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ROLE AND MECHANISM OF ESTROGEN RECEPTOR BETA IN THE PHYSIOLOGY OF THE OVARY AND COLON

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Role and mechanism of estrogen receptor beta in the physiology of the ovary and colon
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By

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Till farmor och farfar

Popular science summary of the thesis

Estrogen is often thought of as the hormone that regulates fertility in females. However, estrogen has a wide spectrum of functions and is important for the health of both females and males. To perform its functions, the estrogen molecule must bind to a receptor, a type of protein. Estrogen receptor beta (ERβ) is one of the proteins that estrogen can bind to. ERβ is a transcription factor, which means that it can bind to DNA in the cells and regulate the expression of genes. By controlling which genes are expressed, estrogen can affect different functions in the body. When the estrogen signaling is disrupted it can lead to the development and progression of certain diseases, including reproductive disorders and cancer.

Estrogen signaling specifically through ERβ has been shown to be important for the function of the ovary, where it helps regulate ovulation, but has also been shown to protect against the development of tumors in the colon. While these effects of ERβ are known, there is still a lack of knowledge regarding exactly how ERβ functions in the ovary and colon. The aim of this thesis was therefore to gain a better understanding of how ERβ regulates genes and consequently affects the function of the ovary and suppresses tumor development in the colon.

The regulation of genes is a process involving a whole complex of proteins, and identifying which proteins interact with each other is an important step toward understanding how transcription factors such as ERβ function. In **paper I** we studied ERβ in the ovary and identified a new interaction partner of ERβ, the protein LRH-1. Both ERβ and LRH-1 bind to the same place on the DNA and can either work together or compete against each other to regulate genes. The identification of this new interaction provides new knowledge regarding how ERβ affects reproduction in females.

As mentioned above, estrogen signaling has also been shown to protect against colorectal cancer development. Other studies have previously shown that it is estrogen signaling through ERβ, specifically, that mediates the protective effects. However, ERβ can be found in several types of tissues and cells, and it has therefore not been clear from which tissue the protective effect of estrogen originates. In **papers II** and **IV** we used mice that lack ERβ specifically in the colon to investigate whether estrogen acts directly in the colon. When ERβ was deleted from the colon of these mice, males developed more tumors and females got larger tumors when colorectal cancer was induced compared to normal mice. This

showed that ERβ in the colon protects against the development of colorectal cancer. We further saw that the protective effects of estrogen were due to reduced inflammation in the colon. Throughout **papers II–IV**, we also saw a clear difference between male and female mice, demonstrating the importance of including both sexes when studying colorectal cancer.

With this research, we have improved our understanding of how ERβ functions in both the ovary and colon. This new knowledge can help us to better understand female fertility and can be used to develop new treatments for the prevention of colorectal cancer.

Abstract

Estrogen regulates a variety of important physiological functions in both males and females, where the regulation of female reproduction and the development of sexual organs are typical examples. The effects of estrogen are predominantly exerted via signaling through the two nuclear receptors estrogen receptor α (ERα) and β (ERβ), or the membrane G protein-coupled estrogen receptor 1 (GPER1). While estrogen signaling is important for human health, dysregulation of signaling can have adverse effects and impact the development and progression of a wide range of diseases including reproductive disorders and cancer.

ERβ has been shown to be highly important for ovarian function by regulating folliculogenesis and ovulation but has also been implied to protect against the development of colorectal cancer (CRC) by mediating the effects of estrogen. Despite the known role of ERβ, there is a lack of mechanistic understanding regarding how ERβ acts under both normal conditions and during disease. The overall aim of this thesis was to characterize the function and molecular mechanism of endogenous ERβ and to decipher its role in the normal ovary as well as its impact on colitis and CRC development. To further understand the role of estrogen signaling in the colon, we also aimed to identify sex differences during CRC development.

In **paper I** we characterized the full cistrome of endogenous ovarian ERβ in the mouse and explored its transcriptional impact. We confirmed a direct role for ERβ in the regulation of essential ovarian functions and identified a novel crosstalk with the nuclear receptor LRH-1.

In **paper II** we induced colitis-associated CRC (CAC) in intestinal epithelial-specific ERβ knockout mice and identified a protective effect by intestinal ERβ against tumor development in both male and female mice. We further characterized sex-dependent effects and proposed an underlying mechanism involving the regulation of TNFα/NFκB signaling.

In **paper III** we expanded the investigation of sex-dependent changes during chemically-induced colitis in wildtype mice and identified a sex-specific response related to inflammatory response. We further found that male mice have an enhanced response to induced colitis.

In **paper IV** the transcriptome of colitis-induced tumors and their immune cell infiltration was explored in wildtype and intestinal epithelial-specific ERβ knockout

mice of both sexes. This showed that sex differences in the transcriptome appear to be dependent on the expression of ERβ. Also, the identified ERβ-dependent changes in the tumor transcriptome of female mice were specifically related to immune response. We corroborated an impact of ERβ on the infiltration of immune cells, especially a reduction of regulatory T cell and NK cell recruitment.

In summary, this thesis provides new mechanistic understanding of the transcriptional role of ERβ in the normal ovary and in the colon microenvironment. This includes the discovery of crosstalk with LRH-1 in the ovary and NFκB in the colon. Our characterization provides a foundation to develop targeted therapies for improved fertility and chemoprevention in CRC. This thesis also highlights the importance of including both sexes in colitis and CRC research to advance our knowledge and improve treatment development.

List of scientific papers

I. **Birgersson M***, Indukuri R*, Lindquist L, Stepanauskaite L, Luo Q, Deng Q, Archer A, Williams C. Ovarian ERβ cistrome and transcriptome reveal chromatin interaction with LRH-1. *Revised manuscript under review*

II. Hases L, Indukuri R, **Birgersson M**, Nguyen-Vu T, Lozano R, Saxena A, Hartman, J, Frasor J, Gustafsson JÅ, Katajisto P, Archer A, Williams C. Intestinal estrogen receptor beta suppresses colon inflammation and tumorigenesis in both sexes. (2020) Cancer Letters. 492:54-62.

III. Hases L, **Birgersson M**, Indukuri R, Archer A, Williams C. Colitis Induces Sex-Specific Intestinal Transcriptomic Responses in Mice. (2022) International Journal of Molecular Sciences. 23(18):10408.

IV. **Birgersson M***, Holm M*, Stepanauskaite L, Gallardo-Dodd C, Hases L, Kutter C, Archer A, Williams C. Intestinal estrogen receptor beta modulates the tumor immune microenvironment in a mouse model of colitis-associated cancer. *Manuscript*

Scientific papers not included in the thesis

V. Hases L*, Archer A*, Indukuri R, **Birgersson M**, Savva C, Korach-André M, Williams C. High-fat diet and estrogen impact the colon and its transcriptome in a sex-dependent manner. (2020) Scientific Reports. 10(1).

VI. Jafferali MH, Khatami K, Atasoy M, **Birgersson M**, Williams C, Cetecioglu Z. Benchmarking virus concentration methods for quantification of SARS-CoV-2 in raw wastewater. (2021) Science of The Total Environment. 755.

VII. Hases L, Stepanauskaite L, **Birgersson M**, Brusselaers N, Schuppe-Koistinen I, Archer A, Engstrand L, Williams C. High-fat diet and estrogen modulate the gut microbiota in a sex-dependent manner in mice. (2023) Communications Biology. 6(1).

VIII. Perez-Zabaleta M, Archer A, Khatami K, Jafferali MH, Nandy P, Atasoy M, **Birgersson M**, Williams C, Cetecioglu Z. Long-term SARS-CoV-2 surveillance in the wastewater of Stockholm: What lessons can be learned from the Swedish perspective? (2023) Science of The Total Environment. 858.

* Equal contribution

Contents

List of abbreviations

3β-HSD	3β-hydroxysteroid dehydrogenase
AF1/2	Activating function 1/2
AOM	Azoxymethane
AP-1	Activator protein 1
APC	APC regulator of WNT signaling pathway
ATAC	Assay for Transposase-Accessible Chromatin
CAC	Colitis-associated colorectal cancer
CD	Chron´s disease
ChIP	Chromatin immunoprecipitation
CIMP	CpG island methylation phenotype
CMS	Consensus molecular subtypes
CRC	Colorectal cancer
Ct	Cycle threshold
CYP19A1	Aromatase
DAI	Disease activity index
DBD	DNA-binding domain
DEG	Differentially expressed gene
DHEA	Dehydroepiandrosterone
DPN	Diarylpropionitrile
DSS	Dextran sodium sulfate
E1	Estrone
E2	Estradiol
E3	Estriol
E4	Estetrol
ERα/β	Estrogen receptor alpha/beta
ERβKO	Full-body ERβ knockout
ERβKO^{Vil}	Intestine-specific ERβ knockout

ERE	Estrogen response element
FAIRE	Formaldehyde-Assisted Isolation of Regulatory Elements
FAP	Familial adenomatous polyposis
FDR	False discovery rate
FOXL2	Forkhead box L2
FPKM	Fragments per kilobase of transcript per million mapped reads
FSH	Follicle-stimulating hormone
GCT	Granulosa cell tumor
GPER1	G Protein-Coupled Receptor 1
HFD	High-fat diet
HNPCC	Hereditary nonpolyposis colorectal cancer
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IF	Immunofluorescence
IHC	Immunohistochemistry
LBD	Ligand-binding domain
LH	Luteinizing hormone
LRH-1	Liver receptor homolog-1
LUC	Luciferase gene
MHT	Menopausal hormone therapy
MSI	Microsatellite instability
NK cell	Natural killer cell
POI	Primary ovarian insufficiency
PPT	Propylpyrazoletriol
RIME	Rapid Immunoprecipitation Mass spectrometry of Endogenous protein
RNA-seq	RNA-sequencing

SCNA	Somatic copy number alterations
scRNA-seq	Single cell RNA-seq
SERD	Selective estrogen receptor degrader
SERM	Selective estrogen receptor modulator
SP-1	Stimulating protein 1
STAR	Steroidogenic acute regulatory protein
TAM	Tumor-associated macrophage
TME	Tumor microenvironment
TPM	Transcripts per million
Treg	Regulatory T cell
UC	Ulcerative colitis
VDR	Vitamin D receptor
WB	Western blot
WT	Wildtype

1 Introduction

1.1 Estrogen signaling

1.1.1 Estrogen hormones

Estrogens are the primary female sex hormones and belong to the steroid hormone family. Although estrogens are primarily thought of as female hormones, they exert a broad spectrum of functions in both males and females. Sex organ development and the regulation of reproduction in females are part of the main effects of estrogens, but they also have effects on the brain (1, 2), bone (3), metabolism (4), cardiovascular system (5), and immune system (6, 7). In females, estrogens take part in the regulation of the menstrual cycle and are responsible for both primary and secondary sexual characteristics (8, 9). Similarly, estrogens play an equally important role in for example sperm maturation and erectile function in males (10, 11).

There are in total four different types of endogenous estrogens, namely estrone (E1), estradiol (E2), estriol (E3), and estetrol (E4). As the most potent type of estrogen, E2 is produced in the ovary and is the predominant estrogen in premenopausal females (12), with circulating levels at a range of around 0.1 to 2.2 nM depending on the stage of the menstrual cycle (13). Premenopausal females have a similar level of circulating E2 as males, at around 0.04 nM and 0.1 nM, respectively (13). E1 is an estrogen primarily produced by extragonadal tissues (such as adipose tissue (14)), with similar levels in pre- and postmenopausal females and males. E1 can be reversibly transformed to E2 in peripheral tissues (12). E3 and E4 are predominantly produced during pregnancy, where E3 is produced by the placenta and E4 by the fetal liver (12, 15).

1.1.2 Estrogen synthesis

The primary site for estrogen synthesis is the ovary in pre-menopausal females. Here, E2 is produced by theca and granulosa cells that enable the conversion of cholesterol to E2 in a cooperative effort, illustrated in Figure 1. As a first step, the steroidogenic acute regulatory protein (STAR) enables the transport of cholesterol into the mitochondrial membrane of theca cells (16). The transport of cholesterol is a rate-limiting step of the E2 synthesis and the expression of STAR is regulated through a process initiated by luteinizing hormone (LH) signaling (17, 18). Steroidogenesis is then initiated by the conversion of cholesterol into pregnenolone by CYP11A1, still in the mitochondria (19). Pregnenolone is the

precursor of all steroid hormones. After being produced in the mitochondria, pregnenolone is transported to the endoplasmic reticulum where the remaining steps in estrogen synthesis take place (20). Pregnenolone is first converted into androstenedione through the actions of CYP17A1 and 3β-hydroxysteroid dehydrogenase (3β-HSD) via either progesterone or dehydroepiandrosterone (DHEA) (21). While the initial steps of steroidogenesis largely take place in theca cells, granulosa cells themselves can produce progesterone from cholesterol. However, granulosa cells cannot convert progesterone to androstenedione and therefore require assistance from theca cells to produce estrogens. For estrogen synthesis to continue, androstenedione needs to diffuse out from theca cells and into the neighboring layer of granulosa cells. In granulosa cells, CYP19A1 (aromatase) converts androstenedione into E1. E1 can finally, through 17β-HSD, be converted into E2. CYP19A1 can also convert testosterone directly into E2. Testosterone in females is produced by 17β-HSD converting androstenedione in the granulosa cells (21). As the expression of both CYP19A1 and 17β-HSD is dependent on follicle-stimulating hormone (FSH) secreted by the anterior pituitary gland, E2 synthesis in granulosa cells is regulated by FSH (21, 22).

