American Society for Reproductive Medicine Classification of Endometriosis," 1997). However, the stages do not correlate with the severity of endometriosis and its prognosis.

2.1.3 Epidemiology and risk factors

Several factors have been associated with endometriosis onset and development (Figure 2, upper part). For example, high levels of circulating E2 in women with early menarche and nulliparity increase risk for endometriosis, while use of oral contraceptives and lactation have been associated with reduced risk (Parasar et al., 2017). Some genetic factors are reported to also contribute to endometriosis, such as family heritability. Moreover, 42 single nucleotide polymorphism were found in association with steroid hormone signaling in endometriosis (Rahmioglu et al., 2023). Epidemiological studies have shown that women with endometriosis have a higher risk for developing certain types of cancer (ovarian cancer and melanoma), cardiovascular and autoimmune diseases (Kvaskoff et al., 2015; Lundberg et al., 2019; Poole et al., 2017). In addition, some environmental factors have been implicated in endometriosis development (Peinado et al., 2020; Simonelli et al., 2017; Wieczorek et al., 2022; Williams et al., 2015). Endocrine disrupting chemicals, commonly found in the environment (e.g. plastics, personal care products), exert estrogenic activity and interfere with hormonal receptors altering steroid signaling pathways. These chemicals have a similar structure to estrogen and they can directly bind to ERs or interact with aryl hydrocarbon receptors, that further bind to estrogen response elements in the nucleus (Shanle

& Xu, 2011). Some other factors, like microbes, have also been associated with inflammatory aspect of endometriosis development (Guo & Zhang, 2024; Muraoka et al., 2023).

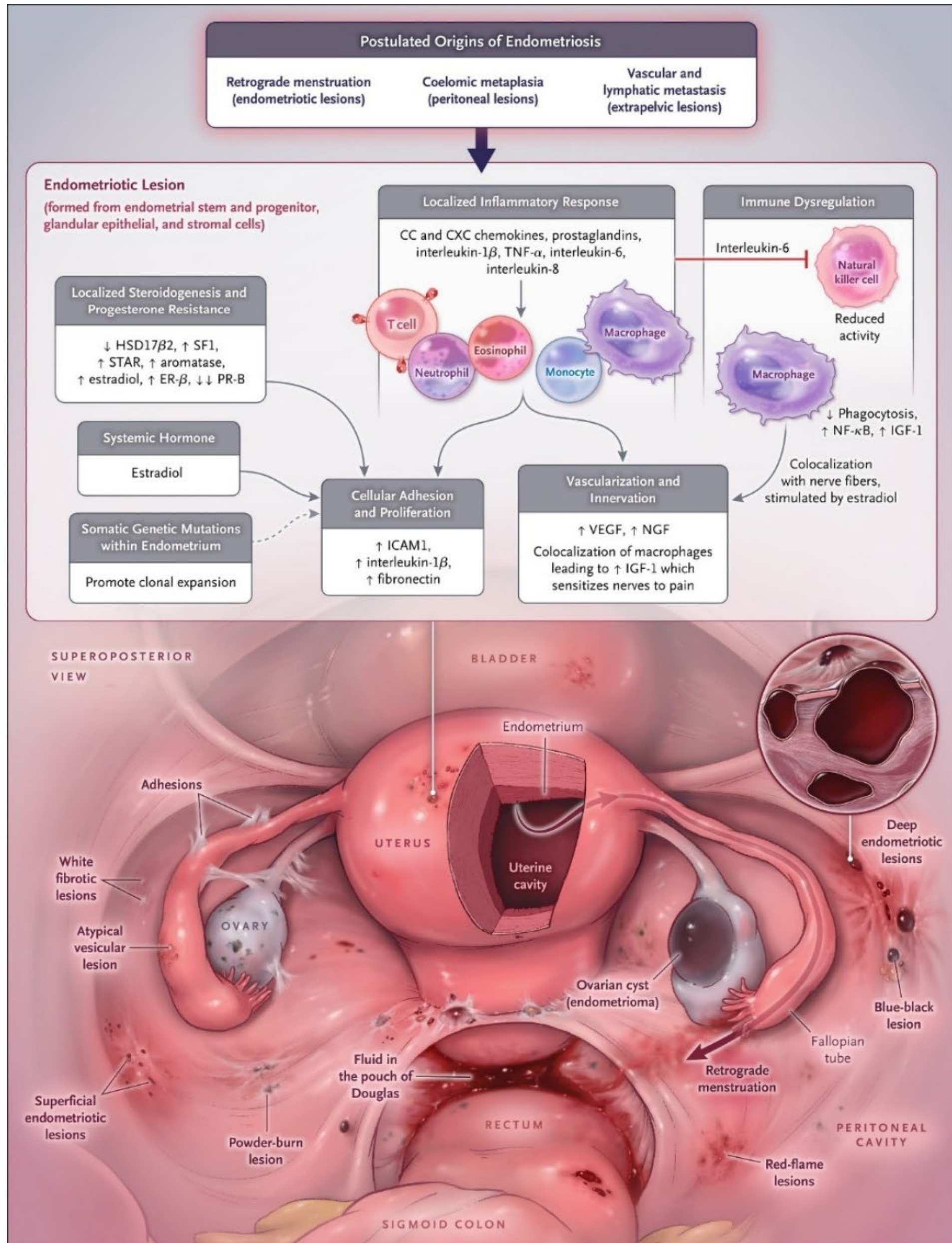

Figure 2. Potential Pathways for the Pathogenesis and Pathophysiological Features of Pelvic Endometriosis. The pathogenesis and pathophysiological features of pelvic endometriosis are complex. Potential origins of the endometriotic lesions include transplan-

tation of endometrial tissue through retrograde menstruation and in situ coelomic metaplasia of the peritoneal lining. Vascular or lymphatic metastasis most likely occurs only rarely, in cases of extrapelvic lesions. Superficial and deep endometriotic lesions are established and maintained through interacting molecular mechanisms that promote cellular adhesion and proliferation, systemic and localized steroidogenesis, localized inflammatory response and immune dysregulation, and vascularization and innervation. The dashed arrow indicates a postulated effect. ER denotes estrogen receptor, HSD17β2 17β-hydroxysteroid dehydrogenase 2, ICAM intercellular adhesion molecule, IGF insulin-like growth factor, NF-κB nuclear factor κB, NGF nerve growth factor, PR progesterone receptor, SF1 steroidogenic factor, STAR steroidogenic acute regulatory protein, TNF tumor necrosis factor, and VEGF vascular endothelial growth factor. Reproduced with permission from Zondervan et al. 2020, Copyright Massachusetts Medical Society, licensed under the Creative Commons Attribution 4.0 International License.

2.1.4 Endometriotic lesions and their origin

Endometriotic lesions are divided into three subtypes: peritoneal (superficial), ovarian (endometrioma), and DIE. Histological analysis of endometriotic lesions shows that they consist of stroma, glands, blood vessels and large areas of fibrotic tissues (Figure 3). Recent single-cell RNA (scRNA) studies revealed that cellular composition of lesions overlaps with the cell types of EuE (Tan et al., 2022). Major cell types include stromal, epithelial, immune and vascular cells. Stem cell niche, present in both stratum basalis and functionalis of EuE, was not identified in EcE. This could be due to technical limitations related to cell loss during tissue dissociation, or their role may be compensated by other cell types as, for example, perivascular cells, known to restore endometrial stroma (Cousins et al., 2022). Another difference is related to the response of lesions to ovarian hormones. Recent findings showed that the menstrual cycles of endometrioma and peritoneal lesions are desynchronized between paired EuE and EcE (Colgrave et al., 2020; Saare et al., 2022). This raises questions of where ectopic cells originate from, how similar they are to eutopic cells, and how microenvironment affects establishment and development of lesions.

There are several theories for endometriosis origin, including retrograde menstruation, coelomic metaplasia, genetic and epigenetic factors, and stem cell theory, Figure 2 (Koninckx et al., 2019; Maruyama, 2022; Sampson, 1927). According to the retrograde menstruation theory, fragments of endometrial tissue fragments, shed during menstruation, implant in the ovaries and/or peritoneum and form lesions. As retrograde flow was later observed in women without endometriosis, the involvement of other processes and factors was proposed (Halme et al., 1984). Among these factors are hormonal imbalance, impact of microenvironment, altered metabolism and immune dysfunction (Bulun et al., 2010; Niu et al., 2023; Rizner, 2009). A model of “seed and soil” suggests that endometrial debris (seed) can implant only on tissues with altered properties, like peritoneum (soil) (Horne & Saunders, 2019). Although there are experimental data on endometriosis model organisms showing potential mechanisms of endometriosis etiopathogenesis, it is still debated how lesions originate in

humans. Each theory/hypothesis may partially answer this question, given various types and locations of endometriotic lesions.

2.2 Pathogenetic hallmarks of endometriosis

The data from various studies on the expression of key molecules involved in processes important for the etiopathogenesis of endometriosis and relevant to our research, are summarized in this (Table 1). These processes are interconnected and altogether highlight the complex nature of endometriosis, explaining the lack of consensus on etiology and pathogenesis of this syndrome. One of the main features of endometriotic microenvironment is its hypoxic condition. Hypoxia effects are central in this review as its role has been widely studied, showing its stimulatory effect on processes involved in the development of endometriosis. There is a challenge in understanding the whole picture of endometriosis pathogenesis, as different endometriosis subtypes exhibit distinct expression patterns of certain molecules. For example, studies of hormonal dysregulation have reported variable expression patterns identified in peritoneal lesions, endometriomas and DIE (Heilier et al., 2006; Rizner, 2009). Moreover, expression analyses of EcE are typically performed in comparison with EuE from women with endometriosis or normal endometrium (NE, from women without endometriosis or healthy volunteers) at various phases or across the menstrual cycle, which further complicates the interpretation of results, as shown in Table 1.

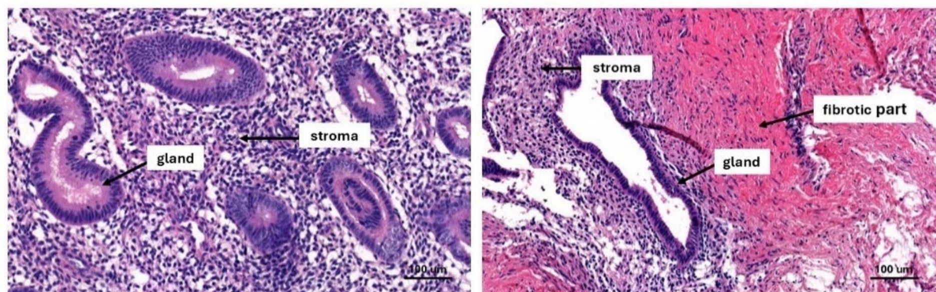

Figure 3. Representative images of haematoxylin and eosin staining of EuE (left) and EcE (right) from a woman with endometriosis, showing morphological differences between paired endometrial tissues. Scale bar 100 µm. Abbreviations: EcE – ectopic endometrium; EuE – eutopic endometrium. The photo was taken by the author.

2.2.1 Hormonal imbalance

E2 in endometrium induces mitotic activity, resulting in cell proliferation, and regulates cell survival and apoptosis, while P4 exerts anti-proliferative and anti-inflammatory activities (Critchley et al., 2020; Yang et al., 2018). Reports on the incidence of endometriosis in adolescents after menarche and in men with prostate cancer treated with high doses of estrogen indicate that E2 plays a role

in the development of endometriosis (Fukunaga, 2012; Hirsch et al., 2020). Moreover, regression of endometriotic lesions after treatment with aromatase inhibitors highlights endometriosis dependence on estrogen activity (Agarwal & Foster, 2015; Razzi et al., 2004). Endometriotic lesions have been shown to exhibit hormonal imbalance reflected by increased ERβ:ERα ratio and decreased expression of PR (Smuc et al., 2007; Xue et al., 2007). The downregulation of the progesterone receptor (*PGR*) results in reduced action of P4 (progesterone resistance) and enhanced activity of E2 via ERβ (Attia et al., 2000; Bulun et al., 2006; Smuc et al., 2007).

Increased levels of E2 induce ectopic cell proliferation via ERβ target genes. High level of estrogen in women with endometriosis is also linked to impaired metabolism and increased production of E2 by local tissues (Rizner, 2009). Aromatase pathway and 17β-HSDs play a key role in local E2 synthesis and metabolism in endometriotic lesions. Aromatase has been shown to be higher expressed at both messenger RNA (mRNA) and protein levels in both EcE and EuE of women with endometriosis compared to NE (Bukulmez et al., 2008; Noble et al., 1996).

E2 is inactivated by 17β-HSD type 2 (encoded by *HSD17B2*), which converts E2 to a less active estrogen form (estrone). The expression of *HSD17B2* has been found to be lower in EcE (Rizner, 2009). In contrast, 17β-HSD type 1 converts estrone to E2 and its overexpression in EcE has been reported in some studies (Rizner, 2009). Notably, the reported aberrant expression of aromatase and 17β-HSDs varies between different subtypes of endometriotic lesions (Heilier et al., 2006; Rizner, 2009). In parallel, P4 function is inhibited in endometriosis due to reduced expression of *PGR* and its inactivation by reductases, AKR1C1 and AKR1C2 (Bulun et al., 2006; Hevir et al., 2011; Rizner et al., 2006). Moreover, impaired P4 signaling leads to enhanced estrogen dominance, as there is no inhibitory activity of P4 on E2 (Yilmaz & Bulun, 2019).

Table 1. Review of selected publications with a focus on processes and representative key molecules involved in the development of endometriosis, discussed in the chapter.

Process(es)	Studied sample(s)	Expression of key molecules	Reference sample(s)	MC phase	Authors, year
Hormonal imbalance	EcE (endometrioma)	Up: ESR2, Down: ESR1	EuE	P, S	Šmuc et al., 2006
	EcE (endometrioma)	Up: ESR2, Down: ESR2	NE	NA	Xue et al., 2007
	EcE (peritoneal ES)	Down: PR	EuE	P, S	Attia et al., 2000
	EcE (endometrioma, peritoneal ES)	Up: CYP19A1/aromatase	EuE, NE	P, S	Bukulmez et al., 2007
Angiogenesis	PF	Up: VEGF & IL-6	PF (controls)	NA	Mahnke et al., 2000
	PF	Up: VEGF	PF (controls)	P, S	McLaren et al., 1996
	EcESCs (endometrioma, peritoneal ES)	Up: IL-8	EuESCs	NA	Hsiao et al., 2014
	EcESCs (endometrioma, peritoneal ES)	Up: HIF-1α, leptin	NESCs	NA	Wu et al., 2007
	EcE (endometrioma)	Up: angiogenin	EuE	P, S	Fu et al., 2018
	HPMC	Up: VEGF-A	HPMC (controls)	P, S	Young et al., 2015
	PF, adjacent peritoneum	Up: TGFB1/TGF-β1, ID1, ID3	PF (controls), peritoneum	S	Young et al., 2014b
Metabolism	EcESCs (peritoneal ES)	Up: PFKP, ENO2, LDHA, Down: DLD	EuESCs (ES and controls)	S	Kasvandik et al., 2016
	EcE (peritoneal ES), peritoneum, HPMC (TGF-β1-induced), PF	Up: PDK-1, SLC2A1/GLUT-1, LDHA, lactate	EuE, peritoneum, HPMC (control), PF (controls)	S	Young et al., 2014a
	EcESCs	Up: PDK1, lactate	EuESCs, NESCs	P, S	Lee et al., 2019

Process(es)	Studied sample(s)	Expression of key molecules	Reference sample(s)	MC phase	Authors, year
Adhesion, fibrosis	EcE (peritoneal ES), EuESCs (exposed to hypoxia and/or TGF-β1)	Up: Integrin α5, integrin αV, integrin β3, and integrin β5	EuE, NE, EuESCs (control)	P	Lin et al., 2018
	ESCL, 11Z and HESCs (co-cultured with activated platelets)	Up: E-cadherin, α-SMA, and F-actin, LOX and CCN2, desmin, SM-MHC	ESCLs, 11Z and HESCs (controls)	NA	Zhang et al., 2016
Inflammation	Endometrial epithelial cell line	Up: IL-1β, TNFα, lipopolysaccharides	controls	NA	Hashimoto et al., 2023
	EcE (endometrioma)	Up: COX-2, PGE2	EuE	P, S	Ota et al., 2001
	EcE	Up: EP3, EP4, FP, PGT	EuE	P, S	Rakhila et al., 2015

Abbreviations: 11Z – endometrial ectopic epithelial cell line; Down – downregulation of gene expression; EcE – ectopic endometrium; EcESCs – ectopic endometrial stromal cells; ES – endometriosis; ESCL – normal endometrial stromal cell line; ESR – gene encoding estrogen receptor; EuE – eutopic endometrium; EuESCs – eutopic endometrial stromal cells; HESC – human endometrial stromal cells; HPMC – human peritoneal mesothelial cells; MC – menstrual cycle; NA – not applicable; NE – normal endometrium; P – proliferative phase; PF – peritoneal fluid; S – secretory phase; Up – upregulation of gene expression.

2.2.2 Hypoxia – master regulator in endometriosis

Hypoxia occurs in cells with reduced oxygen supply and is known to modulate gene expression in physiological and pathological contexts via hypoxia inducible factor (HIF). Hypoxia regulates cell metabolism, growth, apoptosis, cell adhesion and angiogenesis. In EuE, HIF-1α was found to be expressed throughout the menstrual cycle, peaking in the late secretory phase and menstruation, suggesting its role in initiation of the menstruation and further endometrial restoration, demonstrated in mice (Critchley et al., 2006; Maybin et al., 2018). Moreover, the overexpression of HIF-1α in endometriotic lesions indicates its involvement in the development of endometriosis (Wu et al., 2007). And hypoxic microenvironment is suggested to play a key role in endometriosis. Unfavorable conditions without sufficient blood supply trigger the activation of hypoxia-induced processes to sustain ectopic growth and survival of lesions (Hsiao et al., 2015; Wu et al., 2019). Ectopic endometrial stromal cells (EcESCs) show high expression of markers related to hypoxia, cell adhesion and invasion compared to eutopic cells (EuESCs), that may be critical during the establishment of endometriotic lesions (Kasvandik et al., 2016). Additionally, EcESCs had altered energy metabolism with activation of glycolytic over oxidative metabolic pathways (Kasvandik et al., 2016; Young et al., 2014a). Aberrant expression of HIF-1α, PDK1, LDHA as well as increased glucose uptake, oxygen consumption, elevated lactate and adenosine triphosphate (ATP) levels, and in EcE demonstrate hypoxia-mediated metabolic reprogramming in endometriosis (Lee et al., 2019; Zheng et al., 2021). Hypoxia-mediated hormonal regulation in endometriosis is evident by elevated mRNA and protein expression of ERβ in EcESCs exposed to hypoxic conditions (1% O_2) (Wu et al., 2012). Moreover, ERβ expression was reduced after silencing *HIF1A* in these cells, while ERα expression was restored.