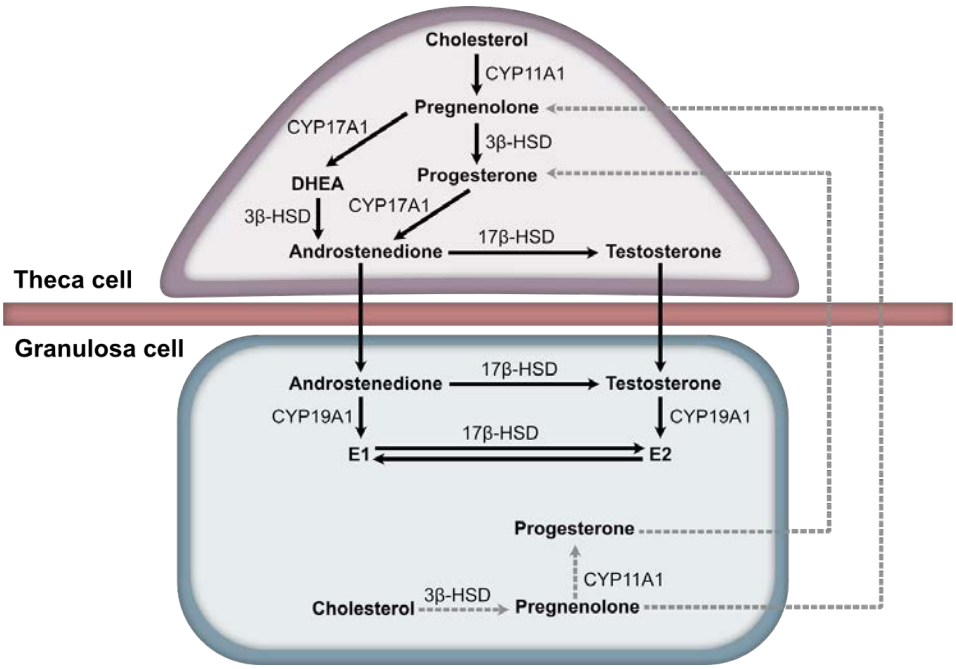

Figure 1. Synthesis of estradiol (E2) by granulosa and theca cells in the ovarian follicle.

In males, E2 is produced in the testis, by a similar process between Sertoli and Leydig cells. In both males and postmenopausal females E1 and E2 are mainly

produced through the conversion of C19 steroids, such as DHEA and androstenedione, by CYP19A1 which is produced in extragonadal tissues such as bone, brain, the adrenal glands, and adipose tissue (12, 14).

Estrogens can also be synthesized by for example plants (23) and enter the body through the diet. These estrogens include phytoestrogens such as isoflavones and prenylflavonoids, which are present at high levels in soy (24) and hops (25), respectively.

1.1.3 Estrogen receptors

Estrogens predominantly exert their effects via estrogen receptors (ER), though some ER-independent mechanisms through enzyme interactions have been observed (26). The existence of estrogen receptors was discovered in 1958 (27), and ERα (*ESR1*) was the first ER to be cloned in 1986 (28, 29), followed by the cloning of ERβ (*ESR2*) in 1996 (30). Both receptors belong to the nuclear receptor superfamily and act as ligand-activated transcription factors. In addition, G Protein-Coupled Receptor 1 (GPER1) was more recently identified as an ER. GPER1 was first recognized as an orphan receptor but was years later shown to respond to estrogen stimulus (31). In contrast to the two nuclear ERs, GPER1 is a transmembrane receptor with a rather low affinity for estrogens (31, 32).

All nuclear receptors, including the ERs, consist of the same functional domains (A/B, C, D, and E/F, illustrated in Figure 2) with their own function to facilitate transcriptional activity (33, 34). The A/B region contains the amino-terminal domain, which enables transactivation of the receptors. Nuclear receptors can bind directly to response elements in DNA through their DNA-binding domain (DBD), which is located in the C region. In the case of the ERs, the DBD binds to the estrogen response element (ERE) (34), a sequence defined as 'GGTCAnnnTGACC' (35). The DBD harbors two zinc fingers that facilitate target sequence binding. The DBD is where ERα and ERβ share the highest degree of identity (95%). In contrast, the ligand-binding domains (LBD) only share 55% identity (30). This domain is in the last E/F region, which also contains binding sites for co-factors (33, 34). The hinge region in the D domain connects the C and E/F regions. This domain can also bind chaperone proteins and contains a nuclear localization signal that allows translocation upon estrogen binding. The receptors also encompass two additional domains, the activating function (AF) domains AF1 and AF2, that are involved in the regulation of transcriptional activity. AF1 is ligand-

independent and located in the amino-terminal domain, while AF2 resides in the LBD and requires ligand binding (34).

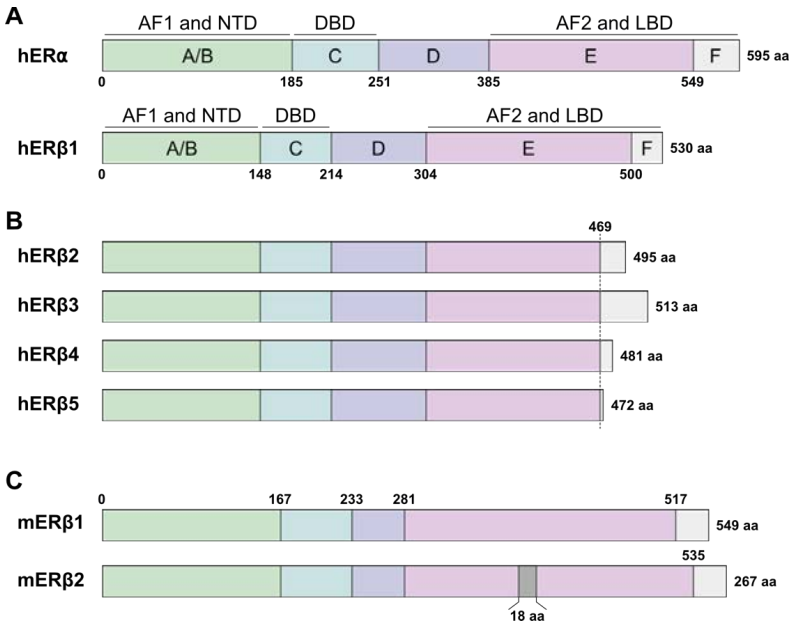

Figure 2. The structure of ERs. (A) The nuclear ERs contain the same five functional domains. **(B)** There are five splice variants of human (h) ERβ, while only two **(C)** have been identified in mouse (m).

Evolutionarily, the ERs are well conserved between species (36). However, the splice variants of *ESR2* are species-specific. In humans, five isoforms of ERβ (ERβ1 to 5) have been identified, while only two major splice variants (ERβ1 and 2) have been found in rodents (37, 38, 39) (Figure 2). Of the five human ERβ variants, only ERβ1 (which is sometimes referred to as the wildtype (WT) variant) has an intact LBD and is, therefore, the only variant capable of binding estrogens (37). In rodents, both splice variants are capable of binding ligands, but ERβ2 (ERβ-ins) has an 18-amino acid insert in the LBD that lowers its affinity for E2 (40).

Despite the similarities between ERα and ERβ, the two nuclear receptors differ in their expression, and ERα is more widely expressed compared to ERβ. In humans, ERα is expressed in several tissues, including testis, uterus, ovary, breast, and bone (41, 42). Meanwhile, the expression of ERβ has been debated, largely due to the usage of unvalidated antibodies. An antibody validation study published in 2017 revealed that the most widely used antibodies were unspecific and that expression of ERβ can be found in only a select number of human tissues, including

the ovary (granulosa cells), testis, spleen, lymph nodes, adrenal gland, and intestine (41).

1.1.4 ER signaling pathways

The ERs can be located in the cytoplasm and in the nucleus. Depending on their subcellular location, the nature of the signaling event can be either genomic or non-genomic. Genomic signaling involves direct or indirect binding of DNA, while non-genomic signaling instead triggers intracellular signaling cascades (Figure 3).

1.1.4.1 Genomic signaling

Genomic signaling is the classical signaling pathway of the nuclear ERs. In response to ligands, the receptors translocate into the nucleus, where they dimerize and can bind directly to chromatin at promoter or enhancer sites containing an ERE. Depending on the co-factors that are subsequently recruited upon DNA-binding, gene transcription can be either activated or suppressed (13). Alternatively, ERs can regulate gene expression without directly binding to an ERE by tethering to other transcription factors. Classical examples of ER interaction partners include stimulating protein 1 (SP-1), activator protein 1 (AP-1), and NFκB complexes (34). In addition to the ligand-activated transcriptional response, ERs can also be activated independently of ligands through phosphorylation. This type of activation can for example be mediated through growth factor receptors (43).

1.1.4.2 Non-genomic signaling

During non-genomic ER signaling, there is no direct interaction with chromatin. Instead, the ligand binding results in activation of intracellular signaling cascades. The subsequent regulation of cAMP and activation of protein-kinases can then activate transcription factors to alter gene expression (34). This is the mode of signaling for GPER1-mediated estrogen signaling (44). There are several different described signaling pathways for GPER1, including the Ras/Raf/MAPK and PI3K/Akt kinase cascade (34, 44). Similar non-genomic pathways have also been described for the other two ERs when located in the cytoplasm (45).

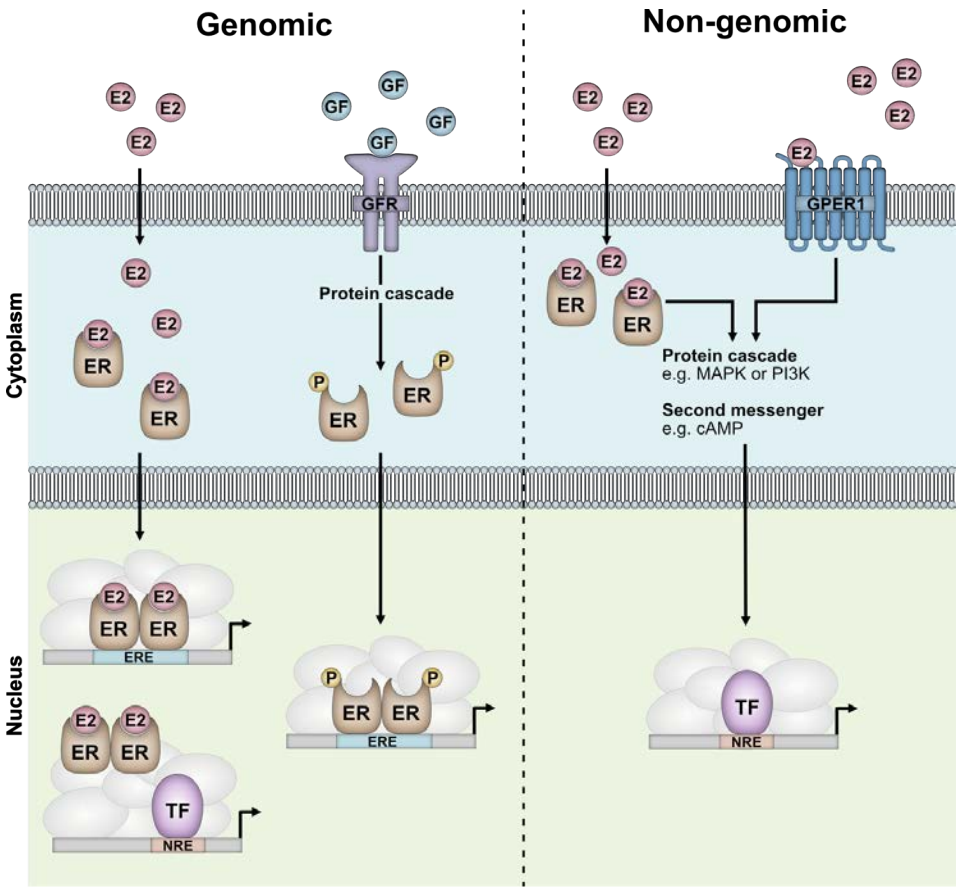

Figure 3. ER signaling pathways. ERs can signal through genomic and non-genomic pathways. Genomic signaling where ERs directly interact with DNA to alter transcription is mainly estrogen-dependent but can also occur independent of ligands through the activation of growth factor receptors. Non-genomic signaling initiates intracellular signaling pathways, which can indirectly alter transcription.

1.1.5 Receptor-selective activation

Due to the difference in the LBD, various estrogenic compounds can have diverse binding capacities for the different ERs, resulting in receptor selectivity (46, 47). Genistein is an example of a phytoestrogen that is more selective for ERβ activation due to a higher affinity for its LBD (48). Like the natural compounds with diverse binding capacities, the difference in the LBD can also be utilized to design receptor-selective compounds. Examples of ERα- and ERβ-selective ligands are propylpyrazoletriol (PPT, 410-fold greater affinity for ERα) (49) and diarylpropionitrile (DPN, 70-fold greater binding affinity for ERβ) (50). By

employing such compounds, unwanted side effects from either receptor can be circumvented, thereby enhancing the potential therapeutic benefit.

Further, selective estrogen receptor modulators (SERMs) are a group of compounds that with tissue selectivity can act as estrogen receptor agonists or antagonists (51). One of the most widely known examples is tamoxifen, used in the treatment of breast cancer. Tamoxifen has an antagonistic effect on breast cancer cells resulting in anti-proliferative effects, while it has an agonistic effect on myometrial cells in the uterus (52). In addition to the SERMs, there are selective estrogen receptor degraders (SERDs) such as fulvestrant, which upon binding block the dimerization of the ERs and subsequently induce degradation of the protein (53).

1.1.6 The role of estrogen signaling in disease

Dysregulation of estrogen signaling can lead to the development and progression of various diseases ranging from reproductive disorders to cancer (54, 55). Estrogen resistance due to loss of function mutations in *ESR1* leads to delayed puberty with associated primary amenorrhea and ovarian cysts in females and affects bone growth in both sexes (56, 57, 58). Estrogens have also been linked to increased immune response and autoimmunity (6, 59). The incidence of autoimmunity is higher in females, although the reasons for this are not completely clear. As sex hormones play a part in the development and activity of the immune system, estrogens are suggested to be a contributing factor (6, 59). Activated estrogen signaling through ERα has been shown to promote the progression of different cancers including breast- and ovarian cancer (60, 61), and ERα is upregulated in these tumors. In contrast, ERβ has been linked to tumor suppressive effects mainly in colorectal cancer (CRC), and its expression is also lost as CRC progresses (62). However, in a subtype of ovarian cancer, namely granulosa cell tumors (GCT), ERβ is highly expressed (41). Yet, the role of ERβ in GCT remains unknown.

1.2 The ovary

1.2.1 The ovarian follicle

The main role of the ovary is to produce steroid sex hormones and to facilitate the differentiation and release of mature oocytes. The ovarian follicle is a structure consisting of the oocyte, granulosa cells, theca cells, endothelial cells, and connective tissue. The resting pool of primordial follicles, an oocyte surrounded

by a single layer of flattened granulosa cells, is established early in life (63, 64, 65). The whole process of development from primordial follicle to pre-ovulatory follicle in humans has been estimated to take approximately one year, or 13 regular menstrual cycles (66) (Figure 4), and starts with the recruitment of non-proliferating follicles. Once recruited the oocyte size will increase and the granulosa cells will become cuboidal and start to proliferate, making up the primary follicle (63, 67, 68). With the continued proliferation of granulosa cells, the secondary follicle will form, with multiple layers of granulosa cells around the oocyte. Once two layers of granulosa cells have formed, theca cells are recruited to the growing follicle (63, 67, 68). When the oocyte is close to fully grown, an antrum will develop. The formation of the antral space is accompanied by a separation of granulosa cells into mural- and cumulus granulosa cells. The mural granulosa cells line the wall of the follicle, while the cumulus cells surround the oocyte (63). In the antral stage, the granulosa and theca cells continue to proliferate, and the vascularization of the follicle will increase. At this stage in development, there is cyclic recruitment of follicles, largely dependent on FSH (69). The selected follicles will continue to grow, while the remaining follicles will become atretic, which is the fate of more than 99% of all initially recruited follicles (70). Of the few follicles selected, one dominant follicle will emerge and reach full maturation as a pre-ovulatory (Graafian) follicle, which is also the major producer of estrogens. In response to gonadotropin surges, the dominant follicle will ovulate and release the mature oocyte. The remaining granulosa and theca cells of the ruptured follicle undergo luteinization to become the corpus luteum (63, 67).

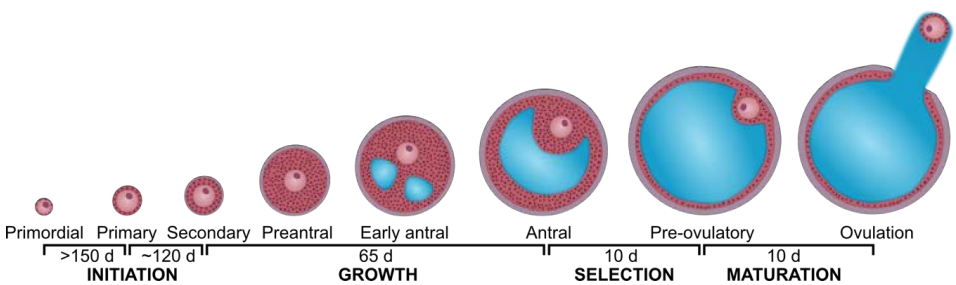

Figure 4. Development of the ovarian follicle. The whole folliculogenesis from primordial follicle to ovulation is a process that takes one year. During folliculogenesis the oocyte grows and matures, a process supported by the surrounding granulosa and theca cells.

1.2.2 The menstrual and estrous cycle

The menstrual cycle in humans, and the corresponding estrus cycle in mice, is regulated by hormones secreted under the control of the hypothalamus. The

chain of regulation starts with the secretion of gonadotropin-releasing hormone from the hypothalamus, which is transported to the anterior pituitary to stimulate the production of FSH and LH. As secreted FSH and LH reach the ovary, they trigger E2 production by the follicles (71, 72), as described in section 1.1.1. The level of produced E2 will in turn regulate the secretion of FSH and LH (71, 73, 74).

A menstrual cycle in humans is on average 28 days (75) and consists of four phases: the menstrual phase, the follicular/proliferative phase, ovulation, and the luteal/secretory phase (Figure 5A). The cycle starts with the follicular phase, which starts on the first day of the period, partly overlapping with the menstrual phase. This is the stage where E2 is the predominant hormone. The high production of E2 in this phase is a result of an upregulation of FSH receptors in the ovarian follicles (21, 22). The increasing levels of E2 provide negative feedback to the anterior pituitary, reducing the levels of FSH and LH (73). As the follicular phase progresses, the follicles will mature, resulting in increased E2 production. The peak in E2 levels will change the feedback to the pituitary from negative to positive. With this, there is an increased secretion of both FSH and LH (76), which is called the LH surge. It is the LH surge that leads to ovulation where the mature follicle ruptures and releases the oocyte (77). Following ovulation, the menstrual cycle moves into the luteal phase, where the luteinization of granulosa and theca cells results in LH-induced progesterone production (78).

While similar mechanisms control the estrus cycle in rodents, the estrus cycle is considerably shorter, lasting only 4-5 days on average (79). Like the menstrual cycle, the estrus cycle also consists of four stages, namely diestrus, proestrus, estrus, and metestrus (Figure 5B). Diestrus is the stage where E2 levels begin to rise. In proestrus, the pre-ovulation stage, E2 levels peak (80), leading to the surge of FSH and LH with subsequent ovulation (81). In the estrus stage, the level of E2 continues to decline until it reaches the basal level. The levels of E2 remain low in metestrus until they start to rise again during diestrus (80). During early diestrus, there is also a rise in progesterone levels, but in contrast to E2, the levels do not continue to rise. Instead, the levels go down at the end of diestrus before peaking right after the E2 peak in proestrus (80).

Altogether the various hormonal changes during the menstrual/estrus cycle regulate female reproduction and fertility, and also affect nonreproductive health, making them highly important for female health overall.

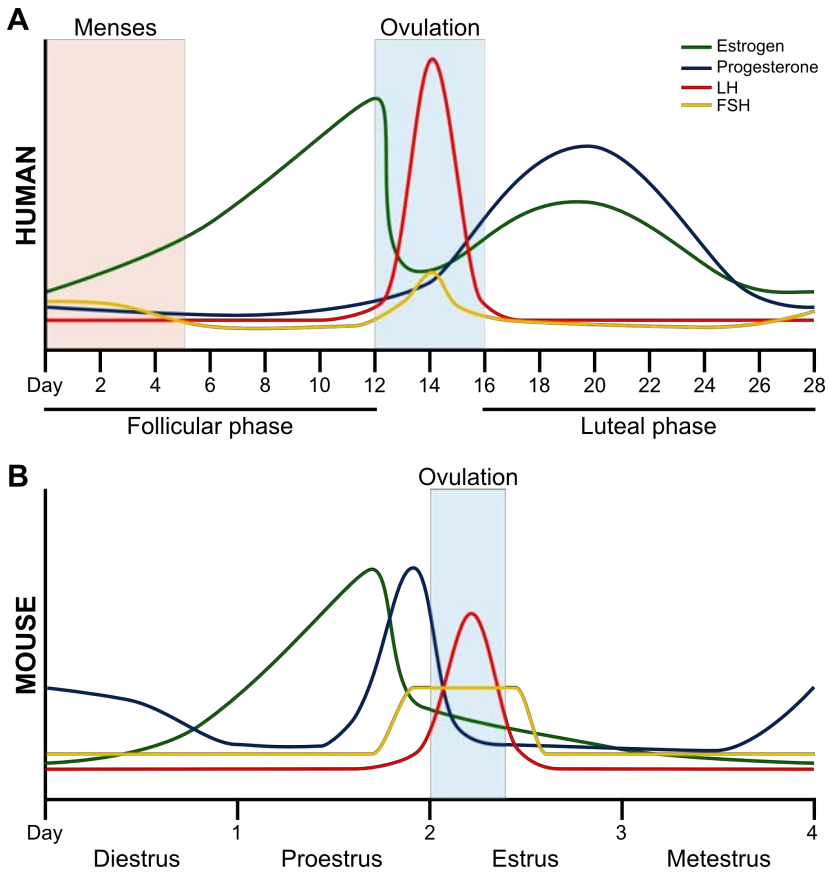

Figure 5. The menstrual and estrus cycle. (A) The menstrual cycle in humans and **(B)** estrus cycle in mice are both coordinated through the secretion of estrogen, LH, FSH, and progesterone.

1.2.3 Ovarian dysfunction

Loss of ovarian function is inevitable with age as females reach menopause and the pool of primordial follicles is depleted (82). However, premature disruption of ovarian function before expected menopause, often called ovarian insufficiency, is relatively common. Exogenous factors such as diet and exercise (83), and treatments including chemotherapy and radiation (84) can affect ovarian function. Other examples of ovarian disruptors are hypothalamic-pituitary disorders, including polycystic ovary syndrome, caused by an endocrine disruption outside of the ovary (85). Finally, ovarian insufficiency can also be caused by factors inside the ovary and is then often termed primary ovarian insufficiency (POI) (86).

The incidence of POI has been estimated to be 3.7% (87). POI is characterized by amenorrhea, deficiency of sex steroid hormones, and high levels of FSH (86). While there are many causes of POI, the condition generally results in the depletion or dysfunction of ovarian follicles. Insufficient initial follicle number and accelerated follicle loss both result in the depletion of follicles and can be caused by 46,XY gonadal dysgenesis (88) and Turner's syndrome (89), respectively. Dysfunction of the follicle on the other hand can be the result of signaling defects caused by mutations in for example the FSH receptor, deficiency in enzymes such as CYP17A1 or CYP19A1, autoimmunity, or early luteinization of pre-ovulatory follicles (90). However, many cases of POI go undefined. Although the proper function of the ovary is an essential factor in female reproduction and health, the mechanism of action behind several aspects of ovarian insufficiency remains unknown.

1.3 Estrogen signaling in the ovary

1.3.1 Importance of ERβ for normal ovarian function

ERβ is the main ER in the follicle. It is highly expressed in granulosa cells and has also been reported to be expressed at lower levels in theca cells (30, 41, 91). The receptor has been shown to be highly important for proper ovarian function, with a direct role in folliculogenesis and ovulation. When knocking out ERβ in rodents, mice become subfertile, with smaller and fewer litters, while rats become completely infertile (92, 93, 94). However, a more recent study shows that mice that lack ERβ also become infertile, but only when they reach 6 months of age (95). Both mice and rats present with fewer large antral follicles and a reduced number of corpus luteum due to the lack of ovulation (94, 96). Further, mice lacking ERβ have an improper thinning of the follicular wall and cumulus-oocyte complex expansion which normally happens during the ovulatory process (96).

Following ERβ knockout, female rats lack an estrus cycle, with an overall reduction in serum E2 levels, and do not respond to exogenous gonadotropins (93). Additionally, the loss of ERβ has been shown to increase the recruitment and activation of primordial follicles, leading to premature ovarian senescence (97). Meanwhile, ERβKO female mice exhibit a reduction in the LH surge with a reduced FSH response (92, 96). While their overall E2 levels are not greatly affected, they display a reduced E2 surge at diestrus (95, 98). Both the E2 surge and fertility can be restored in these mice by transplantation of a WT ovary (98), indicating a primary intraovarian defect as a consequence of ERβ loss. ERβ has further been

shown to regulate downstream gene targets of FSH and LH that have important roles for proper ovarian function (94, 99).