Hypoxia mediates the activation and distribution of protein kinases (PKs) as shown *in vitro* (Goldberg et al., 1997; Lucia et al., 2020). PKs are involved in different cellular processes in healthy tissues and in disease development and progression. cAMP-dependent PK (PKAc), Rho-dependent PK (ROCK), protein kinase B (Akt/PKB), and casein kinase 2 (CK2) are known to influence cell growth, survival, apoptosis, migration and metabolism in physiological and pathological conditions (Kazi et al., 2009; Kim et al., 2021; Lavogina et al., 2021; Zhang et al., 2020a). Altered activity of these kinases was found to support epithelial-mesenchymal transition (EMT), proliferative activity of EuESCs and thereby contributing to endometriosis development (Aghajanova et al., 2010; Huang et al., 2020; Matsuzaki & Darcha, 2015; McKinnon et al., 2016).

2.2.3 Hypoxia and cell metabolism

Cellular metabolism is shaped by activated regulatory pathways (AMP-activated protein kinase, AMPK, signaling and HIF-1 signaling) that orchestrate the network of metabolic pathways to sustain cell proliferation and growth (Chandel, 2015). Metabolic plasticity is defined by metabolic adjustments of cells to

changing environment. In response to hypoxic condition, cells modulate their metabolism according to cellular demands of energy. As oxygen supply is limited in hypoxic environments, oxidative phosphorylation (OXPHOS) becomes inefficient, therefore cells switch to anaerobic glycolysis. The shift between metabolic pathways is evolutionarily conserved, as it allows cells to maintain energy production within low oxygenation conditions. However, in some physiological or pathological settings (rapidly growing healthy or malignant tissues with enhanced cell proliferation), cells switch to glycolysis even in the presence of oxygen supply. This metabolic switch was first described in cancer, known as the Warburg effect or aerobic glycolysis (Vaupel et al., 2019). The advantage of aerobic glycolysis is to produce energy and macromolecules at a higher rate compared to oxidative metabolism.

Recent studies on endometriosis revealed a Warburg-like effect in endometriotic cells, suggesting it as a survival coping mechanism in lesions in response to unfavorable ectopic environment and high energy needs. Kasvandik et al. showed that EcESCs overexpressed glycolysis markers (PFKP, ENO2, LDHA) compared to EuESCs from women with endometriosis and normal endometrial stromal cells from controls (NESCs) from women without endometriosis (Kasvandik et al., 2016). Moreover, they found reduced expression of oxidative metabolic markers at both mRNA and protein levels. In concordance with these findings, a study on non-human primates also revealed reduced oxidative metabolism indicating metabolic reprogramming in endometriosis; however lactate levels did not differ between EuE and EcE (Atkins et al., 2019). Another study has identified an increased oxygen consumption in EcESCs compared to EuESCs and NESCs, while EuESCs and NESCs exhibited similar oxygen consumption levels (Lee et al., 2019). Glucose uptake was similar in EcESCs and EuESCs, while lactate production was higher in EcESCs. PDK1 was overexpressed in EcESCs in a hypoxia-dependent manner. PDK1 inhibits PDH, an enzyme converting pyruvate to acetyl coenzyme A (acetyl-CoA) that is further used in oxidative metabolism, and thus pyruvate is utilized in glycolysis with formation of lactate. The inhibition of PDK1 in EcESCs resulted in a reduced production of lactate. Moreover, EcESCs, treated with inhibitor of PDK1 under hypoxia and oxidative stress, exhibited cell death, which may indicate the anti-apoptotic protective role of PDK1 (Lee et al., 2019). A study by Young et al. identified high levels of *PDK-1*, *SLC2A1* (encodes GLUT-1) and *LDHA* in EcE and elevated lactate in peritoneal fluid from women with endometriosis (Young et al., 2014a). The expression of HIF-1α, PDK-1, LDHA and GLUT-1 has been observed in both stromal and epithelial cells of endometrium. Furthermore, the authors found an overexpression of aerobic glycolysis regulators (HIF1A, PDK-1, LDHA, and GLUT-1) in adjacent peritoneal tissues of endometriotic lesions (Young et al., 2014a). Peritoneal mesothelial cells treated with transforming growth factor beta-1 (TGF-β1) exhibited an elevated production of lactate compared to controls. Similarly, *HIF1A* and its downstream targets *LDHA* and *SLC2A1* were found to be elevated after TGF-β1 induction, indicating TGF-β signaling involvement in endometriosis (Young et al., 2014a).

AMPK signaling is known to sense energy stress in cells, reflected as a high AMP:ATP ratio, and activate catabolic metabolism, while inhibiting biosynthetic processes (Dengler, 2020; Herzig & Shaw, 2018). AMPK has been shown to be activated via LKB1 in cells with increased AMP/ATP ratios after exposure to hypoxic conditions (Tan et al., 2012). Interestingly, inhibition of AMPK signaling through inactivated AMPK induced the Warburg effect in cancer cells, highlighting the regulatory role of AMPK in maintaining metabolic homeostasis (Faubert et al., 2013). Mitochondrial production and accumulation of reactive oxygen species (ROS) induces oxidative modification of AMPK, leading to its activation (Rabinovitch et al., 2017; Zmijewski et al., 2010). Subsequently, AMPK activates the antioxidant system via PGC1-α and/or mitophagy via ULK1, thereby regulating ROS formation and controlling oxidative stress (Zmijewski et al., 2010). Altered AMPK signaling and its involvement in inflammatory processes, apoptosis and metabolic reprogramming have been shown in endometriosis (Assaf et al., 2022). Increased ROS (O_2^-, H_2O_2) were identified in stromal and epithelial cells from EcE (Ngô et al., 2009).

2.2.4 Hypoxia and angiogenesis

A stimulating effect of hypoxia on angiogenesis has been found in different tissues. HIF-1α modulates the expression of cytokines and angiogenic factors that regulate vessel growth from pre-existing blood vessels and induce vascular cell migration (Chung & Han, 2022). Vessel sprouting starts with the induction of the release of matrix metalloproteinase by endothelial cells. Matrix metalloproteinases (MMPs), upregulated and activated under hypoxic conditions, degrade the extracellular matrix (ECM), followed by detachment of perivascular cells, migration of endothelial cells and tube formation (Ben-Yosef et al., 2005; Laschke & Menger, 2018). In tumors, hypoxia-induced angiopoietins recruit perivascular cells to the growing vessels (Krock et al., 2011). Peritoneal macrophages were shown to produce the pro-inflammatory cytokine IL-1β, which stimulates migration and invasion of endothelial cells and induces *VEGF* and *IL-6* expression in IL-1β-treated EcESCs (Lebovic et al., 2000). In endometriosis, hypoxia promotes angiogenesis via IL-6 (shown in a mice model), IL-8, leptin, angiogenin, VEGFs and angiopoietins (Chung & Han, 2022; Guo et al., 2021; Hsiao et al., 2014; Wu et al., 2007). Angiogenin, overexpressed in EcE under hypoxic conditions via downregulation of *COUP-TFII*, has been shown to be exclusively expressed in endothelial cells of EcE compared to EuE (Fu et al., 2018). Elevated levels of VEGF and IL-6 were found in peritoneal fluid from women with endometriosis compared to women without endometriosis (Mahnke et al., 2000; McLaren et al., 1996). VEGF levels are positively regulated by TGF-β signaling, activated under hypoxia (Vissers et al., 2024). Overexpression of *VEGF*, induced by culturing with TGF-β1, was found in peritoneal mesothelial cells, indicating the involvement of the peritoneum in vessel branching in endometriosis (Young et al., 2015). Angiogenic targets of TGF-β signaling, *ID1* and *ID3*, were found to be increased

in adjacent peritoneum of women with endometriosis compared to peritoneum from women without endometriosis (Young et al., 2014b).

2.2.5 Hypoxia, adhesions and fibrosis

The formation of adhesions is typical of endometriosis, affecting the function of organs and tissues that are surrounding or in contact with endometriotic lesions, and often manifesting in various clinical symptoms, including pain (Abd El-Kader et al., 2019; Garcia Garcia et al., 2023). Myofibroblasts primarily originate from fibroblasts, perivascular cells and smooth muscle cells. Activated myofibroblasts produce ECM and contract to rescue tissue integrity until wound resolution and remodeling of damaged normal tissues (Schuster et al., 2023). TGF-β and Rho/ROCK signaling play an important role in fibroblast-to-myofibroblast transition (FMT) and contractile function of myofibroblasts (Sandbo et al., 2009; Tomasek et al., 2006). In pathology, myofibroblasts remain active even after the tissue integrity is restored. The investigation of the role of hypoxia in adhesion revealed changes at protein level, such as the overexpression of HIF-1α and integrins in EcE compared to EuE from women with endometriosis (Lin et al., 2018). EuESCs exposed to hypoxic conditions for 48 hours exhibited higher mRNA expression of HIF-1α and integrins, adhesion and migration abilities. Moreover, EuESCs exposed to hypoxia and co-stimulated with TGF-β1 showed increased integrin-mediated adhesive functions via Smad2 signaling. Treatment of EuESCs with a TGF-β1 inhibitor led to a reduction in migration and adhesion (Lin et al., 2018).

The involvement of TGF-β/Smad signaling in fibrosis was previously studied in various tissues, including intrauterine adhesions (Zhang et al., 2020b). Integrins represent a class of cell adhesion molecules implicated in tissue fibrotic processes (Hu et al., 2023b). They have been shown to activate TGF-β1 in pulmonary fibrosis (Munger et al., 1999). A platelet-associated mechanism of fibrogenesis has been investigated in a baboon model of endometriosis (Zhang et al., 2016b). The study showed that platelets in endometriotic lesions induced the activation of TGF-β/Smad signaling, followed by subsequent stages of EMT, FMT, smooth muscle metaplasia and fibrosis. Further, they demonstrated the involvement of TGF-β1/Smad3 signaling in platelet-driven pro-fibrotic processes in endometriotic cells (Zhang et al., 2016a). The authors identified the activation of migratory and invasive abilities of endometriotic cells, followed by EMT with overexpression of E-cadherin, α-SMA, and F-actin, and FMT with overexpression of *LOX* and *CCN2*. Altogether, these experiments support a probable mechanism of adhesion and fibrogenesis in endometriosis, highlighting the role of TGF-β and Rho/ROCK signaling.

2.2.6 Hypoxia and inflammation

Inflammation is another hallmark of endometriosis that implicates altered hormone production, growth, adhesion and migration of cells. Inflammatory cytokines and mediators, such as IL-1β, TNFα and lipopolysaccharides were found to activate HIF-1α even in the presence of oxygen in endometrial and lung epithelial cells, with the involvement of ROS, PI3K and mitogen-activated protein kinase (MAPK) signaling (Haddad & Land, 2001; Hashimoto et al., 2023). In endometrial epithelial cells, the inflammatory activation of HIF-1α was accompanied by the overexpression of hypoxia markers (*PDK1*, *VEGFA*, *SLCA2A1*, and *LDHA*), and TFs related to EMT, reduced expression of *CDH1* encoding E-cadherin, overexpression of *CDH2* encoding N-cadherin, and enrichment of wound healing and epithelial cell migration pathways, which was further confirmed with a wound healing assay (Hashimoto et al., 2023).

Another group of pro-inflammatory factors is presented by prostaglandins, among which PGE2 plays an important role in endometriosis. PGE2 is known to induce synthesis of aromatase and E2 in endometriotic lesions (Attar et al., 2009; Noble et al., 1997). Elevated expression levels of prostaglandin receptors and transporters and COX-2 (that converts arachidonic acid to prostaglandins) have been identified in peritoneal fluid and EcE of women with endometriosis (De Leon et al., 1986; Ota et al., 2001; Rakhila et al., 2015). Inhibition of COX-2 decreased PGE2 levels and negatively regulated endometriotic cell survival and lesion growth (Olivares et al., 2008).

2.3 Single-cell studies on pathogenesis of endometriosis

The review of single-cell transcriptomic studies on the endometrium and endometriosis is presented in this chapter, highlighting their main focus and findings (Table 2). Recent advances in RNA sequencing methods made it possible to examine transcriptomes of single cell populations. Transcriptome deconvolution method is based on scRNA-seq followed by data analysis. Bioinformatics tools are utilized to process raw sequencing data and identify cell clusters based on expression of specific cell markers, and further downstream analysis depends on the primary focus and research questions. Human endometrium has been recently characterised using scRNA-seq, revealing cell populations and their expression profiles. For example, Queckbörner et al. explored endometrial cell populations with a focus on stromal compartment of NE at the proliferative phase of the menstrual cycle from healthy women (Queckbörner et al., 2021). They characterized expression profiles of ten stromal cell types and two pericyte subtypes, highlighting their role in endometrial repair and immunomodulation. Bunis et al. compared the endometrium from women with and without endometriosis. In EuE, they found an enrichment of cell populations with a pro-inflammatory phenotype and altered immune pathways (Bunis et al., 2022). Another study compared endometrial mesenchymal stem cells isolated from EuE and NE (McKinnon et al.,

2022). They identified altered differentiation, ECM remodeling, focal adhesion and immune response in EuE that may implicate in the development of endometriosis.

Shih et al. examined menstrual effluent from women with confirmed endometriosis, women with endometriosis symptoms, and controls (Shih et al., 2022). In samples from women with endometriosis and symptomatic women they found differences in immune and stromal cell compartments compared to controls, with activation of processes characteristic of endometriosis. The inflammatory profile of endometriosis at the single cell transcriptomic level was studied by Huang et al. in EuE and NE in the proliferative and secretory phases (Huang et al., 2023). They identified impaired pro-inflammatory immune regulation in EuE at the secretory phase. Epithelial cells of EuE had abnormal expression of the receptor of pro-inflammatory cytokine *IL17*, highlighting interactions between epithelial and immune cells. A recent study by Marečková et al. provided analysis of single-cell transcriptome datasets of EuE and NE (Marečková et al., 2024). They introduced the Human Endometrial Cell Atlas, which classified four major cell populations: epithelial, mesenchymal, endothelial, and immune cells. The authors identified TGFβ-mediated stromal-epithelial interactions in the stratum functionalis. TGFβ signaling receptors were expressed in epithelial and stromal cells throughout the menstrual cycle. However, *TGFβ1* and *GDF7* were overexpressed in stromal cells at the proliferative and early secretory phases, indicating a role of TGFβ signaling in proliferative EuE. In addition, they identified an enrichment of macrophages in EuE and their role in endometrial repair, anti-inflammatory processes, and angiogenesis via cell interactions.

Fonseca et al. performed a large-scale transcriptomic study of a variety of tissues from women with and without endometriosis, and generated a single-cell atlas of stromal, epithelial, and microenvironmental cell types identified in the tissues (Fonseca et al., 2023). They examined EuE, unaffected ovaries, endometriomas, superficial and deep peritoneal lesions, and unaffected endometriosis-free peritoneum. The study mainly focused on the epithelial and stromal compartments of EcE. The cellular composition of peritoneal lesions was found to be similar to DIE, but different from endometriomas. Moreover, epithelial and stromal cells in peritoneal lesions and DIE exhibited expression profiles indicative of transitional changes from peritoneal lesions to DIE, suggesting the two endometriosis subtypes are rather continuous stages of the disease. The authors also found an epithelial cell-specific mutation in *ARID1A*, possibly involved in pro-lymphangiogenesis. A study by Ma et al. characterized expression profiles of EcE, EuE and NE (Ma et al., 2021). They revealed a functional shift of fibroblasts from NE to EuE, and further to EcE. The expression profile of fibroblasts in NE was related to cell metabolic processes and showed a progressive change towards EcE with an enrichment of endometriosis-specific processes and altered hormonal regulation. A recent single-cell study by Zhu et al. focused on EcE, EuE, and NE at the secretory phase (Zhu et al., 2023). In all three tissue groups, they identified 16 cell clusters, with myofibroblasts and fibroblasts being the largest cell populations. Epithelial cell heterogeneity was found between EuE and NE,

with upregulation of genes related to immune cytokines and factors related to cell adhesion, migration, inflammation, and angiogenesis. With trajectory analysis, they found a putative transition pathway of myofibroblasts from NE to EuE, and eventually to EcE state. Myofibroblasts in EuE had an enrichment of pathways related to cell proliferation, migration, and steroidogenesis compared to NE, which may indicate their involvement in endometriosis onset. Myofibroblasts in EcE had an enrichment of pathways related to ECM, angiogenesis, mesenchymal development, wound healing, steroidogenesis, and response to TGFβ signaling.

A comprehensive investigation of transcriptomes of peritoneal lesions and endometrioma by Tan et al. indicated the structural complexity and distinct characteristics of EcE when compared to NE and EuE (Tan et al., 2022). Larger proportions of endothelial and perivascular cell populations with a pro-angiogenic profile in peritoneal lesions and adjacent peritoneum, suggest the role of perivascular cells in angiogenesis. The immune component of peritoneal lesions expresses immunomodulatory genes. Shin et al. compared three endometriosis subtypes to NE from external datasets, showing endometriosis-specific altered steroidogenesis, pro-inflammatory and angiogenic expression profiles of EcE (Shin et al., 2023). A recent study by Yan et al. investigated gene expression in EcE (endometrioma), EuE, and NE (Yan et al., 2024). In the stromal cell population of EuE, they found an enrichment of MAPK-related genes and insulin growth factors, suggesting a pro-survival state of stromal cells important for endometriosis development. Epithelial cells of EcE exhibited overexpression of an apoptosis inhibitor, *NNMT*, whose silencing induced apoptosis via activation of FOXO1.

Table 2. Review of single-cell transcriptomic studies on human endometrium and endometriosis.