Although ERβ appears not to be essential for ovarian formation in rodents, there is evidence that ERβ might play an important role in ovarian development in humans. According to a case report in 2018, a woman born with a point mutation in the *ESR2* gene presented with non-detectable gonads. These could later be detected as streaks following treatment with transdermal estrogen therapy (100). While ovarian streaks could be detected after estrogen treatment, no follicles could be detected. This indicates that while ERα can partially support ovarian development, ERβ appears to be needed for proper development and maintenance, especially regarding follicle development. The identified point mutation had a dominant negative effect, disrupting the activity of the WT allele and making it non-functional, without affecting protein stability. The point mutation instead resulted in an impaired signaling where ERβ was unable to drive gene expression (100).

1.3.2 Other key transcription factors in the ovary

Several other transcription factors have important functions in the ovary. The forkhead box transcription family is a group of transcription factors of which several can affect ovarian function, including forkhead box L2 (*FOXL2*) (101). This transcription factor is highly expressed in the ovary and is involved in both ovarian development and folliculogenesis. The GATA family is another group of important transcription factors that act as pioneering factors (102). GATA4 and GATA6 have been identified as crucial for follicle assembly, granulosa cell differentiation, and luteinization (103), and knockout of both GATA4 and GATA6 lead to infertility in female mice (104). Similarly, the transcription factor liver receptor homolog-1 (LRH-1, *NR5A2*) has been shown to be essential for ovulation. Knockout of LRH-1 leads to complete infertility in female mice as a result of failure to ovulate (105, 106). This orphan nuclear receptor is specifically expressed in granulosa cells and has been identified as a major player in the transcriptional network of the ovary (106).

1.4 Colorectal cancer

As described in section 1.1.5, estrogen is involved in the development of diseases relating to the reproductive and immune systems. In addition, the development of certain cancers is influenced by estrogen. This includes CRC, where estrogen exerts a protective effect against cancer development, although the cancer itself is not estrogen sensitive.

1.4.1 Epidemiology

CRC is the third most common cancer type and the second leading cause of cancer-related death worldwide (107). The incidence of CRC is higher in males compared to females and higher in developed nations (107). Although there is an overall increase in CRC incidence worldwide, the incidence in the population above 50 years of age is in fact decreasing at a steady rate in countries such as the US where systematic screening has been implemented (108). On the contrary, the prevalence of CRC is increasing among 20–49-year-olds, especially the incidence of rectal cancer (108, 109, 110). This increase has been linked to economic development which leads to changes such as a higher intake of processed food and a higher degree of obesity (107).

1.4.2 Risk factors

Risk factors for developing CRC include sociodemographic factors, genetic and medical factors, as well as lifestyle and diet factors (111). Regarding non-modifiable risk factors, sociodemographic factors such as being of male sex and increasing age are associated with an increased risk of developing CRC (112). Further, although approximately 80% of CRC cases are sporadic, hereditary factors, including family history and hereditary CRC syndromes, also play a part in the development (113, 114). There are several hereditary CRC syndromes, both non-polyposis and polyposis syndromes. Hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) are the most common hereditary syndromes, and both are characterized by autosomal dominant inheritance (114, 115, 116). HNPCC arises from mutations of DNA mismatch repair genes, most often *MLH1* and *MSH2*, while FAP is caused by mutations in the APC regulator of WNT signaling pathway gene (*APC*) (114, 115, 116). Similarly, patients with inflammatory bowel disease (IBD) are at an increased risk of developing CRC (117). While risk factors such as sociodemographic and genetic factors are non-modifiable, there are risk factors that are also modifiable risk factors. These include diet, alcohol intake, smoking, and body weight (118).

1.4.3 Molecular pathogenesis

The progression of CRC from aberrant crypt to polyp and, ultimately, invasive tumor is estimated to be 10-15 years. The cancerous lesions are a result of the progressive accumulation of both gene mutations and epigenetic alterations, resulting in the activation of oncogenes and repression of tumor-suppressor

genes (111). The molecular pathogenesis of CRC can be divided into two pathways: the adenoma-carcinoma sequence and the serrated neoplasia pathway.

The adenoma-carcinoma sequence underlies around 70-90% of all CRCs and was first described by Fearon and Vogelstein in 1990 (119). It is characterized by initial mutations in the *APC* gene leading to hyperactivation of the WNT signaling pathway, followed by activating mutations of *RAS* and loss-of-function mutations in *TP53* (120) (Figure 6). These mutations are often accompanied by chromosomal instability, which is why this pathway is also referred to as the chromosomal instability pathway (121). In contrast, the serrated neoplasia pathway, which accounts for around 15% of CRC tumors, is associated with activating mutations in *RAS* and *RAF*. This pathway is also characterized by epigenetic instability with a CpG island methylation phenotype (CIMP) and often a high level of microsatellite instability (MSI) as a consequence of methylation in the *MLH1* gene (121, 122).

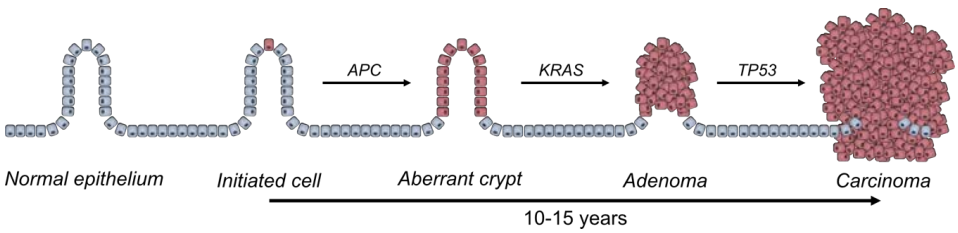

Figure 6. The adenoma-carcinoma pathway. The progression from normal epithelium to carcinoma is a long process involving mutations of *APC*, *RAS*, and *TP53*.

1.4.4 Staging and molecular classification

Once CRC has been diagnosed through assessment of biopsies collected during colonoscopy, the cancer is staged according to the TNM system by the level of local invasion (T), spread to lymph nodes (N), and presence of distant metastasis (M) (123). However, this staging method has limitations (124) and an effort to classify tumors based on molecular data has therefore been made (125). The new classification system is based on transcriptomic data and comprises four different consensus molecular subtypes (CMSs): CMS1 (MSI-Immune), CMS2 (Canonical), CMS3 (Metabolic), and CMS4 (Mesenchymal) (125). The characteristics of each subtype are summarized in Table 1. The CMS1 subtype displays strong immune infiltration and activation, which can be linked to the high rate of MSI that is theorized to produce a large number of antigens that can be recognized by the immune system, resulting in pronounced immune cell infiltration (126). The largest fraction of tumors (37%) aligns with the canonical

subtype (CMS2), with enrichment of *APC* mutations leading to WNT activation. The third subtype, CMS3, has a relatively stable genome but is characterized by marked metabolic deregulation. The metabolic phenotype could be associated with the enrichment of *KRAS* mutations in this subtype, which has been documented to induce metabolic alterations in cancer (127, 128, 129). The last subtype (CMS4, mesenchymal) has high somatic copy number alterations (SCNA) but lacks an enrichment of specific driver genes. It does, however, display prominent TGFβ activation, angiogenesis, and stroma infiltration, and is associated with a more aggressive and metastatic phenotype compared to the other CMSs. In fact, this subtype tends to be diagnosed at a more advanced stage and has worse both relapse-free and overall survival (125). During the development of the new classification system, samples with mixed features were also identified. It is possible that these samples represent a transition phenotype or intra-tumoral heterogeneity (125).

Table 1. Molecular classification of CRC based on the consensus molecular subtypes (CMSs).

	CMS1 MSI-Immune	CMS2 Canonical	CMS3 Metabolic	CMS4 Mesenchymal
MSI	High		Mixed	
CIMP	High		Low	
SCNA		High	Low	High
Mutations	Hypermutations, *BRAF*	*APC*	*KRAS*	
Signature	Infiltration and activation of immune cells	Activation of MYC and WNT	Metabolic deregulation	Stroma infiltration, angiogenesis, TGFβ activation

1.4.5 Location

In addition to molecular diversity, tumor location adds an additional dimension to tumor heterogeneity (Figure 7). Right-sided tumors are more prevalent in females while males more frequently present with left-sided tumors. Unexpectedly, tumors located on the left side also have a better prognosis compared to right-sided tumors (130), despite the fact that males have a higher mortality rate than females (107). The worse prognosis of patients with right-sided tumors has been linked to a reduced response to conventional chemotherapies and targeted treatments. However, due to the high antigenic load (as described in 1.4.4),

patients with right-sided tumors show a promising response to immunotherapy (131). Further, tumors that develop through the serrated neoplasia pathway are often located in the right colon (131). This can further be correlated to the sex difference in location, as sessile serrated adenomas are more prevalent among females (132). Right-sided tumors are therefore often CIMP- and MSI-high with *BRAF* mutations. Left-sided tumors, on the other hand, often have mutations in *APC* and *TP53* (111, 133). Regarding the CMS classification, MSI-immune and metabolic tumors are more frequent in the right colon, while canonical and mesenchymal tumors tend to occur in the left colon (111, 125).

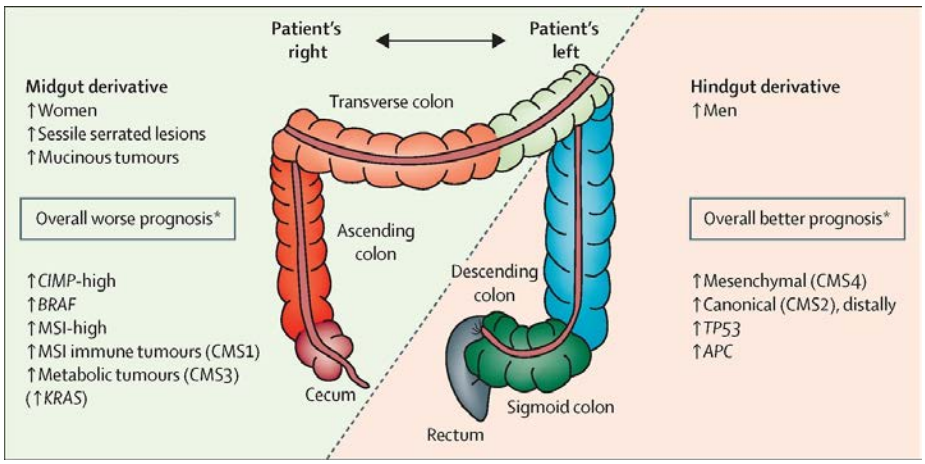

Figure 7. Characteristics of right- and left-sided CRC tumors. The location of the tumor can be associated to differences including sex and tumor type. Note: Reprinted from The Lancet, Vol. 394, Dekker et. al., Colorectal cancer, p 1467-1480., Copyright (2019), with permission from Elsevier.

1.4.6 The role of inflammation in CRC

Ulcerative colitis (UC) and Crohn´s disease (CD) are the main types of IBD and constitute risk factors for developing CRC, as previously mentioned. The gut inflammation in UC is limited to the colonic mucosa, while the inflammation in CD is more spread and can involve any part of the gastrointestinal tract (134). Although genetics, environmental factors, and other host-related factors contribute to the development of IBD, the underlying cause is not yet completely understood (135). There is evidence that T helper 17 cells that secrete IL-17 and IL-23 might play an important role in IBD development, and inhibition of T helper 17 cells can inhibit the inflammatory process and thereby slow down or decrease the development of colitis (136, 137).

Out of all IBD patients, about 20% will develop colitis-associated CRC (CAC) within 30 years of disease onset, and around half of these patients eventually die from CRC (138). IBD patients with a family history of CRC without associated IBD have a 2-fold increased risk of CAC development (139). The basics of the molecular mechanisms, including alterations in genetic pathways such as WNT and β-catenin, TP53, and KRAS, closely resemble the classical adenoma-carcinoma sequence of sporadic CRC (138, 140). The major difference between CAC and CRC is the presence of chronic inflammation, which in combination with genetic alterations makes the disease development faster and multifocal in its nature (140, 141).

1.4.7 Tumor microenvironment

Tumors are surrounded by and mixed with stroma. Stroma includes endothelial cells, fibroblasts, and immune cells and constitutes the tumor microenvironment (TME). The TME can influence tumor progression through different types of crosstalk. Immune cells, for example, play a vital role in CRC tumorigenesis and can influence progression in both a negative and positive manner and are strong predictors of clinical outcome (142, 143).

The presence of tumor-infiltrating T cells is one prognostic marker of clinical outcome. Specifically, infiltration of cytotoxic ($CD8^+$) T cells and a low $CD4^+/CD8^+$ ratio is associated with improved survival and improved clinical outcome (144, 145). On the contrary, regulatory T cells (Tregs) are generally associated with poor prognosis in solid tumors. While a similar association has been seen in CRC, where poor survival has been linked to high infiltration of Tregs into the tumor stroma (146), their role in CRC is debated due to conflicting data (147).

Macrophages are another group of immune cells with prognostic value. These are plastic cells with two major polarization states: pro-inflammatory (also called M1-like) and anti-inflammatory (also called M2-like) (148). The M1-like subtype is generally considered to have antitumor properties as they promote host defense mechanisms. Tumor-associated macrophages, on the other hand, more closely resemble the M2-like phenotype, which is considered pro-tumor due to the secretion of anti-inflammatory cytokines and factors that promote proliferation, angiogenesis, and migration (149). It is, however, worth noting that although the model of two polarization states has been widely used to describe macrophage activation, it has been shown that macrophages are plastic and have a spectrum of polarization modes rather than the two extreme activation states (150). With

the spectrum of polarization, the classification of macrophages becomes more complex, but can also aid in the understanding of their function in health and disease.

Natural killer (NK) cells make up another type of immune cell that is interesting in relation to cancer. With their strong cytotoxic potential and capacity to recognize tumor cells, they have been implicated in antitumor responses, and have been suggested as candidates for immunotherapy (151, 152). In addition to their own cytotoxic activity, NK cells also regulate other immune cells, including T cells (153). The number of NK cells infiltrating CRC tumors is low, but despite this, NK cell and $CD8^+$ T cell infiltration have been associated with a favorable outcome, most likely as a result of crosstalk between the two immune cell types (154).

1.5 The role of estrogen signaling for CRC development

1.5.1 Epidemiological studies

As mentioned earlier, male sex is a risk factor for the development of CRC. Males have both a higher incidence of CRC and an earlier onset of disease compared to females (155). However, as females enter menopause and their circulating E2 levels decline, they present with a worse overall survival compared to males of the same age (156). This sex difference has also been seen in patients with IBD, where males have been reported to have a 60% increased risk of developing CRC compared to females with IBD (157). These effects on CRC development and survival have been, at least partly, attributed to the effects of estrogens. The protective effect of estrogens is further supported by a significantly reduced risk of CRC following treatment with menopausal hormone therapy (MHT), with especially estrogen-only formulations being linked to up to 60% risk reduction (158, 159). Also, oral contraceptives and intake of phytoestrogens have been linked to lower CRC incidences (158, 160, 161, 162, 163).

1.5.2 ERβ signaling in the colon

ERβ is expressed in the colon (41), but its expression is lost in tumors (164, 165). Polymorphism in the *ESR2* gene is further associated with both CRC risk and survival (166, 167), indicating that ERβ may impact CRC development.

A protective role of ERβ has been demonstrated *in vivo*, as full-body knockout of the receptor leads to enhanced tumor formation in female mice using both a chemically (AOM/DSS)-induced CRC model (168) and the APC^{Min} model (169). Comparably, colonic crypt proliferation and tumor formation were reduced

through selective activation of ERβ in an APC^{Min} model (170). Together, this strengthens the notion that ERβ has anti-proliferative and tumor-suppressive effects in the colon. Supporting this, similar molecular mechanisms have been seen by ERβ in human CRC cell lines and xenografts (171, 172, 173, 174, 175).

However, whether these effects on CRC and inflammation are mediated by intestinal epithelial ERβ have not been demonstrated. To be able to realize the potential of utilizing this receptor for preventative treatments, for example treatment with receptor-selective ligands in high-risk groups, it is vital to properly characterize the CRC-protective effects of intestinal epithelial ERβ.

2 Research aims

The aims of this thesis were to characterize the mechanism of endogenous ERβ and decipher its influence on ovarian function, and to characterize the role of intestinal epithelial ERβ in the colon, with a special focus on related sex differences, during colitis and CAC.

Specific aims:

Paper I. To map the endogenous cistrome of ERβ and corresponding transcriptional role in the mouse ovary.

Paper II. To test whether intestinal ERβ mediates protective effects against colon inflammation and tumor formation in mice of both sexes and explore possible sex differences.

Paper III. To define the colon transcriptome during induced colitis in mice and explore sex differences.

Paper IV. To investigate the impact of intestinal ERβ on the transcriptome and tumor microenvironment during colitis-induced tumor formation and explore sex differences.

3 Materials and methods

3.1 Animal experiments

This thesis utilized both full-body ERβ knockout (ERβKO) mice to decipher the role of ERβ in the normal ovary and mice lacking ERβ specifically in the intestinal epithelial cells (ERβKO^{Vil}) to decipher its role in the colon, colitis, and CAC. All work involving animals in this thesis was approved by the Animal Care and Use Committee and/or the local ethical committee of the Swedish National Board of Animal Research.

3.1.1 ERβ knockout mice

Both full-body and tissue-specific ERβ knockout mice were generated by the Cre-lox system, where exon 3 of ERβ is flanked by two loxP sites (ERβ$^{flox/+}$). The floxed mice were then crossed with transgenic mice bearing the Cre recombinase, either with universal expression for a full-body knockout or under the control of the Villin promoter for an intestinal-specific knockout. Villin is expressed in intestinal epithelial cells, and thus, expression of Cre under this promoter results in a knockout in the epithelial cells of the intestine. The deletion of exon 3 creates a frameshift and early stop codon, with a subsequent complete lack of protein expression. The mice were kept on a C57BL/6J background, and corresponding littermates lacking the Cre allele were used as WT controls for ERβKO and ERβKO^{Vil} mice, respectively. Genotypes were confirmed using standard PCR.

3.1.2 AOM/DSS model of colitis-induced colorectal cancer

The AOM/DSS model is a widely used and highly reproducible model to chemically induce CRC in mice. In this model, carcinogenesis is induced by an intraperitoneal injection of the carcinogen azoxymethane (AOM) followed by the administration of the irritant dextran sodium sulfate (DSS) in drinking water. The administered AOM will induce mutagenesis in the colonic epithelium, and together with the epithelial damage caused by DSS, the model resembles features of IBD and will result in relatively rapid tumor formation. This model is very advantageous as the phenotype and molecular changes closely mimic several features seen in human CAC (176). Further, based on the desired stage of disease for the study, the model can be adapted by modifying the administration of DSS, either by altering the concentration, length of treatment, and/or the number of cycles. In paper **II-IV** of this thesis, five- to 10-week-old mice were injected with AOM (10mg/kg body weight) at the start of the experiment. One week after the injection, the mice were

subjected to 2.5% DSS in drinking water for one week followed by two weeks of normal drinking water. The DSS cycle was repeated for a total of three times. The mice were sacrificed 9 or 15 weeks after the start of the first DSS cycle, representing a stage of acute inflammation and a more chronic stage of inflammation with established tumors, respectively. A disease activity index (DAI) was used to track disease progression and evaluate the severity of the response to treatment. The DAI included scores for loss of body weight, stool consistency, and rectal bleeding.

3.1.3 Tissue collection

3.1.3.1 Ovaries

Ovaries were separated from the uterus and cleaned from surrounding fat tissue. The ovaries were then either snap-frozen in liquid nitrogen for RNA applications, fixed for histological applications, or crosslinked for chromatin immunoprecipitation (ChIP). Ovaries were fixed for 24h in 4% formaldehyde, before being stored in 70% EtOH and later embedded in paraffin. For the crosslinking in preparation for ChIP, fresh ovaries were cut into small pieces and crosslinked with 1% formaldehyde for 15 min before quenching with glycine. Following PBS washes, the samples were snap-frozen and stored at -80°C.

3.1.3.2 Colons

After harvesting, the colons were washed and opened along the longitudinal axis. The number of visible adenomas and polyps was counted, and their size was recorded. For downstream RNA applications, either intact pieces of the colon or the colon-epithelial layer, collected by scraping off the epithelial cell layer, were snap-frozen. For histology, whole colons were rolled into a Swiss-roll before fixation as described above. For colitis scoring, sections were stained with hematoxylin and eosin (H&E) and scored by a pathologist. The scoring included severity, degree of ulceration, degree of hyperplasia, and percentage of affected area, which was scored between 0-3 for a total score ranging between 0-12. To avoid bias during scoring, the pathologist was blinded to the mouse genotype and sex.

3.2 Clinical samples

Paper II included clinical samples of colon tumors and matched adjacent non-tumor colon tissue to measure the expression of *ESR2* in the human colon and CRC. These samples were collected from 22 patients (16 females and 6 males)

that underwent surgery in Stockholm. The study was approved by the local ethical review board in Stockholm.

3.3 Cell lines

The use of cell lines provides a well-controlled system that can be used for a wide variety of applications to elucidate molecular mechanisms. While the lack of a microenvironment and complexity in the form of different cell types contributes to the high degree of control, it also has the drawback of not providing the outside influence that can be essential when studying cancer development. In this thesis, human CRC cell lines HT-29 and SW480 were used for *in vitro* studies of ERβ. ERβ was either stably expressed with a lentivirus system or transiently expressed in the cells, depending on the application. The cells were cultured in standard cell culture media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

Both cell lines used in the thesis are derived from colorectal adenocarcinomas. SW480 was obtained from a 50-year-old male with a Dukes' B primary tumor (177) and HT-29 from a 44-year-old female with a more advanced Dukes' C primary tumor (178). Both SW480 and HT-29 are microsatellite stable, but HT-29 is positive for CIMP. They also differ in their mutations. SW480 has mutated *KRAS* (G12V) but lacks the mutations in *BRAF* (V600E), *PIK3CA* (P449T), and *PTEN* seen in HT-29. Although both cell lines do have mutations in *TP53*, SW480 has a double mutation (R273H and P309S) while HT-29 only harbors one (R273H) (179).

3.4 Antibody-based protein detection

3.4.1 Western blot

Western blot (WB) is an extensively used antibody-based method to visualize proteins in a sample. In this thesis, SDS-PAGE was applied, where denatured proteins are loaded on a polyacrylamide gel containing SDS, giving all proteins a negative charge and allowing for the separation of proteins by size during electrophoresis. Following electrophoresis, the proteins are transferred to a PVDF membrane where they can be detected by antibodies. A primary antibody is added to the membrane and binds to the epitope of interest, followed by a secondary antibody conjugated with horseradish peroxidase (HRP) for detection. The addition of enhanced chemiluminescence (ECL) substrate, which reacts with the HRP, gives a luminol-based signal that can be captured by X-ray film or a digital imager and quantified.

In this thesis, WB was used in **paper I** to detect ERβ in mouse ovary samples, and in **paper II** to measure the nuclear translocation of p65.

3.4.2 Immunohistochemistry

Immunohistochemistry (IHC) is another extensively used antibody-based method to visualize proteins in tissues or cells, with the advantage of providing spatial information. In this thesis, an indirect staining method was used to detect proteins of interest. As in WB, a primary antibody was used to detect the antigen. However, instead of an HRP-linked antibody, a biotinylated secondary antibody against the appropriate species of IgG was used. For detection, an avidin-biotin complex with HRP was added that reacts with the substrate 3,3'-diaminobenzidine and produces a colorimetric stain that can be visualized with a light microscope.

IHC was used in **paper I** to confirm the expression and location of ERβ in sections of mouse ovary. Further, in **paper II,** we used IHC to measure proliferation after AOM/DSS treatment by staining for Ki67 in the colon of mice. Lastly, in **paper IV,** IHC for the macrophage marker CD68 was used to study the infiltration of macrophages into inflamed tissues and tumors.

3.4.3 Immunofluorescence

While IHC and immunofluorescence (IF) are similar methods, in IF, the proteins are visualized with a secondary antibody linked to a fluorochrome and visualized with a fluorescent microscope. While IF has certain disadvantages such as loss of signal over time and autofluorescence, an advantage of IF is that it can easily be used for multiplex staining of several proteins.

In this thesis, IF was used in **paper IV** to stain for CD3, FOXP3, and NK1.1 to detect the presence of T cells, regulatory T cells, and NK cells, respectively.

3.4.4 Antibody specificity

While antibody-based methods for protein measurement are important pillars in molecular biology research, they are error-prone, which easily can lead to inaccurate conclusions. While some errors can be avoided by proper blocking of non-specific staining, a major problem relates to antibody specificity. Regardless of the application, it is essential to ensure that the antibody is detecting the intended target, and the antibodies used should therefore be validated for the chosen application before use. This can be further aided by including a negative control, enabling the exclusion of any non-specific staining that may appear. As

an example of this, we used a validated anti-ERβ antibody (mouse monoclonal PPZ0506, R&D Systems, cat no: PP-PPZ0506-00, RRID: AB_2293861) in **paper I** for the detection of ERβ in the mouse ovary and included ovaries from ERβKO mice as negative controls in both the WB and IHC.