Authors, year	**Tissues (menstrual cycle phase)**	**Main findings**
Bunis et al. 2022	EuE and NE (proliferative and secretory phases)	EuE enriched for endothelial cells, epithelial cells, macrophages and plasmacytoid dendritic cells. EuE cell populations exhibited a pro-inflammatory phenotype and altered immune pathways.
Fonseca et al. 2023	EcE: endometrioma, peritoneal lesions, deep infiltrating lesions; EuE; NE; ES-free peritoneum, unaffected ovaries (proliferative and secretory phases)	Pro-inflammatory profile of EcE. Ectopic epithelial and stromal cells in endometrioma had activated complement signaling, ECM reorganization, and immune dysregulation. Spatial transcriptomic analysis revealed expression of *ECM1* and *MMP11* in stromal cells of endometriotic lesions. Epithelial cells of EcE had a mutation in the *ARID1* gene associated with the overexpression of several paracrine factors (*VEGFC*, *CCBE1*, and *FGF2*).
Huang et al. 2023	EuE and NE (proliferative and secretory phases)	Pro-inflammatory cytokines (*IL-1α*, *IL-1β*, *TNF-α*, *VEGF*, *TGF-β*) expressed in macrophages, B cells, NK cells, and T cells of EuE. High proportions of immune cells and presence of immature NK cell subpopulation, epithelial-immune cell crosstalk in EuE.
Ma et al. 2021	EcE: endometrioma; EuE; NE (proliferative phase)	In EcE, stromal FBs exhibited transcriptome related to cell motility, ECM remodeling and angiogenesis, overexpression of *StAR* (E2 synthesis from cholesterol), impaired immune profile (decreased proportion of NK cell population, increase of macrophages and reduced activation of T cells).
Marečková et al. 2024	EuE and NE from 6 scRNA-seq datasets (proliferative and secretory phases)	TGFβ-mediated coordination of stromal and epithelial cells in stratum functionalis. Overexpression of MMPs and inhibin in stromal cells at the proliferative phase, while downregulation of *ESR1* and *PGR* in the secretory phase. Enriched macrophages in EuE with role in endometrial regeneration, anti-inflammatory response and angiogenesis.
McKinon et al. 2022	ESCs from EuE and NE (proliferative and secretory phases)	MSC and stromal cell populations of EuE exhibited divergent differentiation and expression profile related to ECM remodeling, focal adhesion and reactive immune response.

Authors, year	Tissues (menstrual cycle phase)	Main findings
Queckbörner et al. 2021	NE (proliferative phase)	Stromal cell subtypes with EMT, ECM remodeling and tissue regeneration characteristics. Pericyte subtypes exhibiting a classical phenotype and a smooth muscle cell phenotype.
Shih et.al 2022	ME from ES, symptomatic cases and controls (menstruation)	ME from ES and symptomatic cases compared to controls exhibited depletion of uterine NK cells, enrichment for B cells, stomal cell subpopulations expressing MMPs and genes associated with inflammation, fibrosis, senescence, ECM, adhesion and migration.
Shin et al. 2023	EcE: endometrioma, peritoneal lesions, deep infiltrating lesions; NE from public dataset (prolife-rative and secretory phases)	Altered steroid hormonal signaling, immune dysregulation (activation of NK cells and dysregulation of T cells), and pro-inflammatory status of macrophages, pro-angiogenic and inflammatory expression profile of endothelial cells in EcE.
Tan et al. 2022	EcE: endometrioma, peritoneal lesions, adjacent peritoneum; EuE; NE (proliferative and secretory phases)	Distinct population of perivascular cells characteristic of endometriosis, expressing *MYH11* and *STEAP4* cell marker genes in EcE. Activation of angiogenesis regulators in CCL19+ perivascular cell population of peritoneal lesions and adjacent tissues. Immune cells (macrophages and dendritic cells) exhibited immunomodulatory profile, promoting immunotolerance in peritoneal lesions. A novel epithelial cell population (MUC5B+) with progenitor characteristics and high proliferative potential in organoid culture. Cell interactions between MUC5B+ epithelial cells and myeloid cells.
Yan et al. 2024	EcE: endometrioma; EuE; NE (proliferative and secretory phases)	Epithelial cells of EcE showed anti-apoptotic, pro-inflammatory and immunoregulatory profile with T cell interactions. Stromal cells of EuE exhibited the enrichment of genes related to MAPK and insulin signaling.

Authors, year	Tissues (menstrual cycle phase)	Main findings
Zhu et al. 2023	EcE: endometrioma; EuE; NE (secretory phases)	EcE enriched for fibroblasts, macrophages, neutrophils and B cells, and depleted in NK cells. Epithelial cell population of EuE vs NE with activation of genes related to immune cytokines, adhesion, migration, inflammation and angiogenesis. MyoFBs exhibited transition pathway from NE to EuE to EcE, with enrichment of proliferation and migration processes in EuE, and angiogenesis, steroidogenesis, ECM formation and response to TGFβ in EcE. Altered immune function in EcE, related to T cell dysfunction, inflammatory, and proliferating state of macrophages and neutrophils.

Abbreviations: E2 – estradiol; EcE – ectopic endometrium; ESCs – endometrial stromal cells; ECM – extracellular matrix; EMT – epithelial to mesenchymal transition; ES – endometriosis; EuE – eutopic endometrium; FB – fibroblasts; MAPK – mitogen-activated protein kinase; ME – menstrual effluent; MSCs – mesenchymal stem cells; MyoFBs – myofibroblasts; NA – not applicable; NE – normal endometrium; NK – natural killer cells; TGFβ – transforming growth factor beta.

2.4 Diagnostics and treatment

2.4.1 Invasive and non-invasive diagnostics

Diagnosis of endometriosis is usually delayed for seven to nine years due to non-specific symptoms characteristic of other gynecological conditions. In most cases, women are diagnosed during medical examination for other issues like infertility or ovarian cysts. To date, there are no robust diagnostic markers for endometriosis. Routine diagnostics in women with suspected endometriosis include non-invasive imaging, such as transvaginal or transabdominal ultrasound and magnetic resonance imaging. However, these methods are effective only for two subtypes of endometriosis: ovarian and DIE. New techniques, such as magnetic resonance elastography and positron emission tomography/computed tomography are potentially useful for detection of peritoneal lesions. There are some challenges related to visual diagnostics of endometriosis, for example the detection rate also depends on experience of the operator. The benefits of imaging methods are related to the ability to describe and characterize the location and size of lesions for further surgical management as well as follow-up during therapeutic management of endometriosis. Magnetic resonance elastography allows for characterizing tissue composition by measuring and analyzing physical properties of tissues. The positron emission tomography/computed tomography method provides information on the tissue localization of the ^{18}F-FDG tracer used for detection with exposure to low radiation doses, Currently, ^{18}F-FDG tracer is used to detect tissues with high metabolic activity, such as malignant tissues, but it has not been proven to provide robust and consistent detection of endometriotic lesions (Griffiths et al., 2024). Other tracers related to receptors overexpressed in endometriosis are being tested. Artificial intelligence can be applied for improved analysis of visual diagnostic methods as seen in malignancies.

Currently, clinicians and researchers are looking for robust, semi- and non-invasive ways to detect endometriosis. This means searching for potential biomarkers in biological fluids (e.g., saliva, sweat, urine, and blood) with high specificity and high sensitivity. The levels of proteins, metabolites, microRNAs and other biomarkers, previously shown to be altered in endometriosis, have been evaluated to develop non-invasive diagnostic tests. Emerging data from omics studies on endometriosis provide new insight into the biology of endometriotic tissues and the pathogenic mechanisms underlying endometriosis development, with new molecular players that can be further explored in the light of diagnostics and therapeutics. However, the complexity of endometriosis subtypes, linked to their phenotypic and morphological differences, may complicate the search for biomarkers. Currently, biopsies and biological fluids obtained from women with confirmed endometriosis serve as a valuable source to study and identify potential biomarkers. Biomarkers, like metabolites, can be identified using targeted or high-throughput techniques, such as nuclear magnetic resonance spectroscopy and mass spectrometry-based methods (Adamyan et al., 2024). The challenges for biomarkers discovery are related to study methodologies and the dependency

of biomarker expression on the menstrual cycle phase, different subtypes and phenotypes of endometriosis and the impact of treatment (Adamyan et al., 2024). A systematic review of blood biomarkers by Brulport et al. reported four candidates (TBF-α, MMP-9, TIMP-1 and miR-451), associated with ECM organization and inflammation (Brulport et al., 2024). A recent study validating a saliva-based miRNA test has demonstrated the potential of incorporating this test for non-invasive diagnosis of endometriosis (Bendifallah et al., 2023). The test, designed to identify expression of 109 miRNAs with use of next-generation sequencing and artificial intelligence, showed high sensitivity and specificity (96.2% and 95.1%, respectively), however further studies with larger sample size are required to account for confounders and endometriosis recurrences. Future directions for improving diagnostics of endometriosis may involve a combination of powerful and accessible imaging methods and semi- or non-invasive testing using a panel of biomarkers.

2.4.2 Therapeutic and surgical treatment

Current treatment strategies are limited to symptomatic management and/or surgical removal of endometriotic lesions and adhesions. The risk-benefit paradigm is relevant for surgical treatment due to the limited evidence of improvement in symptoms (pain) in women after removal of peritoneal lesions. In addition, surgeries carry risks related to damage of organs and tissues, including the potential for damage of ovary tissues in women with endometriomas. The relapse of lesion growth presents another significant challenge, as some studies even report worsening of symptoms after surgical interventions for recurrent endometriosis (Guo, 2009). Hormone-based medications are prescribed to suppress the activity of hormone-producing organs or block hormone receptors and enzymes. They help to reduce menstrual bleeding, inflammation and menstrual pain, but these symptoms may return once treatment is discontinued. The drawbacks of hormonal treatment are related to the risks and complications from the suppression or withdrawal of hormones. Suppression of sex hormones may impact fertility and cause side effects, such as vasomotor symptoms, weight changes, among others. Considering the effects of current treatment approaches on fertility and quality of life, it is of high importance to develop safer and more effective strategies for endometriosis management.

Proposed non-hormonal therapeutic strategies may focus on targeting key players in pathophysiological processes in endometriosis (Kobayashi et al., 2021). Recent studies have suggested targeting cell metabolism to disrupt lesion growth. Distinct metabolic signatures of lesions in hypoxic microenvironment have been identified, highlighting potential key regulators. For example, proposed metabolic targets include GLUT4, PDH, PDK1, and LDHA. GLUT4, a glucose transporter sensitive to insulin, is overexpressed in ectopic epithelial and stromal cells (McKinnon et al., 2014). LDHA, which converts pyruvate to lactate and sustains aerobic glycolysis, is highly expressed in EcE. Silencing *LDHA* impaired metabolism, reduced cell migration, and induced apoptosis in endo-

metriotic cells (Zheng et al., 2021). A PDK inhibitor, dichloroacetate, which also activates PDH complex, has been shown to alter metabolism of human peritoneal mesothelial cells and lead to inhibited proliferation of co-cultured endometrial stromal cells (Horne et al., 2019). Furthermore, the authors showed that oral administration of dichloroacetate in a mice endometriosis model reduced lesion size and lowered peritoneal levels of lactate. Thus, metabolic drugs may represent an alternative to hormonal treatments of endometriosis. Further research is necessary to uncover the mechanisms underlying the action of metabolic regulators and provide information on specific drugs that can safely and effectively target them.

2.5 Rationale for studies

Given the role of hypoxia in regulating downstream processes in endometriosis development, we decided to explore its impact on the global transcriptome of stromal cells, representing the most abundant cell type in endometriotic lesions. Previous studies have investigated the role of particular genes altered by hypoxia in endometriosis, so we aimed to utilize hypothesis-free mRNA sequencing to identify differentially expressed genes in stromal cells exposed to hypoxia vs normoxia. Additionally, we examined the activity of kinases involved in processes important for endometriosis development.

Another direction of our research investigated metabolic profile of various cell populations in endometriotic lesions, using single-cell transcriptomic analysis. Previous scRNA studies have not covered metabolic alterations in cell populations of endometriotic lesions. In this study, we aimed to profile gene expression related to energy metabolism and steroidogenesis. The results of this study may contribute to further discoveries of drugs for non-hormonal metabolic treatment and provide insights into cell populations that support lesion survival and growth and that can be addressed as potential therapeutic targets.

In previous research, our lab identified latexin (LXN) as a novel candidate gene, highly expressed at both mRNA and protein levels in endometriotic lesions. LXN, an inhibitor of carboxypeptidases A, is expressed in both physiological and pathological conditions, and has been shown to exhibit tumor suppressor properties. In the third study, we aimed to identify LXN expression in EuE throughout the menstrual cycle and assess the impact of *LXN* knockdown on stromal cell proliferation and migration, processes important for endometriosis development.

3. AIMS OF THE STUDY

The main aim of the study was to investigate the pathogenesis of endometriosis from the perspective of metabolic activity, the response to hypoxia and the impact of identified candidate genes on endometriosis development.

The specific aims of the study were:

1. To explore and characterize energy metabolic activity and steroidogenesis in eutopic endometrium and ectopic peritoneal lesions of women with endometriosis at the single cell transcriptomic level.
2. To study the effect of hypoxia on the kinase activity and global transcriptome of primary eutopic and ectopic endometrial stromal cells *in vitro*.
3. To examine the expression of a carboxypeptidase inhibitor *LXN* in endometrial tissues of women with and without endometriosis across the menstrual cycle and its possible roles in the pathogenesis of endometriosis.

4. MATERIALS AND METHODS

4.1 Ethical statement (Studies I–III)

All three studies received approval from the Research Ethics Committee of the University of Tartu (UT) (approvals 276/M-13, 221/M-31, and 333/T-6) and the Regional Ethical Review Board in Stockholm (approval Dnr 2016-02-17, amendment 2022-03774-02). Written informed consent was provided by all enrolled participants. All ethical regulations concerning research with human participants were followed.

4.2 Study participants, sample collection and processing (Studies I–III)

Women with suspected endometriosis, pelvic pain or infertility undergoing laparoscopy at Tartu University Hospital (Tartu, Estonia) were recruited for the studies. Women with confirmed endometriosis, diagnosed according to the revised ASRM classification system ("Revised American Society for Reproductive Medicine Classification of Endometriosis," 1997), were defined as women with endometriosis. Women confirmed to be endometriosis-free, were defined as controls or the Non-ENDO group (Study II and III, respectively). Endometriotic lesions were obtained during laparoscopic surgery, and endometrial biopsies were collected from patients with endometriosis and controls using an endometrial suction catheter (Pipelle, Laboratoire CCD). Recruited healthy fertile volunteers donated endometrial tissue samples and formed a Healthy group (Study III). None of the participants in three studies had received hormonal treatment for at least three months prior to sample collection. The menstrual cycle phases were defined according to self-reported cycle days (Study I and II) or determined according to the methods described by (Saare et al., 2019) for women with or without endometriosis and (Rekker et al., 2018) for healthy women in Study III. Table 3 presents the general characteristics of the participants of three studies.

Table 3. General characteristics of the participants of studies I – III.

	Study I	Study II	Study III
Women with endometriosis (N)	4	72	55
ASRM I – II (N)	3	40	18
ASRM III – IV (N)	1	32	37
Studied tissue type	EuE, EcE	EuE, EcE	EuE, EcE
Age (years, SD)	33 ± 6.4	32 ± 5.3	32 ± 5
BMI (kg/m^2, SD)	21 ± 1.8	23 ± 3.2	23 ± 3.5
Menstrual cycle phase (N)	P (4)	P (16), S (56)	P (16), ES (11), MS (16), LS (12)
	Study I	**Study II**	**Study III**
Controls (N)	NA	24 Non-ENDO	27 Non-ENDO // 12 Healthy
Studied tissue type	NA	EuE	EuE
Age (years, SD)	NA	32 ± 5.6	32.1 ± 5.6 // 30.4 ± 3.7
BMI (kg/m^2, SD)	NA	23 ± 3.9	23.2 ± 3.7 // 22.9 ± 4.6
Menstrual cycle phase (N)	NA	P (5), S (19)	P (5), ES (13), MS (7), LS (2) // ES (12), MS (12)

Abbreviations: ASRM – American Society for Reproductive Medicine classification system; BMI – body mass index; EcE – ectopic endometrium; ES – early secretory phase; EuE – eutopic endometrium; LS – late secretory phase; MS – mid-secretory phase; Non-ENDO – women without endometriosis; N – number; NA – not applicable; P – proliferative phase; SD – standard deviation; // separates data of Non-ENDO and Healthy study groups (Study III).

The collected endometriotic tissue samples were processed as follows: each sample was divided into two portions – one was fixed in 10% formalin for histological evaluation and confirmation of endometriotic tissue, while the other part was preserved in cryopreservation medium containing 1× Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific), 30% fetal bovine serum (FBS, Biowest), and 7.5% dimethyl sulfoxide (DMSO, Hybri-Max, Sigma-Aldrich). The tissue samples in cryopreservation medium were placed into a Nalgene Cryo 1°C 'Mr Frosty' Freezing Container (Thermo Scientific) and stored at −80°C overnight, before being transferred to liquid nitrogen until further use. Haematoxylin and eosin staining was performed at the Tartu University Hospital Pathology Department following standard protocols. A pathologist confirmed the presence of endometriosis-specific morphological features in the lesions.

4.3 Study design (Studies I–III)

The three studies were designed as depicted in Figures 4–6, respectively. Study I was carried out at UT using paired tissues of EuE and EcE (peritoneal lesions) from women with endometriosis (N = 4) at the proliferative phase of the menstrual cycle. The tissue samples were enzymatically dissociated into single-cell suspension, followed by single-cell RNA sequencing and bioinformatic analysis of cell populations and metabolic pathways (Figure 4).

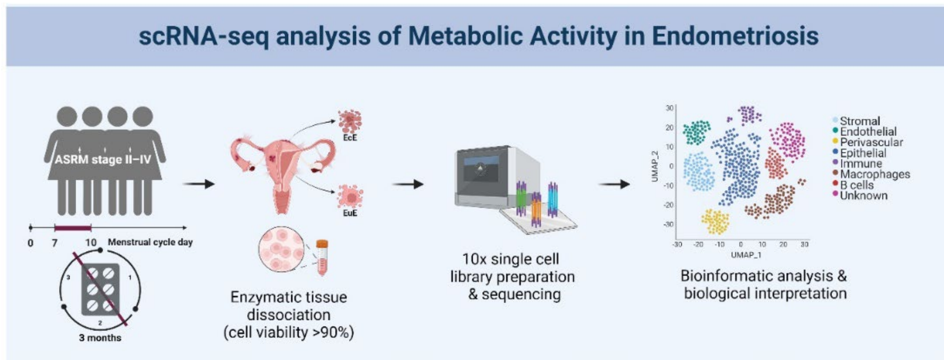

Figure 4. The design of Study I. Abbreviations: ASRM – American Society for Reproductive Medicine classification system; EcE – ectopic endometrium; EuE – eutopic endometrium; UMAP – Uniform Manifold Approximation and Projection. Reproduced with permission from Sarsenova et al., 2024, Communications Biology, licensed under the Creative Commons Attribution 4.0 International License.

In Study II, performed at both UT and Karolinska Institutet (KI), isolated primary stromal cells from EuE and EcE (peritoneal lesions) of women with endometriosis (N = 5) were exposed to hypoxia for 48 h to examine its effect on global transcriptome of cells using mRNA-seq and PK activity (Figure 5). The downstream analysis of TGFBI was performed on spent culture media using ELISA (N = 5) and *ex vivo* on EuE and/or EcE from women with or without endometriosis (N = 72 and N = 24, respectively) using real-time quantitative reverse transcription PCR (qRT-PCR) and immunohistochemistry (IHC) to determine TGFBI mRNA and protein expression in the tissues.