3.5 RNA *in situ* hybridization

RNA *in situ* hybridization utilizes labeled complementary RNA to detect and visualize mRNA in tissue. In this thesis, this was specifically used to detect *Esr2* mRNA in sections of ovary and colon in **papers I** and **II**, respectively. While the ovary has a high expression of ERβ, expression in the colon is low. The low abundance of ERβ protein in the mouse colon is not ideal for detection by IHC. In our case, this is further complicated by the fact that the validated antibody is generated in mice and thus, the corresponding secondary antibody will detect endogenous mouse IgG giving rise to background staining. RNA *in situ* hybridization circumvents this issue and was therefore used to visualize ERβ expression in the colon of WT mice. While this is a minor issue in the ovary due to the high ERβ protein expression, RNA *in situ* it still provided valuable information to exclude any potential non-specific staining in specific cell types where no mRNA was present.

3.6 Quantitative PCR

In quantitative PCR (qPCR), DNA or RNA in the form of cDNA is amplified and measured in real time. Using target-specific primers, a sequence is amplified over several cycles and measured by fluorescence. There are several types of reporters for measuring the amount of amplified DNA and this thesis has applied the use of SYBR Green, a reporter dye that binds all double-stranded DNA. Based on the number of cycles required to reach the signal threshold, a cycle threshold (Ct) value will be given for each reaction and is inversely proportional to the amount of target DNA in the sample. The Ct value can then be used qualitatively to determine the presence of the target or quantitatively to measure the level of expression. Here we have used the ΔΔCt method for calculations of relative levels of gene expression between samples. In the ΔΔCt method, the expression of the gene of interest (reflected by its Ct value) is first normalized to the expression of one or several reference genes, generating a ΔCt value for each sample. The difference in ΔCt between sample groups is then calculated to get the ΔΔCt. Lastly, this is converted to fold change ($2^{-\Delta\Delta Ct}$).

3.7 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) is a method for studying the interaction between DNA-binding proteins, such as transcription factors, and DNA. This can be done in both tissue and cell lines, as in **paper I** and **paper II**, respectively. In this method, proteins are cross-linked to DNA to preserve their binding location. The DNA is then sheared by sonication into fragments, often in the range of 150-300 bp. To isolate the DNA bound by the protein of interest, the protein-DNA complexes are pulled down using a protein-specific antibody. Once pulled down, the cross-linking is reversed, and the resulting DNA can be analyzed, commonly by sequencing or qPCR. While a simple ChIP-qPCR can show whether a particular sequence of DNA has been bound, sequencing of the DNA provides a complete map of binding sites. Still, while it is an accurate map if the proper antibody is used, it does not differentiate between direct binding and binding that comes from interactions with other transcription factors. However, this can be further analyzed experimentally or *in silico*.

As discussed in section 3.4, it is of high importance to use validated antibodies and to include proper controls when using antibody-based methods. Therefore, we once again used the validated ERβ antibody (PPZ0506), which was further validated for ChIP in the lab (180, 181). In addition, negative controls, including mock cells or knockout tissue and IgG ChIP, were used to normalize the results.

When studying the interaction of two DNA-binding proteins, one can perform a ChIP re-ChIP. With this method, once the first pull-down has been done, the eluted protein-DNA complexes are pulled down again with a second antibody. In **paper I** this was done by first performing ChIP for ERβ which was then re-ChIPed for LRH-1. By performing the re-ChIP it is possible to determine whether the two proteins bind to the same DNA fragment, either together or in the same protein complex.

3.8 Transcriptome analysis

3.8.1 Illumina RNA-sequencing

RNA-sequencing (RNA-seq) is one of the primary methods in this thesis and was used in **papers I**, **III** and **IV** to study the transcriptome of ERβ in the mouse ovary and colon and to determine the transcriptional changes during AOM/DSS-induced colitis.

For all studies, library preparation was done with TruSeq Stranded Total RNA and included depletion of rRNA with RiboZero. With this type of library preparation, the

RNA is fragmented and denatured before being converted to cDNA. Once in cDNA form, the 3' ends are adenylated before adapters are ligated to the ends to allow the cDNA to hybridize to the flow cell. The cDNA is then enriched by amplification, followed by a quality check of the final libraries. The produced libraries can then be sequenced.

For sequencing, we used NovaSeq 6000 from Illumina which uses a sequencing-by-synthesis technique. With this technique, fluorescently labeled nucleotides are incorporated one by one into a new complementary DNA strand. After each cycle, the fluorescence is recorded by laser detection. As each nucleotide has a different fluorescent label, the detection gives information about which nucleotide has been incorporated, giving a base-by-base sequence.

3.8.2 Illumina bead array

To study the effect of ERβ on the transcriptome in SW480 and HT-29 cells before and after 2h of TNFα treatment we used the Illumina Whole-Genome Gene Expression Direct Hybridization Array in **paper II**. After converting RNA to cRNA with the Illumina TotalPRep-96 RNA Amplification kit, the cRNA was hybridized to the target-specific probes coated on silica microbeads. Once hybridized, the signal is read by a scanner and the relative expression level is determined by comparing the signal intensity between conditions.

3.8.3 Downstream analysis

3.8.3.1 Differentially expressed genes

After RNA-seq the generated reads were mapped against the mouse genome (GRCm38) using STAR. To generate gene counts as transcripts per million (TPM) and fragments per kilobase of transcript per million mapped reads (FPKM) values FeatureCounts and StringTie were used. To calculate differentially expressed genes, we used DESeq2 with raw counts as input and the Benjamini-Hochberg procedure for false discovery rate (FDR) estimation. For the bead array results, the lumi and limma package was used to determine differentially expressed genes. For both techniques genes were considered to be differentially expressed if FDR$<$0.05 and $\log_2$FC$>$|0.4|. Genes with an average expression below 1 FPKM in both sample groups were filtered out. Gene ontology/biological function was analyzed using Elsevier's Pathway Studio (11.2.5.9) or the DAVID bioinformatics website.

3.8.3.2 Digital cytometry

While bulk RNA-seq is useful to get an idea of global gene expression in samples, it lacks information regarding expression in specific cell types. Thus, some gene expression changes can be missed, or changes seen could be due to changes in cell composition. This is an issue that has been solved with the development of single-cell (sc) RNA-seq. However, in cases where bulk RNA-seq has been performed, new methods have been developed to extract single-cell data as a way of digital cytometry. In **paper I** and **paper IV** we used CIBERSORTx to perform this type of analysis on our bulk RNA-seq data. With this type of method, cell type abundance is estimated based on signatures from previously performed scRNA-seq. While the generated abundance is just an estimation and less robust than actual scRNA-seq, it can still give an idea of changes at cell type level.

3.9 Luciferase assay

While methods like ChIP can show whether a transcription factor binds to a particular sequence, either directly or indirectly, it does not show the effect of the binding in terms of gene regulation. Similarly, RNA-seq and qPCR can show a change in gene expression but do not reveal the underlying transcriptional regulation. With a luciferase assay, it is possible to determine the transactivation activity of a protein at a particular site, including how that activity can be impacted by other proteins. However, the method is limited by the artifactual nature of the assay, where transactivation is limited to a specific DNA sequence, without the context of the whole chromatin structure environment. This was utilized to determine the nature of the crosstalk between ERβ and LRH-1 in **paper I** and how ERβ and NFκB impact each other in **paper II**. An ERE, NR5A-RE, or NFκB-RE was placed upstream of the luciferase gene (LUC), and the level of luciferase was then quantified as a measure of transactivation activity at these sites. In both experiments, a dual reporter assay was used, with a renilla luciferase reporter as an internal control for normalization.

3.10 Organoid crypt formation

As mentioned in section 3.3 one of the disadvantages of using cell lines is the lack of complexity that can hamper the biological relevance. Organoids from patients or animals can help mimic *in vivo* conditions including tissue organization. In **paper II** we used intestinal organoids from WT and ERβKO^{Vil} mice to study the impact of ERβ on TNFα-induced epithelial cell damage. Intestinal organoids from stem cell-containing crypts were grown in Matrigel for 5-7 days and then treated with TNFα

and/or the ERβ selective agonist DPN. After treatment, the number of *de novo* crypt domains was recorded in a blinded scoring. To be noted, this experiment used organoids derived from the small intestine and not colonic crypts, which limits the conclusions that can be drawn regarding the effects of ERβ in the colon.

3.11 Statistical analysis

GraphPad Prism (GraphPad Software Inc.) was used for statistical analysis. The provided data are expressed as mean ± standard error of the mean. When comparing two groups, a two-tailed Welch's t-test or paired t-test was used. For comparison between multiple conditions, one-way or two-way ANOVA followed by Fisher's LSD or Tukey's multiple comparisons test was used. A p-value$<$0.05 was considered statistically significant.

3.12 Ethical considerations

All the papers included in this thesis have involved experimental animal models and in one instance clinical samples (**paper II**), which were all approved by an ethical committee as described in sections 3.1 and 3.2.

Although all findings in animal studies are not directly translatable to humans, significant genetic and physiological similarities between animals and humans make animal models an essential pillar in research. The use of animals provides a robust but modifiable model to study molecular mechanisms in health and disease while preserving the impact of all complex factors that influence the outcome, which cannot yet be achieved through *in vitro* studies. Still, working with animal models entails several ethical considerations, and we therefore implemented the principle of the 3Rs (Replace, Reduce, Refine) when applicable. An example of this was minimizing the suffering of the mice subjected to AOM/DSS treatment. During AOM/DSS treatment, mice are expected to lose weight, develop diarrhea, rectal bleeding, and in the worst case, rectal prolapse. Consequently, proper monitoring and clear guidelines for experiment endpoints are essential. Therefore, the animals were assessed daily during the active treatment cycle, and mice were sacrificed if they lost more than 20% in body weight, developed rectal prolapse, or if their overall health was poor (according to scoring guidelines from KI).

As the clinical samples were collected from patients that were already undergoing surgery, sample collection did not present an inherent additional risk to the patients. However, as required when using human samples, informed consent was

obtained from all patients and data was anonymized to protect the medical privacy of each patient.

While cell lines are usually described as tools that circumvent the ethical issues associated with animal and human tissue, the use of cell lines in research comes with its own ethical considerations. Historically, there has been a lack of complete informed consent during the generation of cell lines. The most famous case is the creation of the HeLa cell line, created using cells unknowingly obtained from Henrietta Lacks in 1951 (182). In such instances, patients have not had complete knowledge of how their samples can potentially be used after collection. Additionally, with the development of today's technologies, sensitive data, including whole genomic data, can easily be generated. Publication of such data can have implications for the patient's biological relatives, which also raises the question of medical privacy.

4 Results and Discussion

This section summarizes the main findings in **papers I-IV**, which together aim to determine the role of estrogen signaling through ERβ in the normal ovary and its impact during colonic inflammation and CRC.

4.1 Paper I: Ovarian ERβ cistrome and transcriptome reveal chromatin interaction with LRH-1

While the ovary is the primary site of ERβ expression, where it has been shown to be important for follicle and oocyte maturation, studies to elucidate the exact mechanism of action of ERβ have been limited. The lack of mechanistic understanding includes characterization of its endogenous genome-wide chromatin binding, identification of its binding partners, and the direct transcriptional impact. In order to map the endogenous cistrome of ERβ we performed ChIP-seq in mouse ovaries and applied corresponding RNA-seq on ovaries from WT and ERβKO mice to determine the direct transcriptional impact of ERβ in the ovary.

First, we confirmed the expression of ERβ in mouse granulosa cells and the lack of expression in ERβKO ovaries. Next, our cistrome data revealed a total of 4875 endogenous ERβ-binding sites in the ovary. The majority of the identified binding sites were located in introns and intergenic regions, with only 5% in promoter regions (-1kb to +100bp from the TSS), in accordance with previously described exogenous ERβ cistromes (181, 183, 184). As expected within these ERβ-bound sequences, the ERE was the most enriched motif. While other well-known ER interaction partners such as GATA, AP-1, and FOXO were found to be highly enriched among the sequences, the NR5A motif, bound by the nuclear receptors SF-1 and LRH-1, was the second most enriched motif and was as abundant as the ERE.

Our transcriptomic data revealed that loss of ERβ in the ovary resulted in 803 differentially expressed genes (DEGs). Further analysis of the genes revealed a downregulation of genes related to estrogen response and cell adhesion, while genes related to male gonad development were upregulated. Assuming a link between ERβ chromatin binding and changed transcriptional regulation upon knockout, we integrated the cistrome and transcriptome to identify the direct transcriptional targets of ERβ. Here we found that 30% of the DEGs had one or multiple sites bound by ERβ. The 245 genes denoted as direct targets of ERβ were

enriched for functions related to response to estrogen, cell differentiation, response to hypoxia, multicellular organism development, and lipid metabolism, all of which are important for proper ovarian function (Figure 8A).

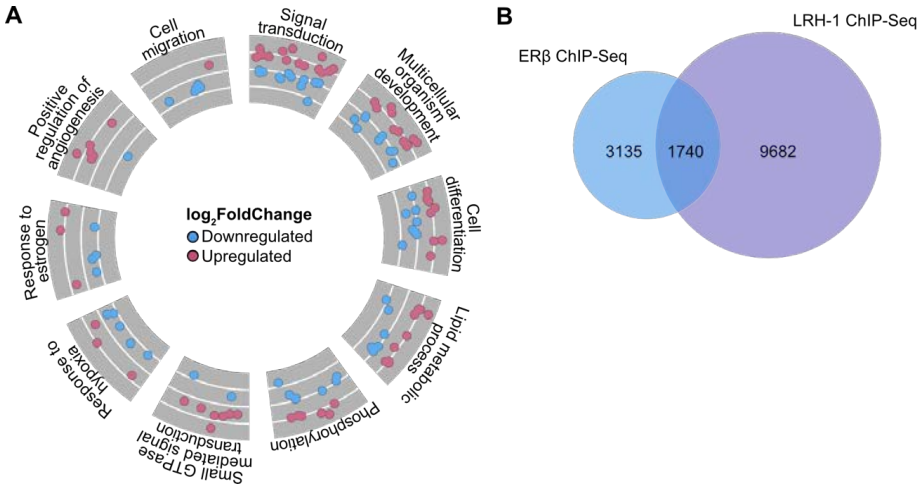

Figure 8. (A) Direct targets of ERβ are enriched for pathways important for ovarian function, including lipid metabolism, response to estrogen, cell differentiation, and response to hypoxia. **(B)** ERβ shares a cistrome with LRH-1.

Lastly, as the NR5A motif was highly enriched among the ERβ-bound sites, indicating that ERβ may function in the same protein complex as SF-1 and/or LRH-1, we explored their potential crosstalk. Comparing the ERβ cistrome to a publicly available LRH-1 cistrome (106), we revealed a large overlap with sites that were bound by both ERβ and LRH-1 (Figure 8B). While the overlap in cistromes indicates a relationship between the two receptors, we wanted to further understand the underlying mechanism to see whether ERβ and LRH-1 bind together at the sites, or if they compete for binding, as illustrated in Figure 9. While the high fraction of co-bound sites with an NR5A motif and no ERE indicate that LRH-1 may act as a pioneering factor for ERβ, we wanted to explore their interaction experimentally. ChIP-reChIP revealed simultaneous binding at an intronic binding site in the *Greb1* gene, while there was no significant enrichment at the intronic binding site in *Fkbp5* and *Cyp11a1*, indicating potential competition at these sites. Using a luciferase assay, we could further show that ERβ and LRH-1 can repress each other's transactivation at their respective response element, suggesting a possible repressive mechanism at the co-bound targets.

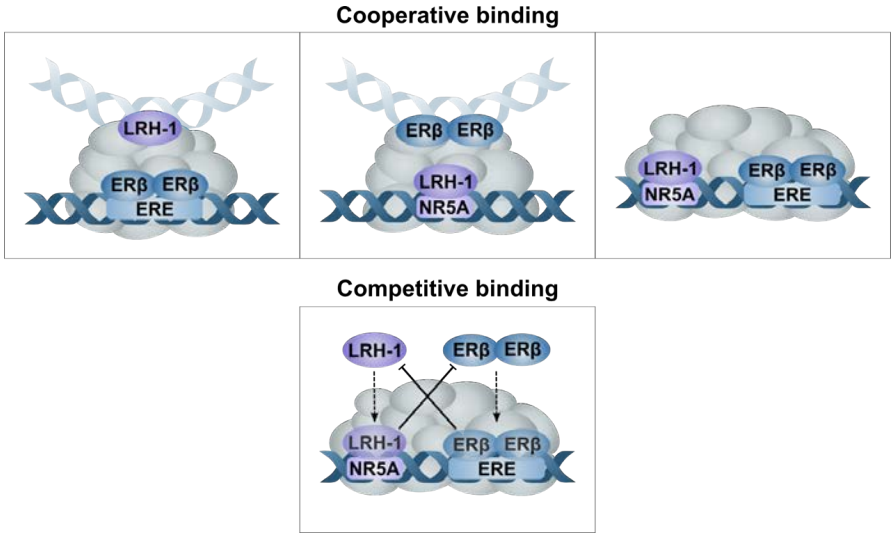

Figure 9. Illustration of proposed binding mechanism on chromatin bound by both ERβ and LRH-1.

In this study, we provide new insight into the endogenous gene regulation by ERβ in the ovary, including a map of the genome-wide endogenous chromatin-binding and regulation of pathways important for ovarian function. We also determine a novel crosstalk with LRH-1, providing new mechanistic understanding of the actions of ERβ in the ovary.

4.2 Paper II: Intestinal estrogen receptor beta suppresses colon inflammation and tumorigenesis in both sexes

Although studies on full-body ERβ knockout mice and treatment with ERβ-selective ligands support the concept that estrogen signaling through ERβ protects against CRC development, the full mechanism has not been thoroughly investigated. To potentially implement ERβ as a treatment target, it is important to understand the underlying mechanism, including the cells responsible for the protective effect. While the extent of ERβ expression has been controversial, it has been detected at low levels in the colon, but also in some immune cells. It is therefore possible that ERβ affects CRC development through the immune system. Whether ERβ in the intestinal epithelial cells, specifically, protects against colitis and CAC has not previously been studied. Further, despite the well-known sex differences in CRC, the majority of studies on estrogen and ERβ have been performed in female mice only. In order to decipher the role of intestinal epithelial ERβ and explore potential sex differences, we induced colitis and CAC through the

AOM/DSS model in male and female mice lacking ERβ specifically in the intestinal epithelial cells (ERβKO^{Vil}).

We first confirmed the expression of ERβ in human and mouse colons by IHC with a validated antibody (PPZ0506) and RNA *in situ* hybridization, respectively. We further demonstrated a loss of expression in human CRC tumors, showing the clinical relevance of ERβ as a chemoprotective target. The protective effect of intestinal ERβ was then confirmed, as the loss of ERβ resulted in significantly more tumors in males and significantly larger tumors in female mice compared to their WT littermates (Figure 10A,B). In addition to the sex differences in tumor formation, we also observed that male mice had a higher degree of colitis. Further, while both male and female mice showed a significant increase in the expression of *Tnfa* and targets of NFκB after treatment, the increase was more prominent in males (Figure 10C). Still, while the males were more susceptible to colitis, female ERβKO^{Vil} mice had more ulcerated areas with delayed healing, which could be linked to the impaired proliferation of the colonic epithelial cells seen in these mice.

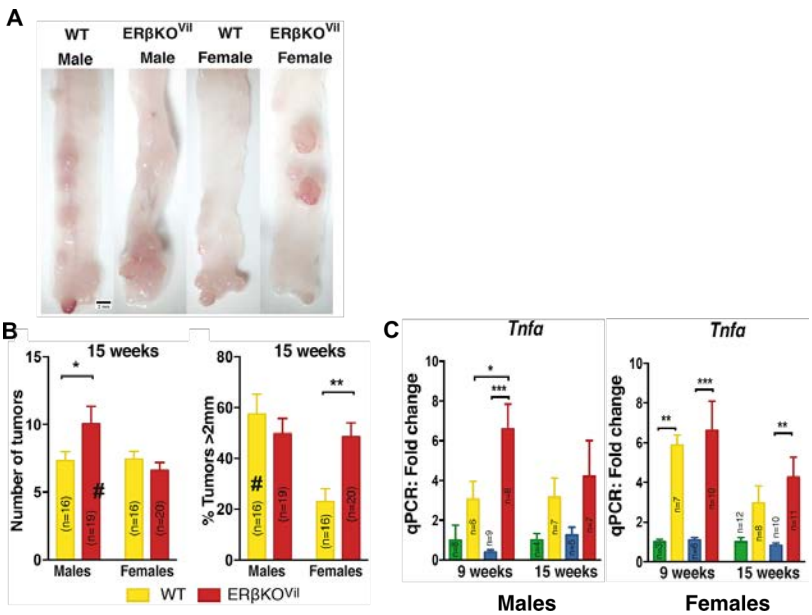

Figure 10. (A,B) Intestinal ERβ protects against tumor development in both sexes. Loss of ERβ leads to an increased tumor size in females and an increased tumor number in males. **(C)** Male mice had a more prominent inflammatory response compared to females.

Next, we demonstrated that ERβ could suppress TNFα signaling both *ex vivo* and *in vitro*, where ERβ activation could protect against TNFα-induced epithelial damage and modify the transcriptomic response. This further emphasizes the

protective effect of ERβ expressed specifically in the colon epithelial cells. While TNFα enhanced transactivation by ERβ in a luciferase assay, neither the transactivation of NFκB nor the translocation of p65 was affected by ERβ. However, we were able to show through ChIP-qPCR that ERβ could bind to several cis-regulatory regions of key NFκB regulators. The regulation of the NFκB regulators *Atf3* and *Bcl3* was further confirmed *in vivo*.

To conclude, we demonstrate a sex-dependent protection by intestinal epithelial ERβ against colitis and CRC *in vivo*. We further propose a mechanism involving crosstalk with NFκB, as illustrated in Figure 11. In this mechanism, the increased levels of TNFα lead to increased activity of ERβ, which in turn will attenuate the formation of adenomas through inhibition of the TNFα/NFκB signaling pathway.

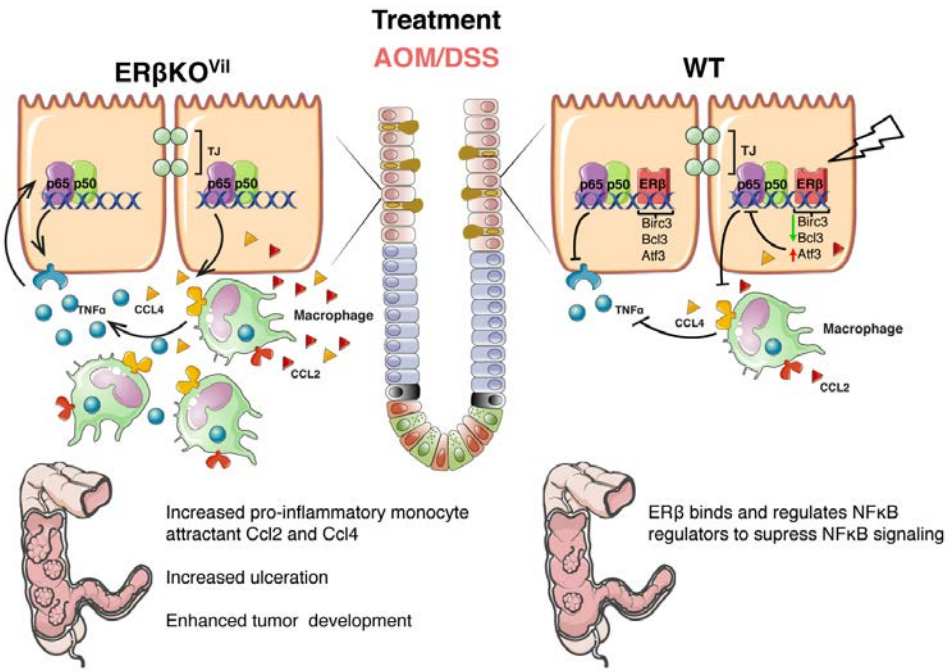

Figure 11. Illustration of the proposed mechanism of how intestinal ERβ protects against CRC development. In the presence of ERβ, there is reduced TNFα signaling following direct regulation of NFκB regulators. The subsequent reduction in NFκB-induced inflammatory signaling results in less recruitment of TNFα-secreting macrophages and reduced tumor formation. Note: the image contains elements that were modified from Servier Medical Art (https://smart.servier.com/), licensed under a Creative Commons Attribution 3.0 Unported Liscence.

4.3 Paper III: Colitis induces sex-specific intestinal transcriptomic responses in mice

In **paper II** we demonstrated clear sex differences in tumor development, including larger tumors and increased expression of certain cytokines in males compared to females. We have also previously demonstrated sex differences in the colon transcriptome of mice both at baseline and in response to a high-fat diet (HFD) (185). While the transcriptional changes during AOM/DSS treatment have been previously characterized, few studies have attempted to define sex-specific target genes despite the known sex differences in CRC. To characterize the sex-specific transcriptome during colitis and improve the understanding of sex differences seen in CAC, we performed a detailed analysis of colon epithelial cells from AOM/DSS-treated mice of both sexes. The analysis was done at the same time points as in **paper II**, at 9- and 15-weeks after treatment initiation.

First, we established that the colon transcriptome differed between the sexes at baseline, with genes enriched for functions in cell proliferation, cell migration, and oxidation-reduction process. Next, we were able to confirm previously defined changes, including the induced expression of *Tnf*, *Ccl4*, *Il1b*, *Nos2*, and *Ptgs2*, validating both the treatment and the transcriptomic analysis. While both sexes displayed an induced immune and inflammatory response to AOM/DSS treatment, males had an overall stronger transcriptional response. During both the acute and chronic phase of inflammation, the male-specific response included the enrichment of genes related to NFκB signaling, an important player in the inflammatory response. Another interesting finding was the enrichment of functions related to circadian rhythm, an important regulator of colonic function which when disrupted contributes to both IBD and CRC (Figure 12A). Specifically regulated genes included an upregulation of *Cry1* and *Arntl2*, and a downregulation of *Nr1d1* (Rev-erbα), *Cry2*, *Per2*, and *Per3*, all part of the core circadian clock (Figure 12B). We further note a difference in the regulation of steroid hormone receptors between the sexes. At 9 weeks, several nuclear receptors were downregulated only in male mice. Several of these genes have been implicated in colitis and/or CRC, including *Vdr*, *Nr5a2* (*Lrh-1*), and *Nr1h3* (*Lxrα*).