Study III was performed at UT and the University Hospital of Münster and focused on the investigation of mRNA (qRT-PCR) and protein (IHC) expression of LXN in EuE and/or EcE from women with and without endometriosis (N = 55 and N = 39, respectively) in different phases of the menstrual cycle, and the effect of *LXN* silencing on stromal cell viability (MTT assay) and migration (Transwell migration assay) using endometrial stromal cell line St-T1b (Figure 6).

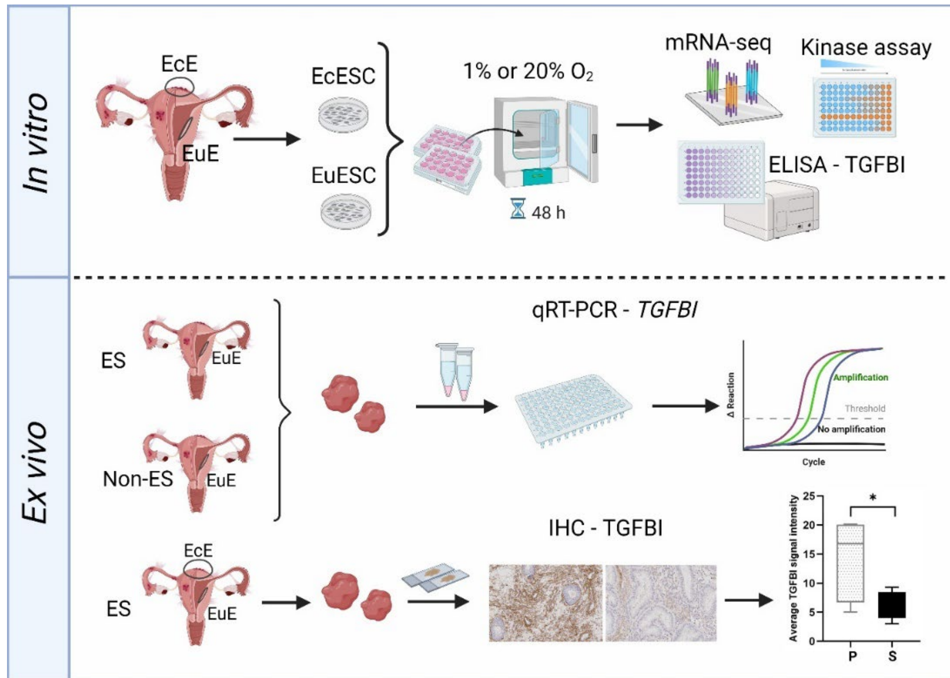

Figure 5. The design of Study II. Abbreviations: EcE – ectopic endometrium; EcESCs – ectopic endometrial stromal cells; ELISA – enzyme-linked immunosorbent assay; ES – endometriosis; EuE – eutopic endometrium; EuESCs – eutopic endometrial stromal cells; Non-ES – non-endometriosis; IHC – immunohistochemistry; P – proliferative phase; qRT-PCR – quantitative reverse transcription PCR; S – secretory phase; TGFBI – transforming growth factor beta induced. Reproduced with permission from Sarsenova et al., 2024, Frontiers in Endocrinology, licensed under the Creative Commons Attribution 4.0 International License.

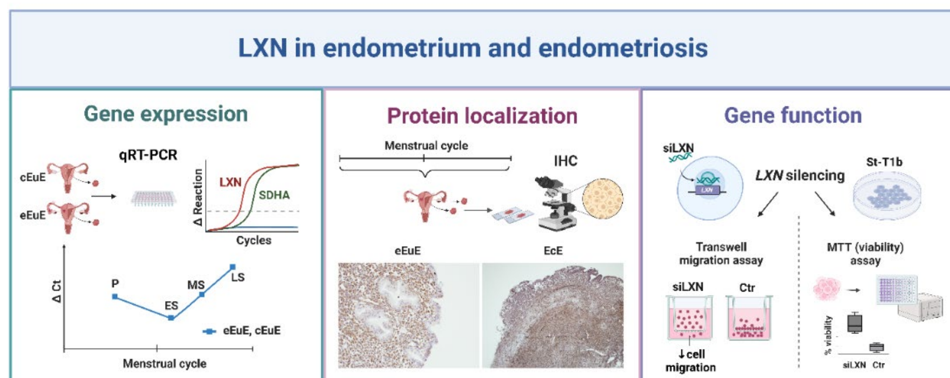

Figure 6. The design of Study III. Abbreviations: Ct – threshold cycle; Ctr – control (no added siLXN); EcE – ectopic endometrium; ES – early secretory phase; cEuE – eutopic endometrium from controls (women without endometriosis); eEuE – eutopic endo-

metrium from women with endometriosis; IHC – immunohistochemistry; LS – late secretory phase; LXN – latexin; MS – mid-secretory phase; P – proliferative phase; qRT-PCR – quantitative reverse transcription PCR; siLXN – small interfering RNA targeting *LXN*; SDHA – succinate dehydrogenase; St-T1b – immortalized human endometrial stromal cell line. Reproduced with permission from Sarsenova et al., 2024, Genes, licensed under the Creative Commons Attribution 4.0 International License.

4.4 Single-cell isolation from tissue biopsies (Study I)

Cryopreserved EuE and EcE tissues were thawed, washed and enzymatically dissociated using collagenase I, DNase, and Dispase II in DMEM during 1 hour of incubation at 37°C on a rotating shaker, as previously described (Sarsenova et al., 2024). The single-cell suspension was filtered through a 30-µm cell strainer, centrifuged at 300×g for 5 minutes. The cell suspension was treated with an ACK lysis buffer (Gibco) to remove the red blood cells. Next, cells were resuspended in DMEM, filtered again, and counted with Trypan blue on an automated counter. Live cells were isolated using the MACS dead cell removal kit (Miltenyi Biotec), and the cells with > 90% viability were washed and resuspended in BSA/DPBS at a concentration of 700–1200 cells/µl.

4.5 Chromium 10x single-cell capturing, library preparation & sequencing (Study I)

Single-cell RNA libraries were prepared using the 10x Chromium Next GEM Single Cell 3’ reagent v3.1 kit (Dual index, 10x Genomics, CG000315 Rev C), following the manufacturer’s guidelines (10x Genomics). Single-cell suspensions, 3’ gel beads, and reverse transcription reagents were applied onto a 10x Chromium microfluidic chip to target 3,000 cells per sample. The samples were processed in a 10x Chromium controller to create Gel Beads-in-emulsion. For subsequent steps, including cell lysis, first-strand complementary DNA (cDNA) synthesis, amplification, and purification, we followed the manufacturer’s instructions to generate barcoded full-length cDNA. Library preparation for all samples was done simultaneously using the construction kit to minimize batch effects. cDNA and single-cell library quality were assessed with an Agilent 4150 Tape Station (Agilent Technologies). The dual-indexed libraries were pooled and sequenced (pair-end) with NovaSeq PE150 (Illumina) to achieve 35,000 reads per cell.

4.6 Bioinformatic analysis (Study I)

Bioinformatic analysis of obtained scRNA data was performed by a PhD student in bioinformatics (Ankita Lawarde). Within the analysis, we performed scRNA-seq data treatment of quality control, normalization, doublet cell removal, sample integration, and cell-type clustering. Reads alignment was done with GRCh38

reference genome and processed using Cell Ranger (v7. 0. 0) to create count matrices. Data analysis was performed using the Seurat package, and cells at high complexity (> 0.80) with unique molecular identifier counts more than 500 and expressing more than 200 genes per cell were kept, while cells containing more than 15% of mitochondrial reads were excluded. Genes expressed in less than 3 cells were filtered out. Normalization was performed for each sample, adjusting for mitochondrial expression and cell cycle phase, followed by clustering, Uniform Manifold Approximation and Projection visualization and doublets removal (McGinnis et al., 2019). We used a similar pipeline of bioinformatic analysis for the external dataset (Huang et al., 2023).

For cell cluster annotations, we applied two methods to identify and label major cell types in our integrated dataset. The FindAllMarkers function from the Seurat package was used to detect differentially expressed genes for each cell cluster with default settings. We validated markers using a list of cell markers from the literature (Garcia-Alonso et al., 2021; Ma et al., 2021; Tan et al., 2022; Wang et al., 2020; Zou et al., 2021), Cell Marker database (Hu et al., 2023a), Panglao database (Franzén et al., 2019) and scRNA-seq database of Human Protein Atlas. A refined list of marker genes was selected based on criteria of $\log_2$ fold change ($\log_2$FC) > 1, adjusted p-value (p_{adj}) < 0.05, PCT.1 ≥ 0.7 higher expression of a cell marker in a specific cell cluster while PCT.2 ≤ 3 lower expression in other cell clusters. The two-sided Fisher's exact test was applied for statistical comparisons of total cell counts and cell proportions by cell cycle phases between EcE and EuE. Our dataset was used as the reference for unbiased cell type annotation in the external dataset using SingleR (v 2.6.0) from Bioconductor.

We used pseudobulk analysis (DESeq2 v.1.40.1, R package) for aggregating scRNA-seq counts from individual samples to provide population-level gene expression profiles (Love et al., 2014). This strategy made comparisons between conditions (EuE vs EcE) more powerful and biologically relevant, using four different samples in each group to estimate overall biological variability rather than of an individual cell. clusterProfiler (v.4.8.1) R package was applied in each cell population for gene and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses of differentially expressed genes (DEGs) (p-value < 0.05, $\log_2$FC ≤ –0.40 or ≥ 0.40) for our and external datasets (Wu et al., 2021a; Yu et al., 2012). The analysis of 12 metabolic pathways and steroidogenesis pathways were performed using the same R package. The single-cell pathway analysis was applied to rank metabolic pathways for each cell population in EcE vs EuE by calculating Q value for each pathway (Bibby et al., 2022). Q value indicates a difference in each pathway activity between EcE and EuE, taken as two conditions. To evaluate the metabolic pathway activity across patients, we analyzed the overrepresentation of these pathways using Gene Set Variation Analysis (v. 1.52.2, Bioconductor package), a non-parametric unsupervised method (Hänzelmann et al., 2013). We also assessed the activity of TFs associated with the metabolic genes in each sample of EuE and EcE using decoupleR (v. 2.10.0), mapping each TF to its target genes (Badia-i-Mompel et al., 2022).

4.7 Stromal cell isolation and culturing (Study II)

For stromal cell culturing experiment (Study II), stromal cells were isolated from eutopic (EuESCs) and ectopic (EcESCs) endometrial tissue biopsies. The tissues were washed to remove debris and blood cells in fresh medium, followed by enzymatic digestion in 5 ml of phenol-red free DMEM media with collagenase I and DNase on a shaking incubator, at 37°C for 1 hour. Next, the cell suspension was filtered using a 30 µm strainer. The filtered cells were resuspended in 10 ml of culture medium and sedimented for 10 min in a tube, these steps were repeated 3 times. The supernatant containing stromal cells was collected, resuspended with 2 ml of DMEM and transferred onto 6-well plate. Then stromal cell cultures were purified by selective adherence to the bottom of wells on the 6-well plate in incubator at 37°C. For hypoxia experiment, cells were plated and cultured at 37°C under 20% O_2 and 5% CO_2 for 24 hours, followed by incubation of half of the replicates under hypoxic conditions (1% O_2) for 48 hours in the culturing medium.

4.8 mRNA-seq and qRT-PCR (Studies II and III)

For analysis of mRNA expression (mRNA-seq and qRT-PCR experiments), total RNA was isolated using RNeasy mini kit (Qiagen). Further, RNA samples were treated with DNase to remove genomic DNA.

To explore global transcriptome of stromal cell cultures exposed to hypoxia or normoxia using mRNA-seq (Study II), we generated cDNA libraries from 10 ng of mRNA using Smart-seq2 protocol (Picelli et al., 2014) with further fragmentation, tagging with Dual Index barcodes, cDNA amplification, purification and sequencing. Next, raw RNA-seq data were treated using FastQC (Brown et al., 2017) and MultiQC (Ewels et al., 2016) programs with further alignment to human reference genome GRCh38. mRNA expression was analyzed using DESeq2 R package (Love et al., 2014), genes with 10 counts or more in one experimental group remained, and differentially expressed genes were considered to be those with $log_2FC > 0.5$ or < 0.5 and Padj < 0.05. Pathway enrichment was analyzed with online tool g:Profiler (Kolberg et al., 2023).

For qRT-PCR (Study II and III), cDNAs were synthesized from RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The analysis of expression levels of *TGFBI* and *LXN* was carried out using 5x HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne) or 2× SYBR Select Master Mix (Applied Biosystems), 200 ng of cDNA, and primers to *TGFBI*, *LXN* and a reference gene *SDHA*. The relative expression and mRNA expression FC were calculated using $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

4.9 Protein kinase assay and ELISA (Study II)

As the cell count was different across EcESCs' and EuESCs' cultures, the total protein concentration in lysates was measured and equalized by diluting the samples to the lowest measured concentration within the set of samples measured on the same day. PK activity was analyzed in lysates from each cell culture as previously described (Lavogina et al., 2021). Briefly, PK activity was assessed using in-house synthesized photoluminescent probes to PKAc, ROCK, Akt and CK2, and with displacing compounds for each PK (Enkvist et al., 2011; Lavogina et al., 2021; Vahter et al., 2018), followed by measuring time-delayed photoluminescence with PHERAstar multi-mode reader (BMG Labtech). The secreted TGFBI level was measured in culture media from each sample using EHTGFBI (BIGH3) human ELISA kit (Invitrogen). The measured level was also normalized to the total protein values in the corresponding lysate sample to compensate for the number of secreting cells.

4.10 Immunohistochemistry (Studies II and III)

To localize and/or analyze protein expression in tissues, IHC was performed using antibodies against TGFBI (Study II) and LXN (Study III) and DAB detection kit. Shortly, the tissue sections of EuE and EcE underwent the consequent steps of deparaffinization in xylene, rehydration in ethanol and water, antigen retrieval, blocking nonspecific binding, and incubation with primary antibody (rabbit anti-TGFBI antibody, Invitrogen, or mouse monoclonal anti-Latexin antibody, ThermoFisher Scientific) and respective secondary antibodies. Then, tissue sections were dehydrated, and mounting medium was applied for further scanning and obtaining microphotographs. The semi-quantification of TGFBI signal intensity was carried out by calculating relative DAB intensity using ImageJ Fiji package (v. 1.54k) (Fuhrich et al., 2013; Schindelin et al., 2012).

4.11 siRNA transfection, MTT and migration assays (Study III)

To assess the role of LXN in endometrial stromal cell viability and cell migration, immortalized human endometrial stromal cell line St-T1b was transfected with small interfering RNA targeting *LXN* (siLXN) or control siRNA. After 24 h incubation, the decreased *LXN* expression in siRNA-treated cells was confirmed with qRT-PCR. Next, the cells were incubated with MTT to measure optical density to determine cell metabolism. To evaluate the effect of *LXN*-silencing on cell migration, cells were incubated in serum-free migration medium in 8.0 µm pore size Transwell chambers (Falcon) for 36 h, followed by fixation and staining of

cells for further quantitative analysis by counting migrated cells in both siLXN and controls.

4.12 Statistics and Reproducibility (Studies I–III)

In Study I, we used two-sided Fisher's exact test for comparison of cell proportions between EuE and EcE. The significant differential expression was determined in the studies by false discovery rate (p_{adj}) < 0.05 and/or $p < 0.05$. The comparison of groups in Study II (PK assay and qRT-PCR analyses) was performed using unpaired two-tailed t-test with Welch's correction. The Wilcoxon Mann-Whitney test was applied to evaluate statistical significance of results from IHC quantitative analysis (Study II). The one-way ANOVA or Student's t-test were applied to assess statistical significance in Study III. Data analysis and visualization in all three studies were performed in R and/or GraphPad Prism 9.0.0 (San Diego, CA).

5. RESULTS

5.1 Study I: Metabolic activity and steroidogenesis in endometriosis

5.1.1 Metabolic activity in major cell types of paired endometriotic lesions and eutopic endometrium

We performed scRNA-seq of paired EuE and EcE from four women with endometriosis at the proliferative phase, followed by bioinformatic analysis of the obtained data. It included quality control and doublets removal, followed by the analysis of expression of cell type-specific marker genes in 14,817 cells (7,279 from EuE and 7,538 from EcE). Based on the analysis, we identified nine major cell clusters in both EuE and EcE with perivascular, stromal, endothelial and immune clusters being the largest in both study groups, as shown on Figure 7A–B. In addition, we applied this analysis to the external dataset (Huang et al., 2023) and identified the same nine cell types in EuE from women without endometriosis.

In each cell population we examined the activity of 12 metabolic pathways (listed in Figure 8A) and identified high numbers of statistically significant DEGs in perivascular, stromal and endothelial cell populations (Figure 8A). Among the 12 metabolic pathways, the highest differences between EcE and EuE were identified in the activity of AMPK signaling, HIF-1 signaling, glutathione metabolism, oxidative phosphorylation, and glycolysis using single-cell pathway analysis (Figure 8B). As the highest differences in metabolic activity between EcE and EuE were detected in perivascular, stromal and endothelial cells, further analysis was focused on these three cell populations. The analysis of TFs associated with metabolic genes showed the activation of TFs related to cell proliferation and survival (*ATF1*, *MECOM*, *ETS2*, *NFYA*, *XBP1*), EMT in stromal cells (*DNMT1*, *EZH2*, *HNF1B*, *MYB*), inflammation, angiogenesis and cell adhesion (*NR2F2*, *NCOR2*) in EcE compared to EuE. With further analysis of DEGs, we identified enrichment of pathways related to the activation of these processes in EcE.

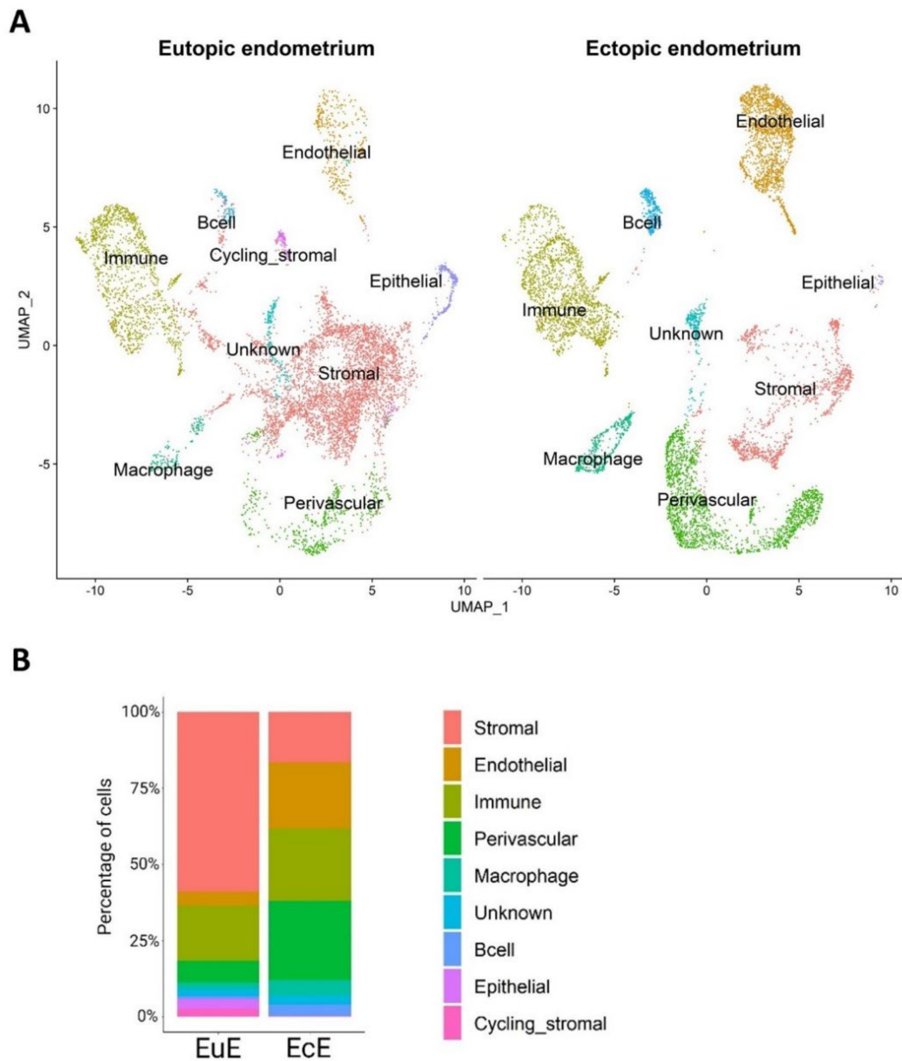

Figure 7. Overview of nine major cell populations of EcE and EuE. **A**. Uniform Manifold Approximation and Projection (UMAP) plot of nine major cell clusters. The cycling stromal cell cluster in EcE was represented by few cells and due to the low cell count and wide distribution of the dots representing this cluster, the cluster name is omitted from the figure to avoid confusion with the cluster localization. **B**. Bar plots representing the relative ratios of nine major cell populations in EcE and EuE. Perivascular, endothelial, and immune cell populations were larger in EcE compared to EuE, while stromal cell population was larger in EuE. Abbreviations: EcE – ectopic endometrium; EuE – eutopic endometrium. Reproduced with permission from Sarsenova et al., 2024, Communications Biology, licensed under the Creative Commons Attribution 4.0 International License.

The differential expression analysis of metabolic pathways, organized into five groups by function, revealed up- and downregulation patterns in perivascular, stromal and endothelial cells of EcE compared to EuE (Table 4). In EcE, we observed altered signaling of two regulatory metabolic pathways: AMPK and HIF-1 signaling. We found AMPK-mediated activation of genes related to glucose uptake (*TBC1D1*, *GLUT4*), while activators of glycolysis were inhibited (*PFKFB3*, *PFKFB4*) in perivascular and stromal cells. Besides, there was an upregulation of genes associated with fatty acid (FA) uptake and oxidative metabolism (*CPT1A*, *MLYCD*), as well as inhibitor of oxidative metabolism (*PDK1*). The analysis of activity of glycolytic pathways in EcE revealed an upregulation of key glycolytic genes, like *ALDOA*, *PGAM*, *ENO2* and *PFKP*. Hexokinases were downregulated in perivascular and stromal cells, but their role to maintain glycolysis was likely taken by upregulated *GCK*. In EcE, we also identified an overexpression of genes encoding lactate dehydrogenase enzymes involved in aerobic glycolysis via conversion of pyruvate to lactate.

Moreover, we identified an upregulation of genes associated with conversion of FA and pyruvate to acetyl-CoA (*ACADL*, *CPT1A*, *CYP2U1*, *ACADS*, *PDHA*, *PDHB* and *DLD*), and key genes of tricarboxylic acid cycle, TCA cycle, (*IDH3B*, *IDH2*, *PCK1*, *SDHC*, *SUCLA2*, *SUCLG1* and *SDHD*). Perivascular cells of EcE showed an upregulation of 24% of genes related to OXPHOS complexes, in stromal and endothelial cells, some of OXPHOS genes were upregulated, while others were downregulated. In addition, in EcE we found differential regulation of genes related to biosynthetic metabolism (e.g., biosynthesis of FAs, steroids, proteins and nucleotides), and inhibition of key regulators of gluconeogenesis, glycogen and sterol synthesis. Similarly, some genes associated with cell cycle progression and cell survival were both up- and downregulated in EcE. Additionally, we identified HIF-1-mediated activation of genes involved in angiogenesis in endometriotic lesions (*VEGFA*, *ANGPT1*, *ANGPT4*, *IL6*, *IL6R*, *FLT1*). The metabolic analysis of EuE from women with endometriosis (our dataset) and EuE from women without endometriosis from external dataset (Huang et al., 2023) identified only few DEGs, suggesting similar metabolic activity in both tissue types.

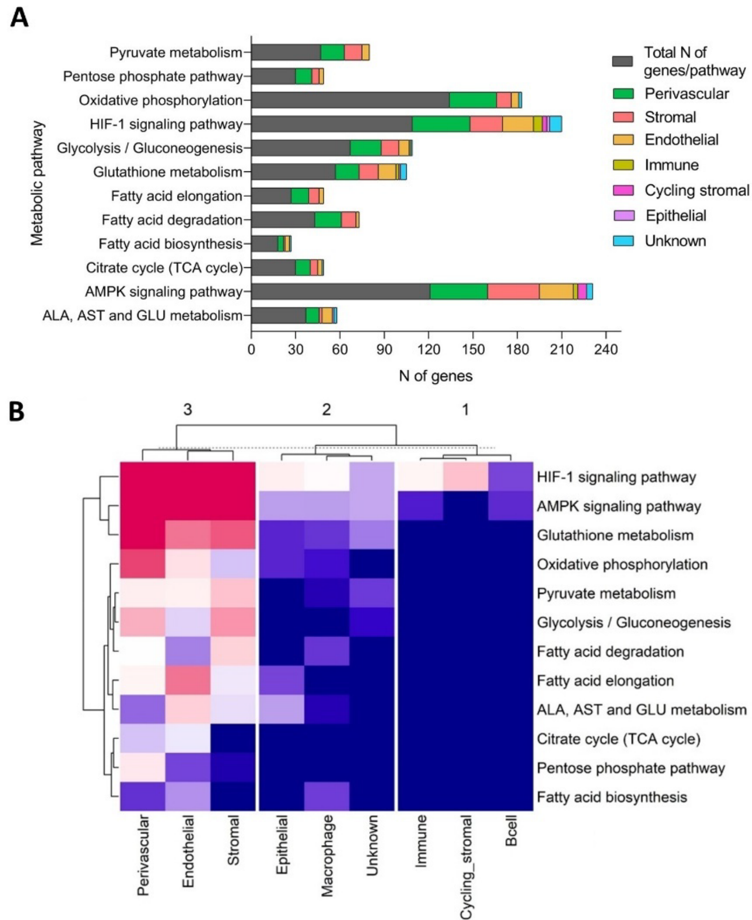

Figure 8. Altered metabolic activity primarily in perivascular, stromal and endothelial cell populations of EcE vs EuE. **A**. A bar plot of the numbers of DEGs in 12 metabolic pathways across nine major cell types (color-coded) and the total number of genes in a given pathway (in dark grey). **B**. A heatmap showing the Q values of 12 metabolic pathways across 9 cell types of EcE compared to EuE. Q value refers to a pathway difference between EcE and EuE, considered as two conditions. A higher Q value corresponds to a higher rank of the pathway. The lower Q value is depicted in blue, the higher Q value in red. Abbreviations: ALA – alanine; AMPK – AMP-activated protein kinase; AST – aspartate; GLU – glutamate; HIF-1 – Hypoxia-inducible factor 1; N – number; TCA – tricarboxylic acid cycle. Reproduced with permission from Sarsenova et al., 2024, Communications Biology, licensed under the Creative Commons Attribution 4.0 International License.

Table 4. Representative differentially expressed genes of metabolic pathways in perivascular, endothelial, and stromal cell types of ectopic endometrium compared to eutopic endometrium. Reproduced with permission from Sarsenova et al., 2024, Communications Biology, licensed under the Creative Commons Attribution 4.0 International License.

		DEGs[a] (log_2FC)		
Pathway group	**Gene expression in EcE**	**Perivascular cells**	**Stromal cells**	**Endothelial cells**
Regulatory pathways	Upregulated	*CAMK2G* (1.74), *PDK1* (1.03), ***CDKN1A*** (3.81), ***CDKN1B*** (1.23), *VEGFA* (0.86), ***ANGPT1*** (3.89), *ANGPT4* (7.42), ***IL6*** (6.77), ***IL6R*** (3.09), *CAB39L* (1.90), *PRKAA1* (0.51), ***PRKAA2*** (3.24), ***PRKAG2*** (2.47), ***TBC1D1*** (2.19), ***GLUT4*** (3.08), *CD36* (4.48), ***CPT1A*** (1.51), *MLYCD* (0.95), ***ACACB*** (0.93), ***EEF2K*** (2.24), ***CCND1*** (1.65)	*CAMK2A* (1.58), ***CDKN1A*** (1.55), ***CDKN1B*** (0.86), ***BCL2*** (1.07), ***ANGPT1*** (1.87), *FLT1* (*VEGFR-1*, 2.45), ***CAB39L*** (0.85), ***PRKAA2*** (1.24), ***PRKAG2*** (1.38), ***TBC1D1*** (1.86), ***GLUT4*** (3.16), ***CPT1A*** (1.07), ***ACACB*** (0.89), ***EEF2K*** (1.13), ***CCND1*** (2.36)	*CAMK2D* (1.28), ***CDKN1B*** (1.39), ***IL6*** (3.97), ***IL6R*** (1.92), ***PRKAG2*** (2.04), ***ACACB*** (2.47), ***EEF2K*** (0.68), ***CCND1*** (–0.93)
	Downregulated	***ANGPT2*** (–0.66), *TEK* (–1.88), ***PP2A*** (–0.66), *PFKFB3* (–1.33), ***PFKFB4*** (–2.84), ***SREBF1*** (–1.20), ***CREB5*** (–1.9), *HMGCR* (–0.77), ***RPTOR*** (–0.41), ***EIF4EBP1*** (–0.82), ***CCNA2*** (–3.65)	***PP2A*** (–0.97), ***PFKFB4*** (–1.90), ***SREBF1*** (–0.86), *LIPE* (–1.04), *SCD* (–1.51), ***CREB5*** (–1.53), *GYS1* (–0.58), ***RPTOR*** (–0.59), ***EIF4EBP1*** (–0.90), *ULK1* (–1.18)	***BCL2*** (–0.98), ***ANGPT2*** (–1.45), ***PFKFB4*** (–1.59), ***SREBF1*** (–1.97), ***CREB5*** (–1.77), ***CCNA2*** (–1.90)
Glycolytic metabolism	Upregulated	*ALDOA* (0.89), *ALDOC* (1.71), ***ENO2*** (1.64), ***PFKP*** (0.91), *PGAM2* (6.17), *GCK* (1.64), *ASPA* (2.68). ***LDHA*** (0.99)	***ENO2*** (2.60), ***PFKP*** (1.15), ***LDHA*** (1.39), *LDHD* (3.30)	***ENO2*** (1.54), *LDHC* (2.83)
	Downregulated	***HK1*** (–0.68), *HK2* (–2.45), ***GPT2*** (–1.48)	***HK1*** (–0.84)	***GPT2*** (–1.65), *LDHB* (–0.64)

		DEGs[a] (log_2FC)		
Pathway group	**Gene expression in EcE**	**Perivascular cells**	**Stromal cells**	**Endothelial cells**
Oxidative metabolism	Upregulated	*PDHA1* (0.67), *PDHB* (0.63), ***DLD*** (0.50). ***ACADL*** (7.76), ***CPT1A*** (1.51), ***CYP2U1*** (1.06). *IDH3B* (0.65), *PCK1* (1.86), *SDHC* (0.49), ***SDHD*** (0.77), *SUCLA2* (0.48), ***SUCLG1*** (0.52). ***GPX3*** (5.67), ***MGST3*** (1.96)	***DLD*** (0.57), ***ACADL*** (4.98), ***CPT1A*** (1.07), ***CYP2U1*** (1.61), ***SDHD*** (0.64), ***SUCLG1*** (0.48), *COX17* (0.92), *NDUFA4L2* (3.67), *NDUFB9* (0.64), *UQCRFS1* (0.43), ***GPX3*** (3.77), ***MGST3*** (1.26)	*ACADS* (0.88), ***SDHD*** (0.57), *ATP6V1G2* (2.67), *COX7A2L* (0.57), ***MGST3*** (0.52)
	Downregulated	***IDH2*** (–1.21), ***GPX7*** (–2.12), ***PGD*** (–1.52)	***IDH2*** (–0.70), *NDUFA6* (–0.56), *NDUFB11* (–0.58), *COX5A* (–0.51), *COX6C* (–0.69), *UQCRQ* (–0.55), ***GPX7*** (–1.10), ***PGD*** (–0.89)	*ME1* (–1.63), ***IDH2*** (–1.81), *LHPP* (–0.79), *UQCRC1* (–0.65), ***GPX7*** (–1.13), ***PGD*** (–0.92)
Biosynthetic pathways	Upregulated	***ACACB*** (0.93), *OLAH* (3.90), ***HACD1*** (2.86), *HACD4* (1.51), ***HSD17B12*** (1.29), *TECR* (0.59), *PGM2* (1.42), ***PRPS1*** (1.26), ***RBKS*** (1.99), *TKTL1* (2.80)	***ACACB*** (0.89), ***ELOVL2*** (3.38), ***HACD1*** (2.30), ***HSD17B12*** (0.79), ***PRPS1*** (0.62), ***RBKS*** (0.65), *TKTL1* (1.87)	***ACACB*** (2.47), *ACSL5* (0.69), ***ELOVL2*** (4.09), *ELOVL7* (7.36), ***RBKS*** (1.63)
	Downregulated	*ACSBG1* (–2.77), *ELOVL2* (–2.95), ***ELOVL4*** (–1.31), ***ELOVL6*** (–0.79), *HACD3* (–0.68), ***PGD*** (–1.52), *PRPS2* (–0.87), *PGLS* (–0.42)	***ELOVL4*** (–0.94), ***ELOVL6*** (–1.01), ***PGD*** (–0.89)	*ACSF3* (–0.52), ***PGD*** (–0.92)

[a] Statistically significant values ($p_{adj} < 0.05$). Negative log_2FC (fold change) corresponds to the reduced gene expression, positive log_2FC corresponds to the increased gene expression in ectopic cells. DEGs found in more than one cell type are marked in bold. Abbreviations: DEGs – differentially expressed genes; EcE – ectopic endometrium.

5.1.2 Steroidogenesis in perivascular, stromal and endothelial cells of endometriotic lesions

As steroid hormones affect tissue metabolism, we extended our analysis to examine the expression level of steroidogenic genes. In EcE, we found a decreased expression of *PGR* and *HSD17B2*, involved in the formation of active P4, while inactivators of P4 were upregulated (*AKR1C1*, *AKR1C2*), as shown in Table 5. The transcriptomic changes of estrogen signaling in EcE were characterized by downregulation of *ESR1* and *HSD17B2*, involved in E2 conversion to a less

potent estrone, and upregulation of *ESR2* and *HSD17B8*, involved in interconversion of estrone and E2. Additionally, we identified a differential regulation of genes related to androgen, steroid, and cortisol synthesis and metabolism. The analysis of steroidogenic genes between EuE from women with endometriosis (our dataset) and EuE from women without endometriosis from external dataset (Huang et al., 2023), revealed no difference in steroidogenesis.

Table 5. Statistically significant differentially expressed genes coding for proteins involved in the regulation of synthesis, conversion and metabolism of steroids in perivascular, endothelial, and stromal cell types of ectopic endometrium compared to eutopic endometrium. Reproduced with permission from Sarsenova et al., 2024, Communications Biology, licensed under the Creative Commons Attribution 4.0 International License.

		Cell type, log_2FC^a ($p_{adj} < 0.05$)			
Related steroid	**Gene**	**Peri-vascular**	**Stromal**	**Endo-thelial**	**Gene function**
Progesterone	*PGR*	–1.94	NS[b]	–4.66	Progesterone receptor
	AKR1C1	NS	2.06	2.33	Progesterone inactivation
	AKR1C2	NS	2.51	NS	Progesterone inactivation
	HSD17B2	NS	NS	–0.48	Formation of active progesterone
Estrogen	*ESR1*	–3.12	–1.14	–4.72	Estrogen receptor alfa
	ESR2	1.84	NS	NS	Estrogen receptor beta
	HSD17B8	1.00	NS	NS	Interconversion of estrone and estradiol
	HSD17B2	NS	NS	–0.48	Estradiol conversion to estrone
Cholesterol	*CYP11A1*	–3.98	NS	NS	Steroid hormone precursor
Cortisol	*HSD11B1*	NS	2.55	NS	Cortisone conversion to cortisol
	HSD11B2	–1.60	NS	NS	Cortisol conversion to cortisone
Androgen	*AR*	–0.93	NS	NS	Androgen receptor
	SRD5A1	–0.75	NS	NS	Testosterone conversion to DHT
	SRD5A3	–1.01	NS	NS	Testosterone conversion to DHT
	HSD17B6	NS	5.53	NS	Androgen catabolism
Ketones	*HSD17B11*	NS	1.16	1.18	Ketone metabolism

[a]Negative log_2FC (fold change) corresponds to the reduced gene expression, positive log_2FC to the increased gene expression in ectopic cells. [b]NS – statistically non-significant value ($p_{adj} > 0.05$).

5.1.3 Differential expression of cell cycle genes in EcE vs EuE

As cell growth and proliferation are processes interconnected with metabolic activity, we examined proportions of perivascular, stromal and endothelial cells of EuE and EcE in each cell cycle phase. In EuE, we found over 60% of cells expressing genes related to G1 phase of cell growth, 16% to 23% of cells in S phase of DNA synthesis and only 9% to 16% of cells in G2/M phase related to cell division/mitosis (Figure 9). By contrast, in EcE we identified higher proportions of cells in S phase (23% – 43%), G2/M phase (21% – 30%) and lower proportions in G1 phase (36% – 50%) compared to EuE. The statistical significance of observed differences between EuE and EcE proportions of each cell type was confirmed with Fisher's exact test, with exception for stromal cell population in G2/M phase.

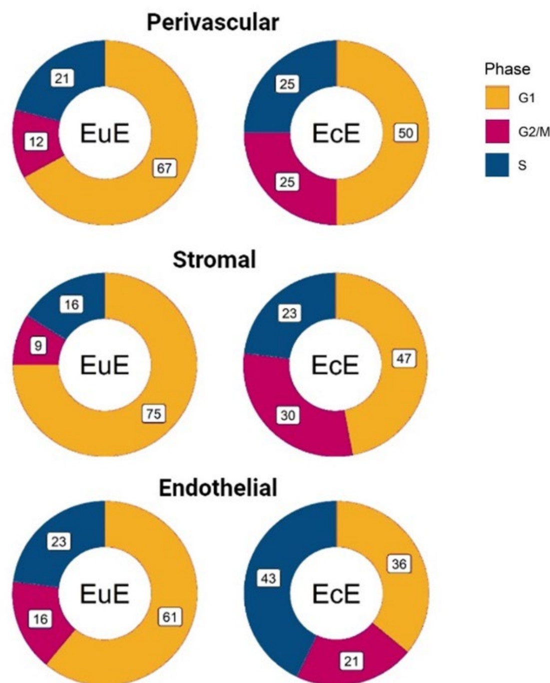

Figure 9. The proportions of perivascular, stromal and endothelial cells in cell cycle phases in eutopic endometrium (EuE) and ectopic endometrium (EcE). G1 – cell growth phase (in yellow), S – DNA synthesis phase (in blue), and G2/M – checkpoint and mitosis phase (in red). The numbers in white squares correspond to the percentages of the cells in each cell cycle phase. Reproduced with permission from Sarsenova et al., 2024, Communications Biology, licensed under the Creative Commons Attribution 4.0 International License.

5.2 Study II: The effect of hypoxia on transcriptome and protein kinases in endometriosis

5.2.1 Hypoxia-mediated alterations in transcriptome and PK activity of eutopic and ectopic endometrial stromal cells

For this study, we isolated primary EuESCs and EcESCs and exposed them to hypoxia (1% oxygen) or normoxia (20% oxygen) for 48 hours. To evaluate the effect of hypoxia on the activity of four PKs of interest (Akt, CK2, PKAc, and ROCK), we used photoluminescent probes to the PKs and measured their time-delayed photoluminescence. The activity of PKAc and Akt was reduced in EcESCs cultured under hypoxia (Figure 10A, $P < 0.05$ and $P \leq 0.01$, respectively). There was no statistically significant difference in CK2 and ROCK activity between culture conditions in both EcESCs and EuESCs.

As we were interested in the impact of hypoxia on global transcriptome of stromal cells, we performed mRNA-seq followed by the differential expression analysis in comparison groups, as shown on Figure 10B. The analysis revealed 116 overexpressed DEGs in EuESCs under hypoxia, mostly associated with glycolysis/gluconeogenesis, biosynthesis of amino acids, carbon metabolism, and HIF-1 signaling pathways. In EcESCs exposed to hypoxia, the transcriptome was not different from that of EcESCs cultured under normoxic condition, with a single statistically significant DEG, *TGFBI*. The comparison of EuESCs vs EcESCs under hypoxia showed 20 DEGs, while in normoxia conditions there were 163 DEGs between EcESCs and EuESCs. The upregulated DEGs in the latter comparison were associated with focal adhesion, PI3K-Akt signaling, ECM-receptor interaction pathways, and anatomical structure morphogenesis, cell adhesion, cell migration, blood vessel morphogenesis, and blood vessel development biological processes. We also compared two conditions representing more physiological settings of both endometriotic lesions and EuE: EcESCs cultured in hypoxia vs EuESCs in normoxia. This analysis showed 569 DEGs, the upregulated DEGs were related to sugars and amino acids metabolism pathways, glycolysis, ECM-receptor interaction, focal adhesion, and PI3K-Akt signaling pathways, and wound healing, blood vessel development, and actin cytoskeleton development biological processes. When we compared DEGs from all comparison groups, we found that *TGFBI* was upregulated in all comparisons (Figure 10C).

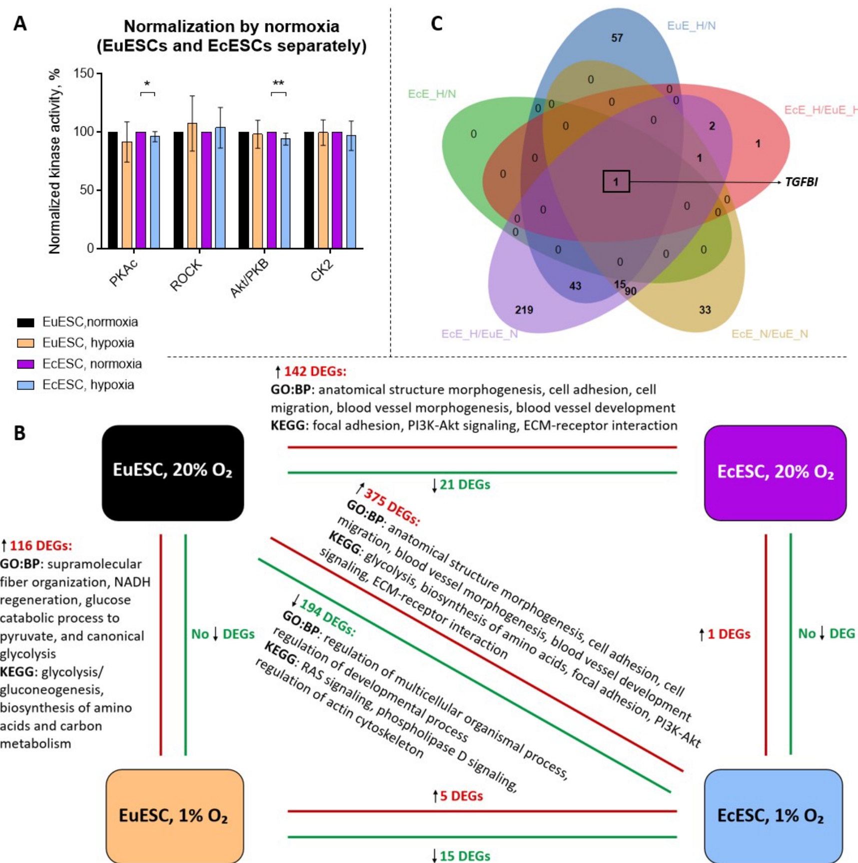

Figure 10. Protein kinase activity and mRNA profiles in EuESCs or EcESCs incubated in different oxygenation conditions. **A**. The normalization was done by normoxia in EuESCs and EcESCs separately, set to 100%. The PKs of interest are listed below the graph and the types of cells and incubation conditions are on the right. Each column shows the mean ± standard deviation for samples obtained from 5 different patients. Asterisks indicate comparisons (t-test with Welch's correction): ** $P \leq 0.01$, * $P \leq 0.05$; only statistically significant comparisons are shown. **B**. The representative biological processes (GO:BP) and enriched pathways (KEGG) in the comparison groups. The analysis is based on statistically significant DEGs, $P_{adj} < 0.05$. The arrows next to the DEGs indicate upregulation (red) or downregulation (green). **C**. Venn diagram depicting overlapped upregulated DEGs between comparison groups. The analysis is based on statistically significant DEGs, $P_{adj} < 0.05$. Abbreviations: Akt/PKB – protein kinase B; CK2 – casein kinase 2; DEGs – differentially expressed genes; EuESCs – eutopic endometrial stromal cells; EcESCs – ectopic endometrial stromal cells; EcE_H/N – EcESCs exposed to hypoxia vs normoxia; EuE_H/N – EuESCs exposed to hypoxia vs normoxia; EcE_H/EuE_H – EcESCs vs EuESCs both exposed to hypoxia; EcE_N/EuE_N – EcESCs vs EuESCs both exposed to normoxia; EcE_H/EuE_N – EcESCs exposed to hypoxia vs EuESCs exposed to normoxia; PKAc – cAMP-dependent PK; ROCK – Rho-dependent PK. Reproduced and modified with permission from Sarsenova et al., 2024, Frontiers in Endocrinology, licensed under the Creative Commons Attribution 4.0 International License.

5.2.2 *In vitro* and *ex vivo* TGFBI expression in endometriosis

Previous studies have demonstrated that TGFBI is a secreted protein (Janša et al., 2021; Janša et al., 2023). To investigate whether TGFBI protein expression in stromal cells correlates with its mRNA expression pattern, we looked at the levels of secreted TGFBI in cell culture media. In line with findings at mRNA level, we identified higher levels of secreted TGFBI in EuESCs exposed to hypoxia vs normoxia and in EcESCs (Figure 11A). Since stromal cell cultures represent a simplified model of EuE and EcE, we decided to explore TGFBI expression in available *ex vivo* models. Thus, we checked TGFBI mRNA and protein levels in EuE and EcE tissue samples from women with or without endometriosis in the proliferative and secretory phases of menstrual cycle using qRT-PCR and IHC. In EuE from women with and without endometriosis, *TGFBI* mRNA level was higher in the proliferative phase (Figure 11B). TGFBI protein signal in EuE was also stronger in the proliferative phase compared to secretory phase (Figure 11C & D). In paired EcE and EuE in the secretory phase, TGFBI protein expression was stronger in lesions (Figure 11D & F). The immunostaining revealed TGFBI localization to the stroma and around the blood vessels of both EuE and EcE (Figure 11C & F).

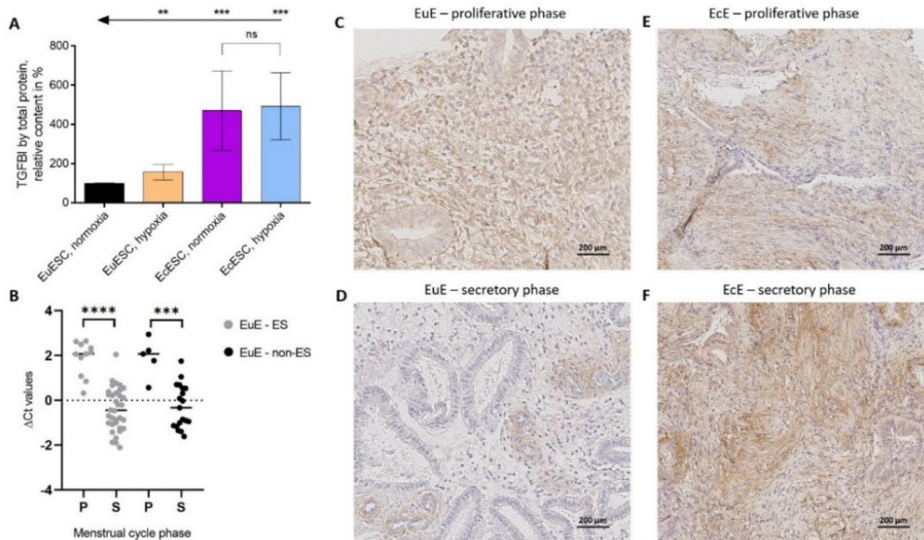

Figure 11. TGFBI mRNA and protein expression in eutopic and/or ectopic endometria from women with or without endometriosis. **A**. Secreted TGFBI protein levels in spent cell culture media relative to the total protein content in the corresponding cell lysates. For each patient, the data was normalized to the relative TGFBI content measured for the EuESCs incubated in normoxia (= 100%). Each column shows the mean ± standard deviation for samples obtained from 5 different patients. Arrows and asterisks indicate pairwise comparisons (t-test with Welch's correction): *** $P \leq 0.001$, ** $P \leq 0.01$. **B**. TGFBI mRNA expression in eutopic endometrial tissues from women with (light grey)

or without (black) endometriosis (N = 45 and N = 24, respectively) in proliferative (P) and secretory (S) phases of menstrual cycle. ΔCt values correspond to the relative expression level of TGFBI, the thick line is the median. An unpaired two-tailed t-test with Welch's correction was applied, **** P value < 0.0001, *** P value < 0.001. **C** – **F**. TGFBI protein localization in EuE and EcE from women with endometriosis at the proliferative (C, E) and secretory (D, F) phases of the menstrual cycle. Scale bar 200 μm. Abbreviations: EcESCs – ectopic endometrial stromal cells; EuESCs – eutopic endometrial stromal cells; EuE – eutopic endometrium; EcE – ectopic endometrium; ES – endometriosis; non-ES – non-endometriosis; ns – not significant. Reproduced with permission from Sarsenova et al., 2024, Frontiers in Endocrinology, licensed under the Creative Commons Attribution 4.0 International License.

5.3 Study III: Carboxypeptidase inhibitor LXN in endometrium and endometriosis

5.3.1 LXN expression across the menstrual cycle in eutopic and ectopic endometrium

Previous transcriptomic and proteomic studies of our lab identified higher expression of a carboxypeptidase inhibitor LXN in endometriotic lesions. In this study, we aimed to investigate LXN in the context of endometrium and its relevance to endometriosis development. First, we analyzed mRNA expression of *LXN* in endometrial tissues across the menstrual cycle using qRT-PCR. We examined EuE and/or EcE from three groups of women: with endometriosis, without endometriosis and healthy volunteers. In EuE of women with and without endometriosis (Figure 12A), we found menstrual cycle-specific expression pattern with the highest *LXN* level in the late secretory phase and lowest in the early secretory phase. In EuE of healthy women in their early vs mid-secretory phase, we found a higher *LXN* expression in the mid-secretory phase (Figure 12B). In paired samples of EuE and EcE from women with endometriosis in the proliferative and mid-secretory phase, we found higher *LXN* expression in EcE vs EuE in both phases (Figure 12C).

To localize LXN protein within endometrial tissues, we performed IHC staining of EuE and EcE in the proliferative, early and late secretory phases. In line with mRNA data (Figure 12A), immunoreaction in endometrial stroma revealed higher LXN expression in the proliferative and late secretory phases compared to the early secretory phase (Figure 12D). The trend of higher *LXN* expression in EcE vs EuE (Figure 12C) was also observed at protein level, with stronger LXN signal in endometriotic lesions.

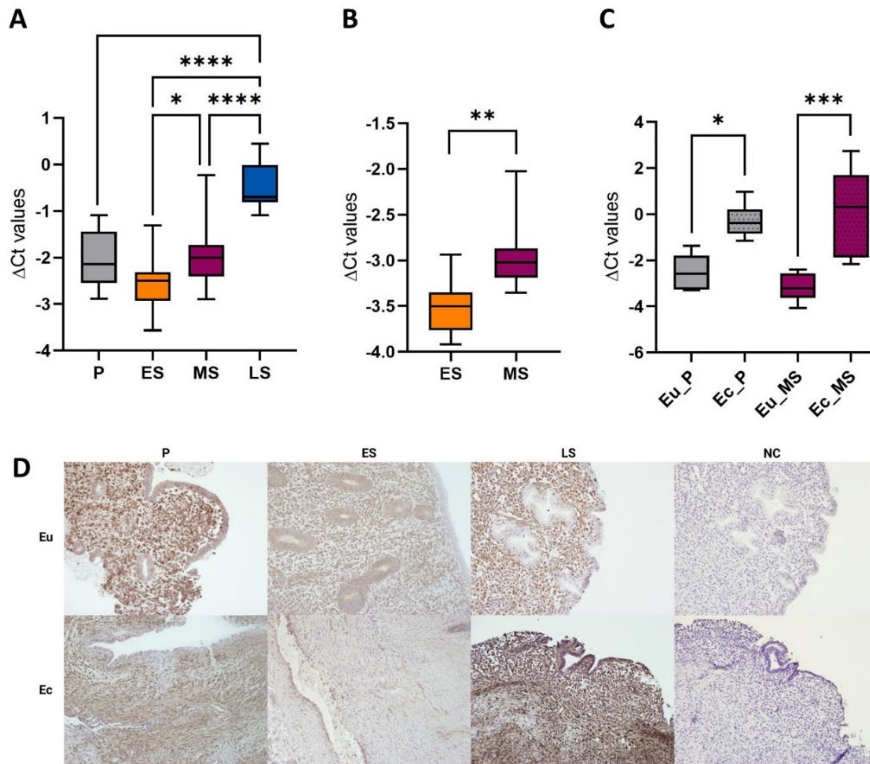

Figure 12. LXN expression in endometrium and endometriotic lesions. **A**. Expression of *LXN* in endometrium throughout the menstrual cycle (in a combined group of ENDO, n=34 and non-ENDO, n=27). **B**. Expression of *LXN* in endometrium of healthy women in ES (n=12) and MS phases (n=12). **C**. Expression of *LXN* in paired eutopic and ectopic endometrium of endometriosis patients (n=12). Statistical difference is shown only for comparisons where it is significant. Y-axis shows relative LXN mRNA level normalized by SDHA (ΔCT). One-way ANOVA was applied, **** correspond to p value < 0.0001, *** p value < 0.001, ** p value < 0.01, * p value < 0.05. **D**. Representative IHC images of LXN expression in eutopic (Eu) and ectopic (Ec) endometrium in different menstrual cycle phases. Original magnification ×200. Abbreviations: Ec – ectopic endometrium, ES – early-secretory phase, Eu – eutopic endometrium, LS – late-secretory phase, MS – mid-secretory phase, NC – negative control, P – proliferative phase. Reproduced with permission from Sarsenova et al., 2024, Genes, licensed under the Creative Commons Attribution 4.0 International License.

5.3.2 *LXN* silencing affects cell viability and migration

Considering the higher expression of LXN predominantly in the stromal compartment, we decided to further explore its role in cell metabolism (viability) and migratory capability, both important for endometriosis development. For that, first we silenced *LXN* in endometrial stromal cell line St-T1b, which resulted in successful reduction of *LXN* expression. Then, we performed Transwell migra-

tion assay, which revealed that stromal cells transfected with siLXN had reduced migration through the Transwell membrane compared to controls (Figure 13A–B). Next, we performed MTT assay to assess cell metabolism as a readout of cell viability, which showed 1.7-fold increased cell viability of siLXN transfected stromal cells compared to controls (Figure 13C).

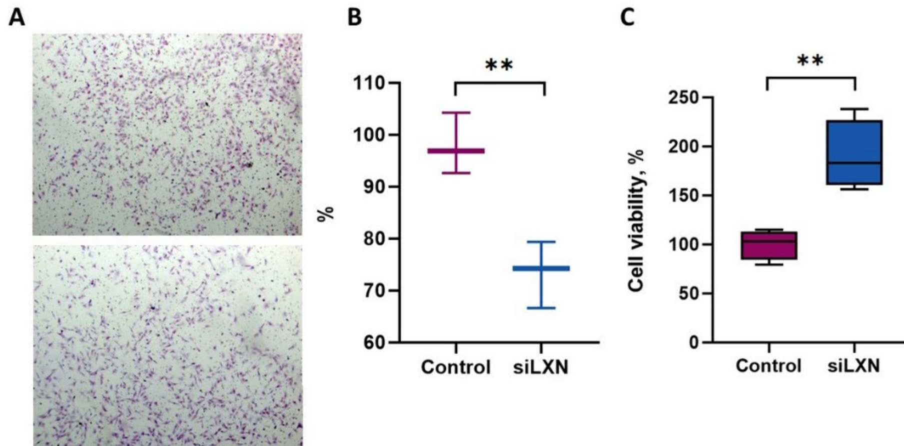

Figure 13. The silencing of *LXN* affects the migration and viability of endometrial stromal cells. **A**. Migration capability of St-T1b cells treated with control (upper panel) or *LXN*-siRNA (siLXN, lower panel) was assessed by Transwell assay, and the migrated cells were quantified (original magnification ×50). **B**. The migration ability of siLXN treated cells is calculated as a percentage of control mean. **C**. The viability of St-T1b cells after transfection with *LXN*-siRNA and control siRNA. The viability of the control cells was defined as 100%. ** correspond to p value < 0.01. Abbreviations: LXN – latexin; siLXN – small interfering RNA targeting *LXN*; siRNA – small interfering RNA; St-T1b – immortalized human endometrial stromal cell line. Reproduced with permission from Sarsenova et al., 2024, Genes, licensed under the Creative Commons Attribution 4.0 International License.

6. DISCUSSION

Metabolism and hypoxia in endometriosis have been mainly studied through the key metabolic or hypoxia markers. In Study I and Study II, we performed global transcriptome profiling of endometrial tissues at the single-cell level and of primary stromal cells, respectively, to explore metabolic or hypoxia-driven signatures of ectopic and eutopic endometria.

6.1 Metabolic activity and steroidogenesis of endometriotic lesions differ from that of eutopic endometrium

Previous metabolic research on endometriosis has primarily focused on a specific set of key metabolic genes or profiling specific cell types, like primary stromal or mesothelial cells (Kasvandik et al., 2016; Lee et al., 2019; McKinnon et al., 2014; Young et al., 2014a; Zheng et al., 2021). Recent scRNA-seq transcriptomic studies of endometriosis have characterized cell populations and their interactions, immune and inflammatory status, fibrosis and angiogenesis in endometriotic lesions (Fonseca et al., 2023; Huang et al., 2023; Tan et al., 2022; Zhu et al., 2023). Considering cellular heterogeneity and unique endometriotic microenvironment, it is also important to investigate the metabolism at a single-cell level to better understand lesion development and growth. However, metabolic profile of endometriotic lesions have not yet been covered.

In Study I, we identified nine major cell types in endometrium, in concordance with previous scRNA studies (Garcia-Alonso et al., 2021; Ma et al., 2021; Queckbörner et al., 2021; Tan et al., 2022), and explored metabolic profile of endometriotic lesions at single-cell transcriptomic level. We revealed variability in metabolic activity across different cell types in EcE compared to EuE. The differences in metabolic pathways were mostly observed in perivascular, stromal and endothelial cells, while immune cells and epithelial cells showed subtle difference between EcE and EuE. Therefore, further analysis of transcriptomic metabolic profiles focused on perivascular, stromal and endothelial cell populations.

AMPK signaling and HIF-1 signaling are evolutionary conserved pathways, known to regulate cell metabolism in response to energy and substrates demands under different oxygenation conditions (Garcia & Shaw, 2017; Herzig & Shaw, 2018; Vander Heiden et al., 2009). In endometriosis, AMPK has been shown to participate in inflammatory process and apoptosis, while HIF-1 promotes angiogenesis and survival in lesions (Assaf et al., 2022; Wu et al., 2019). In Study I, we identified dynamic metabolic processes in EcE that were activated or inhibited under the regulation of AMPK and/or HIF-1 signaling pathways (Figure 14). Specifically, we found an upregulation of AMPK-regulated genes involved in the uptake of substrates for glycolysis and oxidative metabolic pathways, key genes of the TCA cycle, and genes involved in conversion of pyruvate to lactate or

acetyl-CoA. This expression profile indicates a co-activation of glycolytic and oxidative ways of energy metabolism. A similar process of co-activation, referred to as a "hybrid" use of glycolysis and OXPHOS has been shown in cancer and proposed as a concept of "waves" occurring in cancer at the transcriptomic level (Bellance et al., 2009; Jose et al., 2011; Smolková et al., 2011; Yu et al., 2017).

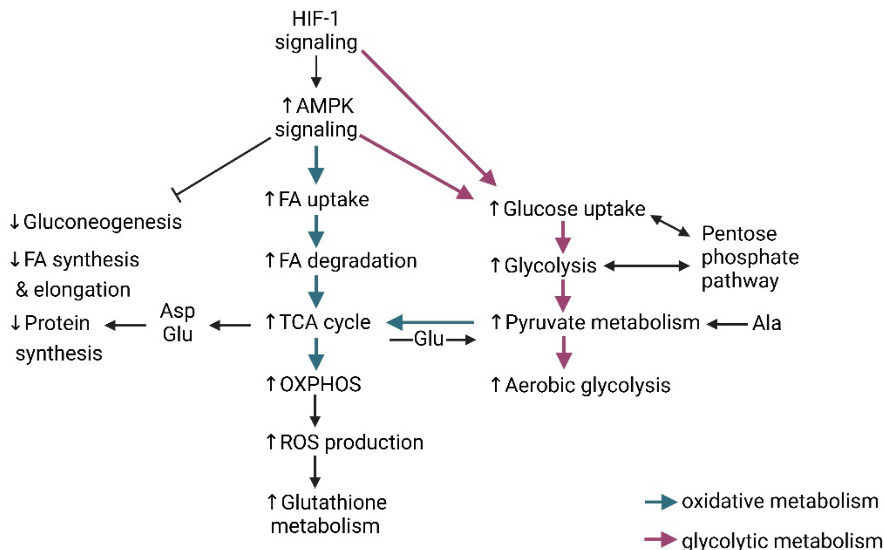

Figure 14. Interconnection between metabolic pathways involved in cellular metabolism. HIF-1 signaling and AMPK signaling are regulatory pathways for the metabolic pathways involved in glycolytic and oxidative metabolism, biosynthesis of macromolecules and nucleic acids. Pink arrows represent glycolytic metabolism, blue arrows represent oxidative metabolism. Abbreviations: Ala – alanine; AMPK – AMP-activated protein kinase; Asp – aspartate; FA – fatty acid; Glu – glutamate; HIF-1 – hypoxia-inducible factor-1; OXPHOS – oxidative phosphorylation; ROS – reactive oxygen species; TCA – tricarboxylic acid cycle. Reproduced with permission from Sarsenova et al., 2024, Communications Biology, licensed under the Creative Commons Attribution 4.0 International License.

The observed transcriptomic co-activation of glycolytic and oxidative metabolism was predominantly observed in perivascular cells of EcE. The endometrial stroma comprises stromal, epithelial and perivascular/vascular compartments, where stromal, perivascular and endothelial cells actively interact with each other (Zhu et al., 2023). Perivascular cells have been shown to play role in endometrial stroma regeneration and mediation of angiogenesis (Cousins et al., 2018; Cousins et al., 2022; Spitzer et al., 2012). Thus, considering the unfavorable environment of lesions with the need for blood vessel sprouting to support their growth, it is likely that perivascular cell niche plays a crucial role in lesion development and growth. Therefore, expectedly perivascular cells would employ various pathways

to produce energy. Additionally, their proximity to blood vessels may offer them advantages in using oxidative metabolism, compared, for example, to stromal cells.

The metabolism of stromal cells, representing a major compartment of endometrium, has been previously studied *ex vivo* and *in vitro* (Kasvandik et al., 2016; Lee et al., 2019; Young et al., 2014a). These studies showed that stromal cells utilize glycolysis over oxidative pathways, which resembles the Warburg effect observed in cancer or highly proliferative cells (Kasvandik et al., 2016; Vaupel et al., 2019; Young et al., 2014a). In our *ex vivo* study, we observed in ectopic stromal cells, and to a lesser extent in endothelial cells, the upregulation of genes involved in both glycolytic and oxidative pathways. Overall, the less pronounced activation of metabolic pathways in these two cell types compared to perivascular cells, may indicate similar metabolic activity in both EuE and EcE during the proliferative phase of the menstrual cycle, due to active tissue growth and vascularization (Gambino et al., 2002).

Steroid hormones regulate metabolic response in endometrial tissues, e.g. E2 is involved in endometrial tissue growth, angiogenesis and cell survival (Kazi et al., 2009; Monsivais et al., 2014; Monsivais et al., 2016). Previous studies have shown that EcE exhibits P4 resistance, increased E2 synthesis and E2 activity mediated via ERβ (Bulun et al., 2010; Delvoux et al., 2009; Maekawa et al., 2019; Rizner et al., 2006; Rizner, 2009; Zeitoun et al., 1998). Consistent with these findings, our transcriptomic analysis of steroidogenesis also revealed progesterone resistance (*PGR*, *AKR1C1*, *AKR1C2*) and enhanced estrogen signaling (*ESR1*, *ESR2*, *HSD17B2*, *HSD17B8*), primarily observed in perivascular cells of EcE. The differences in metabolic activity and steroidogenesis in perivascular cells of EcE vs EuE are shown in Figure 15. The link between cell metabolism and the cell cycle has been well established, as dividing cells have high energy demands. The majority of stromal cells of EuE at the proliferative phase of the menstrual cycle were found to be in G1 phase of macromolecular synthesis (Lv et al., 2022; Wang et al., 2020). McKinnon et al. reported that around 70% of isolated endometrial stromal cells were in G1, with only 15% present in either the G2/M or S phases (McKinnon et al., 2022). We also identified more than 60% of eutopic perivascular, stromal and endothelial cells in G1 phase. In EcE on the other hand, we found a greater percentage of cells expressing S and G2/M phase genes.

The transcriptomic analysis of energy metabolism and steroidogenesis in EuE from women with endometriosis compared to controls (women without endometriosis from external dataset) showed no major difference, suggesting similar metabolic profiles in the same tissue type regardless of presence of endometriosis. It is then likely that the observed difference in metabolic activity of ectopic cells arises from the difference in microenvironment (intrauterine vs peritoneal).

Generally, cells may use multiple pathways simultaneously or adjust their metabolism in response to environmental changes or shifts in energy or substrate demands. Thus, it is important to consider that cellular metabolism can rapidly change, meaning that observed processes may represent only the current metabolic state. Additionally, metabolic activity of cells in endometriotic lesions may depend on various factors, such as lesion localization, conditions of microen-

vironment (e.g. hypoxia), and interactions with neighboring cells and tissues. Given the lack of available comprehensive model organism of endometriosis, we need to apply different techniques to deepen our understanding of the mechanisms underlying lesion growth. In summary, Study I revealed that perivascular cell population in EcE exhibits altered metabolic activity and may represent a potential target for non-hormonal metabolic therapies in endometriosis management. The findings need to be further explored using powerful techniques like spatial metabolomics that profiles proteins and metabolites with capturing information on interactions between single cells (Hu et al., 2023c).

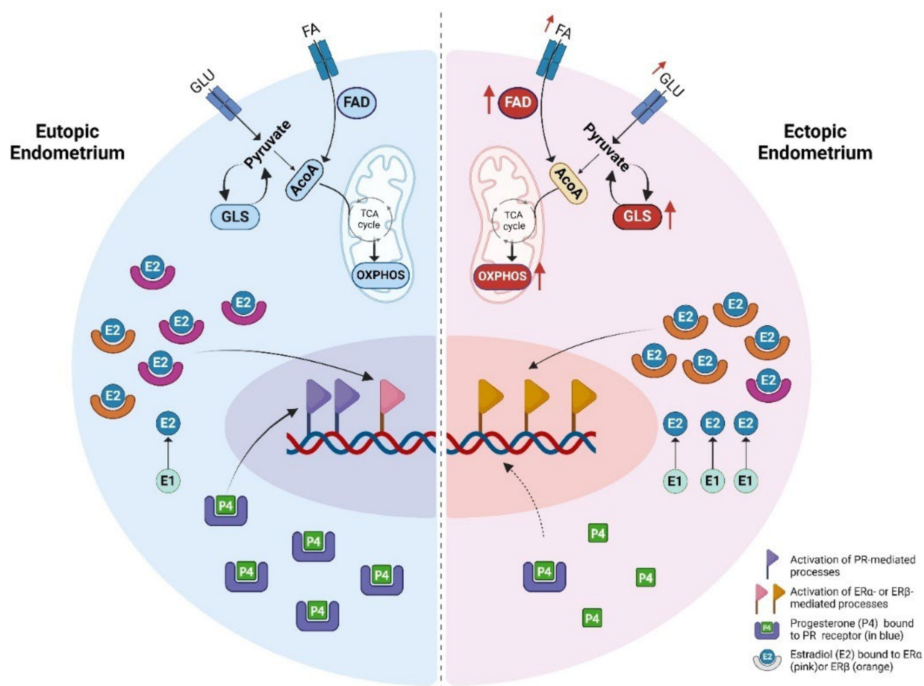

Figure 15. A proposed model of interconnected mechanisms of steroid hormone regulation and altered cellular metabolism in perivascular cells of ectopic endometrium compared to eutopic endometrium. In the ectopic endometrium (a half of a cell depicted on the right side) the following changes are observed compared to eutopic endometrium (on the left side): reduced production of progesterone receptor (PR, blue) contributing to progesterone (P4, green) resistance; decreased level of ERα and increased level of ERβ (in pink and orange, respectively) and increased conversion of estrone (E1, light blue) into estradiol (E2, blue), leading to a nuclear activation of aberrant set of estrogen target genes; upregulation of genes related to increased glucose and FA uptake, activated FA degradation (FAD), and simultaneously increased OXPHOS and glycolysis (GLS) activities. Abbreviations: FA – fatty acids, GLU – glucose, ACoA – acetyl-CoA, nuclear gene expression is indicated with flags, black arrows refer to sequence of the processes, red arrows refer to the activation of processes. Reproduced with permission from Sarsenova et al., 2024, Communications Biology, licensed under the Creative Commons Attribution 4.0 International License.

6.2 Hypoxia alters transcriptome of endometrial stromal cells and activates TGFBI axis in endometriosis

Hypoxia is a well-known driver of altered gene expression underlying hallmark processes in endometriotic lesions, among which angiogenesis and fibrosis play an essential role in endometriosis development and progression (Gambino et al., 2002; Vissers et al., 2024; Wu et al., 2019; Zhu et al., 2023). *In vitro* studies on stromal cell population have shown overexpression of key genes, like HIF1A, PDK1, and LDHA, in EcE at transcriptomic and protein levels, with increased uptake of substrates, production of metabolites and ATP (Lee et al., 2019; Zheng et al., 2021). In addition to HIF-1 signaling, TGF-β signaling, known for its pro-angiogenic and pro-fibrotic action in lesions, has been found to be activated in EcE (Ferrari et al., 2009; Lin et al., 2018; Young et al., 2017; Zhang et al., 2016a; Zhang et al., 2016b).

To explore hypoxia-driven changes in gene expression and cellular responses relevant in the context of lesion growth and survival, in Study II we profiled transcriptomes of EuESCs and EcESCs exposed to hypoxia. In addition, we checked the activity of four PKs, previously shown to be implicated in endometriosis pathogenesis. While previous research reported activating effect of hypoxia on PKAc and Akt (Lucia et al., 2020; Simko et al., 2017; Stegeman et al., 2012), we observed their slightly reduced activity in EcESCs exposed to hypoxia, which may be related to inhibitory effect of TGFBI on these kinases (Wen et al., 2011; Zhang et al., 2009). The observed subtle effect of hypoxia on kinase activity might be due to experimental conditions. We found that hypoxia caused changes in the transcriptome of primary stromal cells from EuE. Particularly, the eutopic cells activated glycolytic pathways with elevated expression of aerobic glycolysis and hypoxia markers, consistent with previous reports (Lee et al., 2019; Zheng et al., 2021). In contrast, stromal cells from EcE exhibited only a single gene, *TGFBI*, differentially expressed between oxygenation conditions. The weak/minor difference in mRNA expression between ectopic cells under hypoxia and normoxia may indicate a prior transcriptomic adjustment of ectopic cells to their hypoxic microenvironment. This adaptation likely persisted and resulted in similar gene expression profiles of isolated primary cells regardless of oxygen levels during *in vitro* culturing. In line with that, it has been previously demonstrated that two key hypoxia markers, HIF-1α and LDHA, were equally highly expressed in ectopic endometrial stromal cells under both normoxic and hypoxic conditions (Zheng et al., 2021).

Further, we compared ectopic cells cultured in hypoxia or normoxia to eutopic cells in normoxia and found activated processes of cell adhesion, cell migration, blood vessel morphogenesis, and blood vessel development. Previous studies have shown that under hypoxia, both eutopic and ectopic cells exhibited transcriptomic activation of genes associated with angiogenesis and tube formation (Fu et al., 2018; Hsiao et al., 2014; Wu et al., 2007). The processes of cell migration and angiogenesis, important for the establishment and growth of endometriotic lesions, are induced partially by TGF-β signaling (Chung & Han, 2022;

Ferrari et al., 2009; Lin et al., 2018; Omwandho et al., 2010; Young et al., 2015). Notably, we found that a target of TGF-β signaling, *TGFBI*, was a common DEG across all comparison groups, indicating the potential involvement of TGF-β signaling in these processes. In cancer, TGFBI was found to be associated with increased cell migration and glycolysis (Costanza et al., 2019). Although there are controversial findings on the activities of TGFBI in different tissues, it has been shown to play a role in angiogenesis, cell adhesion and fibrosis (Lee et al., 2021; Liu et al., 2021; Yang et al., 2022). TGFBI role in fibrosis has been demonstrated in lung tissue (Yang et al., 2022). In cultured lung fibroblasts, TGFBI, induced by TGF-β1, promoted collagen I and α-SMA expression, while silencing *TGFBI* reversed this pro-fibrotic process (Yang et al., 2022). Additionally, ROCK signaling was shown to be activated by TGF-β signaling in relation to pro-fibrosis in different tissues (Wu et al., 2021b; Xie et al., 2022). In our study, we found slightly elevated activity of ROCK under hypoxia in ectopic and eutopic cells. TGFBI has been recently reported to be elevated in serum and peritoneal fluid of women with endometriosis vs controls (Janša et al., 2021; Janša et al., 2023). We found that the level of secreted TGFBI in stromal cell cultures was increased under hypoxic condition and in ectopic vs eutopic cells, suggesting that TGFBI secreted by stromal cells at endometriotic lesion sites facilitates angiogenesis and fibrosis in endometriosis.

As cell culturing represents an isolated cell population, we also examined TGFBI mRNA and protein expression in EuE and/or EcE tissue biopsies from women with and without endometriosis. Quantitative analysis of mRNA expression in EuE showed elevated *TGFBI* levels in the proliferative phase, suggesting pro-angiogenic role of TGFBI in proliferating endometrium. A higher expression of TGFBI protein in proliferative phase, in particular in the stroma and around the blood vessels, aligns with its role in vessel growth. A stronger TGFBI staining in EcE compared with EuE may refer to its pro-fibrotic and angiogenic role in endometriosis.

To summarize, this study demonstrates the effect of hypoxia on gene expression with distinct influence on EuESCs vs EcESCs, highlighting the adapted transcriptome of ectopic cells to the hypoxic microenvironment. In addition, we identified increased TGFBI mRNA and protein levels under hypoxia and in ectopic vs eutopic cells that support its role in angiogenesis and fibrosis in endometriosis development. These findings emphasize the importance of further investigation of TGFBI function using functional assays and more physiologically relevant models of endometriosis.

6.3 LXN expression is menstrual cycle dependent and affects endometrial cell viability and migration

LXN has been studied in cancer and some other diseases, showing its involvement in the negative regulation of cell growth and proliferation, promoting apoptosis in cancer and acting as a tumor suppressor (Li et al., 2011; Ni et al., 2014; Xue et al., 2016). In murine hematopoietic stem cells, LXN inhibited proliferative activity and induced apoptosis, while *LXN* knockdown resulted in increased cell survival (Liang et al., 2007; Liang & Zant, 2008; Liu et al., 2017). Recent findings revealed a positive effect of *LXN* deletion on intestinal tissue regeneration, but also promoting inflammation in colitis (Li et al., 2020; Wang et al., 2024). However, LXN role in reproductive tissues and organs in health and disease is still largely unexplored.

In the previous endometriosis transcriptomic and proteomic studies performed in our lab, LXN was found to be elevated in ectopic stromal cells. To understand its role in endometrial tissue, in Study III we explored LXN expression in EuE and EcE with further investigation of its role in stromal cells viability and migratory ability. As gene expression in endometrial tissues is affected by the menstrual cycle phase, we examined *LXN* expression in EuE and/or EcE in different phases. Our results showed that mRNA expression was menstrual cycle-dependent; moreover, our findings of higher *LXN* expression in late secretory phase and in ectopic tissues may suggest a potential role of LXN in decidualized cells and endometriotic lesions. At the protein level, LXN expression was more prominent in endometrial stroma, consistent with a previous finding of *LXN* expression in endometrial stromal cells induced by macrophages (Eyster et al., 2010), which may indicate that *LXN* overexpression is partially influenced by microenvironment.

To test LXN involvement in stromal cell migration and viability, we performed *LXN* knockdown in endometrial stromal cell line St-T1b. This resulted in reduced migratory capacity and enhanced cell viability, suggesting a dual role of LXN. In particular, it includes increasing migratory potential of endometrial cells, supporting Sampson's theory, and inhibition of cell metabolism and viability, referring to its tumor suppressor function. The inhibitory effect of *LXN* silencing on cell migration was previously observed in prostate epithelial cells (Oldridge et al., 2013). The increased viability of *LXN*-silenced endometrial stromal cells indicates that the overexpression of LXN may have a negative effect on cell viability, as described in cancer studies. Gene expression in endometrium varies by the phase of menstrual cycle and regulated by ovarian hormones that affect cell viability and proliferation. EuE exhibits higher proliferative activity during the proliferative phase, whereas endometriotic lesions exhibit low proliferation regardless of menstrual cycle phase (Béliard et al., 2004; Critchley et al., 2020). Thus, these results may indicate a potential regulatory effect of LXN on endometrial cell proliferation and survival dependent on menstrual cycle phase and tissue type.

6.4 Study limitations and future directions

We acknowledge that our studies have some limitations. The main limitation in all three studies we faced with is the limited number of suitable samples from women with confirmed endometriosis. In Study I, the relatively small sample size included paired samples from only four women with confirmed endometriosis. Nevertheless, we identified major cell types and captured metabolic heterogeneity in both tissue types. With bioinformatic analysis that compared biological samples rather than single cells, we showed consistent results of metabolic activity within study groups accounting for contribution from each patient. In Study II, due to a limited availability of paired eutopic and ectopic endometrial samples, we could not perform statistical analysis of TGFBI mRNA and protein expression in the proliferative vs secretory phases. In Study III, we were not able to evaluate the statistical significance of IHC results of paired samples or perform other methods of protein quantification. In addition, the type of endometriotic lesions used in qRT-PCR analysis of paired samples (Study III) was limited to endometriomas, as peritoneal lesions were not available. However, our previous proteomic study on peritoneal lesions showed high expression of LXN in lesions, suggesting a correlation of these results with LXN expression at mRNA level.

Additionally, in Study I we did not include control EuE samples from women without endometriosis, therefore we performed the same analysis on external dataset that demonstrated no major difference in steroidogenesis and metabolic activity between EuE from women with endometriosis compared with controls. Also, we identified a very small population of epithelial cells most probably due to their loss during experimental part of the study, similar issues were previously reported due to technical challenges. Another limitation of this study is that the findings are based solely on the analysis of transcriptomic data. However, further studies may explore metabolic activity in more diverse groups of patients and controls accounting for interpopulation variability with functional assays for validation of results.

Study II has another limitation related to a limited number of PKs, as we used previously developed in-house synthesized probes to this set of kinases. In the transcriptomic analysis we identified a relatively small number of DEGs in some of comparison groups, that could be likely influenced by interpatient variability within groups impacting the statistical significance of comparisons. Future research may focus on larger study groups to explore the transcriptomic pattern induced by hypoxia both *in vitro* and *ex vivo*, and further investigate and functionally validate the findings of TGFBI role in endometriosis as well as its downstream targets and interactors.

Considering above-mentioned limitations of Study III, further research may include functional analysis of LXN expression (including co-culturing with other cell types like macrophages or mesothelial cells from peritoneum) using larger sample size and more complex model systems (e.g. assembloids, animal models with modified LXN expression) to elucidate its role in endometriotic lesion development.

7. CONCLUSIONS

According to the main findings of the studies presented in this thesis, the following conclusions can be drawn:

1. Perivascular, stromal and endothelial cells of EcE exhibited co-activation of glycolytic and oxidative metabolism, along with altered steroidogenesis, suggesting a coping mechanism to sustain the high energy demands in lesions. Metabolic co-activation was predominantly observed in perivascular cell population, known for its role in endometrial stroma repair and angiogenesis, making these cells a potential target for metabolic treatment of endometriosis.
2. Exposure to hypoxia induced specific response in EuESCs and EcESCs, indicating distinct default transcriptomic signatures of stromal cells in EuE and EcE. Ectopic cells exhibited hypoxic transcriptome profile regardless of oxygen concentration, while eutopic cells under hypoxic condition show similarity with ectopic cells.
3. We identified an upregulation of *TGFBI* gene in EuESCs under hypoxia and in EcESCs, with its emerging pro-angiogenic and pro-fibrotic role in endometriosis pathogenesis.
4. LXN expression in endometrial tissues is menstrual cycle-specific, being the highest in the late secretory phase. Silencing *LXN* in stromal cells reduced cell migration but promoted cell viability demonstrating its potential role in these processes critical to endometriosis onset and development.

SUMMARY IN ESTONIAN

Endometrioosi rakuline profiil ja molekulaarsed mehhanismid

Endometrioos on reproduktiivses eas naistel esinev sage hormoonsõltuv ja krooniline põletikuline haigus, mis mõjutab umbes 190 miljonit naist kogu maailmas. Endometrioos avaldub kliiniliselt sageli kroonilise vaagnavalu, düsmenorröa ja subfertiilsusena. Seda haigust iseloomustab endomeetriumi taolise koe paiknemine kolletena väljaspool emakat, kõige sagedamini kõhukelmel ja munasarjades. Usaldusväärsete mitteinvasiivsete markerite puudumise tõttu lükkub diagnoosi saamine sageli mitmeid aastaid edasi, samas kui ravi piirdub hormonaalse ravi ja endometrioosikollete kirurgilise eemaldamisega.

Endometrioosi tekke kohta on küll mitmeid teooriaid, nagu retrograadse menstruatsiooni ja tsöloomi metaplaasia teooriad ning arvatakse, et tähtsust omavad nii geneetilised kui ka epigeneetilised tegurid, aga selle haiguse täpne etioloogia on siiani ebaselge. Lisaks võivad endometrioosi teket mõjutada häired hormonaalses tasakaalus, mikrokeskkonna mõju, rakkude muutunud ainevahetus ja immuunfunktsiooni häired. Endometrioosi kolletel ja eutoopilisel endomeetriumil on ka erinev molekulaarne signatuur, mis võimaldab kollete ellujäämist ja kasvu emakavälises asukohas. Oluline tegur on ka hüpoksia, mis mõjutab kollete rakkude metabolismi, kasvu, adhesiooni ja angiogeneesi. Hüpoksia-seoselised, adhesiooni ja invasiooni markerid on kollete stroomarakkudes muutunud võrreldes endomeetriumi stroomarakkudega ja energia metabolismis toimunud muutused on viinud aeroobse glükolüüsi suurenenud kasutamiseni sarnaselt Warburgi efektiga vähirakkudes. Lisaks on kolletes täheldatud muutunud steroididhormoonide signaaliülekannet, mis väljendub progesterooni resistentsuse ja östrogeeni domineerimisena.

Tänapäevased suure läbilaskevõimega tehnoloogiad, nagu transkriptoomika, proteoomika ja metaboloomika, võimaldavad läbi viia erinevate kudede põhjaliku analüüsi nii koe kui ka üksikute rakkude tasemel. Nende tehnoloogiate kasutamine endometrioosi patogeneesi uurimiseks hõlbustab haiguse patobioloogia sügavamat mõistmist ja pakub potentsiaalseid rakendusi endometrioosi diagnostikaks ja raviks.

Uurimistöö fookus

Antud doktoritöö põhieesmärk oli uurida endometrioosi patogeneesi rakkude metaboolse aktiivsuse ja hüpoksiale reageerimise perspektiivist ning tuvastada valitud kandidaatgeenide mõju endometrioosi arengule.

Uuringu täpsemad eesmärgid olid:

1. Iseloomustada endometrioosi põdevate naiste endomeetriumi ja endometrioosi kollete rakupopulatsioonide metaboolset aktiivsust ja steroidogeneesi protsesse üheraku transkriptoomika tasemel.
2. Uurida hüpoksia mõju *in vitro* tingimustes endomeetriumi ja endometrioosi kollete primaarsete stroomarakkude kinaaasi aktiivsusele ja transkriptoomile..

3. Uurida karboksüpeptidaasi inhibiitori lateksiini (LXN) ekspressiooni endometrioosiga ja ilma endometrioosita naiste endomeetriumis menstruaaltsükli jooksul ja selle geeni võimalikku rolli endometrioosi patogeneesis.

Materjalid ja meetodid

Kõik uuringud olid kooskõlastatud Tartu Ülikooli inimuuringute eetikakomiteega ja Karolinska Instituudi vastava komiteega. Kõik värvatud osalejad andsid uuringus osalemiseks kirjaliku teadliku nõusoleku ning kõik uuringud viidi läbi järgides vastavaid eeskirju.

Uuringu I jaoks koguti neljalt menstruaaltsükli proliferatsioonifaasis olevalt kinnitatud endometrioosiga (ASRM II–IV staadium) naiselt endomeetriumi ja peritoneaalsete kollete paarisproovid. Koeproovidele rakendati üheraku RNA sekveneerimise (scRNA-seq) metoodikat ja rakupopulatsioonid tuvastati bioinformaatiliselt Seurat integratsiooni- ja klastrianalüüsi abil. Kaheteistkümne metaboolse raja transkriptoomilist aktiivsust, steroidogeneesi raja geene ja rakkude proportsioone rakutsükli eri faasides analüüsiti kasutades pseudohulk diferentsiaalekspressiooni analüüsi ja radade analüüsi.

Uuringus II analüüsiti hüpoksia tingimustes toimuvaid transkriptoomilisi muutusi ja proteiinkinaaside (PK) aktiivsust endomeetriumi ja endometrioosi kollete stroomarakkudes. Stroomarakud eraldati viie naise kudedest, mis olid kogutud menstruaaltsükli sekretoorses faasis. Enne analüüside teostamist kasvasid primaarsed rakukultuurid 48 tundi kas hüpoksia (1% O2) või normoksia (20% O2) tingimustes. Analüüsisime PK-de aktiivsust (PKAc, ROCK, Akt ja CK2) ja teostasime transkriptoomi sekveneerimise (Smart-seq2), et uurida hüpoksia mõju geenide avaldumisele. Sekreteeritud TGFBI taset mõõdeti ELISA meetodil (viie naise paarilised proovid), mRNA (45 endometrioosiga naist ja 24 kontrolli) ja valgu (24 endometrioosiga naise bioloogilised proovid) taset kudedes uuriti kasutades vastavalt kvantitatiivset pöördtranskriptsiooni-PCR (qRT-PCR) ja immuunohistokeemiat (IHC).

Uuringus III hinnati *LXN* ekspressiooni endomeetriumi ja kollete kudedes qRT-PCR (34 endometrioosiga naist ja 27 kontrolli) ja IHC (9 endometrioosiga naist) meetoditega, lisaks hindasime LXN funktsionaalset rolli *in vitro*, kasutades rakkude migratsiooni ja elujõulisuse teste.

Tulemused

1. Nii endomeetriumis kui ka endometrioosi kolletes tuvastati üheksa peamist rakuklastrit: strooma-, endoteeli-, epiteeli-, perivaskulaarsed-, immuun-, tsükleerivad stroomarakud ja B-rakud ning tuvastamata rakkude klaster. Suurimaid erinevusi endomeetriumi ja kollete rakkude metaboolsete radade aktiivsuse vahel täheldati perivaskulaarsetes, strooma ja vähemal määral endoteelirakkudes. Kõige olulisemad erinevused nende rakkude metaboolses aktiivsuses hõlmasid AMPK ja HIF-1 signaaliülekandeid, glutatiooni metabolismi,

oksüdatiivset fosforüülimist (OXPHOS) ja glükolüüsi/glükoneogeneesi. Kollete perivaskulaarsetes ja stroomarakkudes oli toimunud glükolüüsi ja oksüdatiivse metabolismi koosaktiveerimine koos glükoosi ja rasvhapete omastamise, püruvaadi metabolismi, rasvhapete oksüdatsiooni ja TCA tsükliga seotud geenide ülesreguleerimisega. Lisaks leidsime kollete perivaskulaarsetes ja endoteelirakkudes oluliselt madalama progesterooni retseptori ekspressiooni ning östrogeeni retseptori 1 ekspressioon oli madalam kolletes nii perivaskulaarsetes, strooma- kui ka endoteelirakkudes.
2. Kinaasi test näitas, et endometrioosi kollete rakkudes langesid hüpoksia mõjul cAMP-sõltuva PK ja Akt aktiivsused, samas Rho-sõltuva PK aktiivsus suurenes. Transkriptoomi analüüs näitas, et hüpoksia reguleeris endomeetriumi stroomarakkudes aeroobse glükolüüsiga seotud geene ja HIF-1 signaaliülekandega seotud geenide ekspressiooni, samas kui kolde rakkudes eskpresseerus hüpoksia tingimustes erinevalt ainult üks geen – *TGFBI*. *TGFBI* ekspressioon oli kõrgem ka hüpoksiaga kokku puutunud endomeetriumi rakkudes võrreldes normoksia tingimustega ja normoksia tingimustes kolde rakkudes võrreldes endomeetriumi rakkudega ning need erinevused olid detekteeritavad ka rakkude söötmes mõõdetud sekreteeritud TGFBI tasemetes. Endomeetriumi koe biopsiate korral varieerusid *TGFBI* mRNA ja valgu tasemed sõltuvalt menstruaaltsüklist, olles proliferatsioonifaasis kõrgemad. TGFBI lokaliseerus stroomas ja veresoonte ümbruses, tugevam oli valgu ekspressioon kolletes võrreldes endomeetriumiga.
3. *LXN* geeni mRNA ekspressioon endomeetriumis oli menstruaaltsüklist sõltuv, olles madalaim varases sekretsioonifaasis ja kõrgeim hilises sekretoorses faasis ning oluliselt kõrgem endometrioosi kolletes võrreldes endomeetriumiga. IHC kinnitas LXN ekspressiooni endomeetriumi stroomarakkudes ja *in vitro* testid näitasid, et *LXN*-i vaigistamine vähendas tõhusalt endomeetriumi stroomarakkude migratsioonivõimet, tõstes samal ajal rakkude elujõulisust. Seega, LXN võib olla seotud endometrioosi tekkega, reguleerides endometriootiliste stroomarakkude proliferatsiooni ja migratsioonivõimet.

Järeldused

Käesolevas doktoritöös esitatud uuringute tulemuste põhjal võib teha järgmised järeldused:

1. Endometrioosikollete perivaskulaarsed, strooma- ja endoteelirakud näitasid glükolüütilise ja oksüdatiivse metabolismi koosaktiveerumist ja steroidogeneesi muutusi, mis viitavad ilmselt kollete suure energiavajadusega toimetuleku mehhanismidele. Metaboolset koaktivatsiooni täheldati valdavalt perivaskulaarsete rakkude populatsioonis, mis on tuntud oma rolli poolest endomeetriumi strooma parandamises ja angiogeneesis, muutes need endometrioosi ravi potentsiaalseks mittehormonaalseks sihtmärgiks.
2. Kokkupuude hüpoksiaga indutseeris nii endomeetriumi kui kolde stroomarakkudes spetsiifilise vastuse, mõjutades mitmete oluliste geenide ja radade avaldumist. Sõltumata hapniku kontsentratsioonist ektoopilistele rakkudele

oli iseloomulik hüpoksiline transkriptoomiprofiil, kuigi eutoopilised rakud näitasid hüpoksia tingimustes ektoopilistele rakkudele iseloomikku profiili.

3. *TGFBI* geeni avaldumine sõltub nii stroomarakkude päritolu asukohast kui ka kultiveerimisel kasutatud hapnikutasemest. TGFBI võiks olla potentsiaalne endometrioosi terapeutiline sihtmärk tema rolli tõttu fibroosis ja angiogeneesis.
4. *LXN* ekspressioon endomeetriumi kudedes on menstruaaltsükli spetsiifiline, olles kõrgeim hilises sekretoorses faasis. *LXN*-i vaigistamine stroomarakkudes vähendas rakkude migratsiooni, kuid soodustas rakkude elujõulisust, näidates selle geeni potentsiaalset rolli neis endometrioosi tekke jaoks kriitilistes protsessides.

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