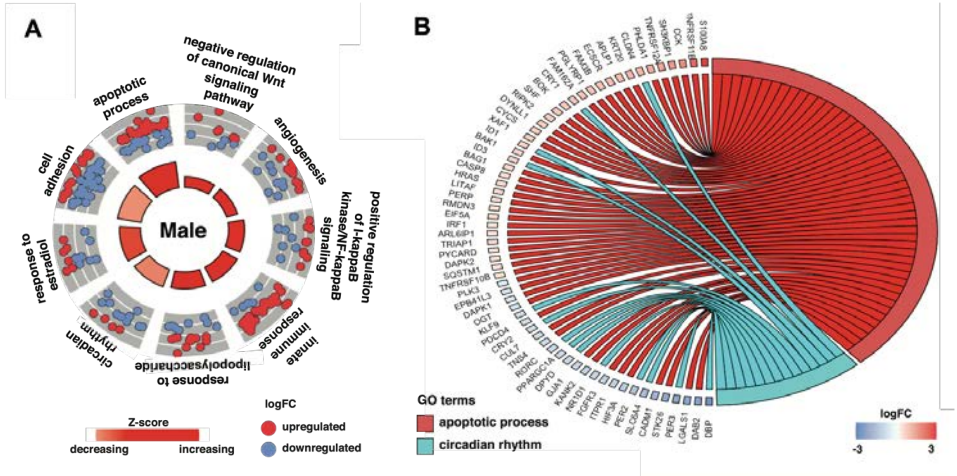

Figure 12. (A) Male-specific genes were enriched for regulation of NFκB signaling as well as circadian rhythm. **(B)** Specific regulation of circadian rhythm genes included the core genes *Cry1, Arntl2*, *Nr1d1* (Rev-erbα), *Cry2*, *Per2*, and *Per3*.

By directly comparing the male and female transcriptome at the two time points, we demonstrate decreased sex differences during acute inflammation compared to baseline and chronic inflammation. Further analyzing all genes that were differentially expressed between the sexes identified three sets of genes with higher expression in males compared to females. These gene sets were enriched for functions that included response to hypoxia, NFκB signaling, negative regulation of cell proliferation, cell chemotaxis, and lipid metabolic process.

Lastly, we investigated the transcriptomic changes along the course of the AOM/DSS treatment by comparing the acute and chronic inflammatory stages. As expected, inflammatory genes were often upregulated at 9 weeks and partly or completely repressed to baseline levels after 15 weeks of treatment. In **paper II,** the DAI showed a rapid response to DSS in females, which was resolved at a faster pace compared to males, in line with a previous study where a rapid resolution of inflammation protected females against systemic inflammation (186). This is also reflected in the transcriptome, where males showed a response related to adaptive immune response at 9 weeks, while the same response was seen in females only after 15 weeks.

Altogether, this data demonstrates clear transcriptomic differences in the response to colitis based on sex, including increased inflammatory signaling and dysregulated circadian rhythm in males. Although this result may not fully reflect the transcriptional changes in human colitis and CRC development due to species

differences and cause of inflammation, it emphasizes the necessity of considering both sexes when characterizing disease mechanisms.

4.4 Paper IV: Intestinal estrogen receptor beta modulates the tumor immune microenvironment in a mouse model of colitis-associated cancer

The chronic inflammation induced by colitis creates a tumor-promoting environment. An important influence in the TME is the infiltrating immune cells including T cells, NK cells, and tumor-associated macrophages (TAMs). A high infiltration of immune cells is generally associated with a favorable outcome in CRC patients, but the location and type of immune cell also appear to impact survival. With the observed differences in tumor formation and the indication of increased immune infiltration seen in **paper II**, we wanted to further explore how ERβ influences the microenvironment both at the transcriptional level and regarding immune cell composition. Here, we investigated the transcriptional changes induced by AOM/DSS treatment in non-neoplastic tissue and tumors and stained for NK cells, macrophages, T cells, and regulatory T cells to profile the impact of ERβ.

In agreement with the results in **papers II** and **III**, male mice had a stronger transcriptional response during the progression from inflamed colon to tumor, indicating an enhanced sensitivity to AOM/DSS treatment compared to female mice. While many of the transcriptional changes identified between the inflamed colon and tumors were commonly regulated within all groups, we noted an impact by both sex and intestinal ERβ on a selection of genes. The condition-specific genes were enriched for distinct pathways, suggesting an impact by intestinal ERβ and sex on the pathways in colitis-induced tumor formation.

As we found distinct transcriptomic differences between male and female mice at 15 weeks of AOM/DSS treatment in **paper III**, we next explored whether these differences were affected by the loss of intestinal epithelial ERβ. In both the inflamed epithelium and tumors, lack of intestinal ERβ diminished the sex differences seen in WT mice, implicating a role for ERβ in regulating sex-dependent genes. This notion was further supported by the lack of differential expression in tumors of WT mice where the expression of ERβ has been lost.

Despite the lack of ERβ expression in tumors, we still noted an impact of the intestinal knockout on the transcriptome in tumors from female mice (Figure 13A). Loss of intestinal ERβ resulted in higher levels of *Il6* and *Ccl4* in female tumors,

supporting the findings from **paper II**. Further, female tumors also displayed a decreased expression of vitamin D receptor (*Vdr*), shown to protect against tumor development (187, 188), as well as a downregulation of genes enriched for immune cell-related pathways (Figure 13B). Through cellular deconvolution, tumors from female ERβKO^{Vil} mice were noted to have a higher fraction of tumor cells compared to WT females. The increased expression of inflammatory genes and decrease in *Vdr* together with a higher abundance of tumor cells following loss of intestinal ERβ support a more aggressive phenotype and might explain the increased tumor size in ERβKO^{Vil} females. While the tumors themselves no longer express ERβ, the adjacent tissue still maintains expression and could thus impact the microenvironment, including the recruitment and activation of immune cells.

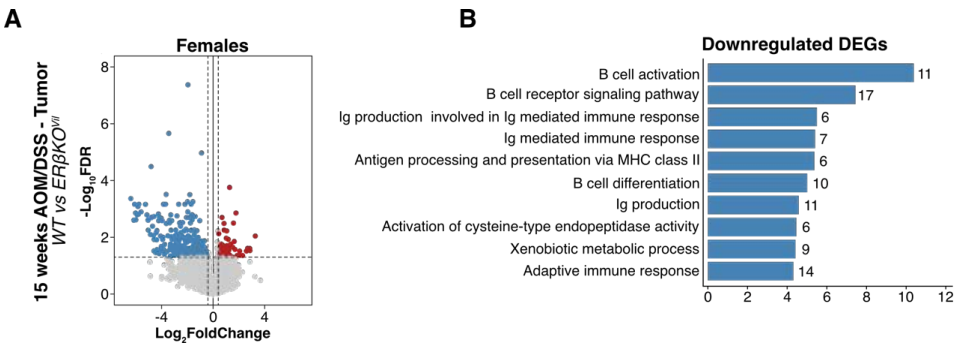

Figure 13. (A) Loss of intestinal ERβ impacted the tumor transcriptome in female mice. **(B)** The downregulated genes were enriched from immune cell-related pathways, including B cell activation and differentiation.

In agreement with the link to immune response seen in the transcriptome, we observed a change in immune cell infiltration upon loss of intestinal ERβ. In **paper II**, we demonstrated an increased expression of *Ccl2* and *Ccl4* in ERβKO^{Vil} mice, which is one of the recruiting factors of circulating monocytes, and hypothesized that it may lead to increased macrophage infiltration. This was partly reflected in the number of infiltrating CD68^{+} cells, where infiltration into ERβKO^{Vil} tumors was significantly increased compared to non-neoplastic tissue, which was not seen in WT mice. While there was an increased infiltration also into WT tumors compared to vehicle-treated tissue, there was a trend of higher infiltration into ERβKO^{Vil} tumors compared to WT tumors. On the contrary, tumors from ERβKO^{Vil} mice had a significantly lower infiltration of T cells compared to WT mice (Figure 14A,B). A high infiltration of T cells has been linked to better outcomes and has a strong negative correlation with stemness. Stemness in turn has been linked to

immunosuppression in tumors and reduced NK cell infiltration. In line with this, NK cell infiltration was significantly increased in WT tumors while there was a complete lack of infiltration into ERβKO^{Vil} tumors (Figure 14C,D). The reduced T cell infiltration and the impaired NK cell infiltration in ERβKO^{Vil} mice may therefore be linked to stemness, indicating that ERβ suppresses the development of immunologically cold tumors.

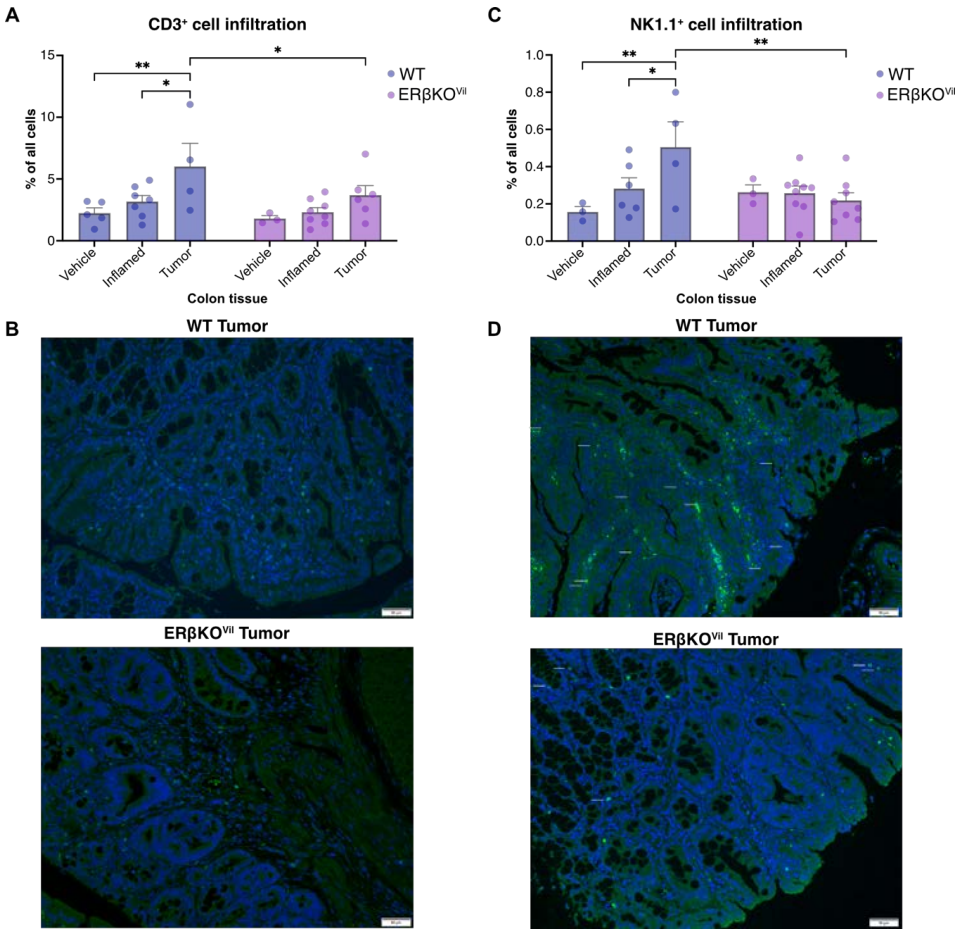

Figure 14. Loss of intestinal ERβ altered the tumor microenvironment by reducing the infiltration of **(A,B)** T cells and **(C,D)** NK cells into tumors.

To conclude, these data show that intestinal ERβ impacts the carcinogenic process at the transcriptional level and specifically modulates the tumor transcriptome in female mice. We further provide evidence that ERβ modulates the tumor immune microenvironment, where loss of intestinal ERβ promotes an immunosuppressive TME.

5 Conclusions

The work presented in this thesis has primarily focused on characterizing the mechanism, role, and function of ERβ in its normal setting in the ovary and colon, as well as during colitis and CRC, and the associated sex differences. The major findings of the thesis are summarized below:

- ERβ has a direct role in regulating genes involved in several functions essential for normal ovarian function, including regulation of the FSH receptor and genes involved in the diestrus E2 surge.

- Endogenous ERβ in the ovary has a shared cistrome with LRH-1, where the receptors can bind simultaneously at certain sites and also have the ability to repress each other's transcriptional activity.

- Intestinal epithelial ERβ protects against tumor development in a sex-dependent manner. Loss of intestinal ERβ leads to larger tumors in female mice and an increased number of tumors in males.

- The protective mechanism of intestinal ERβ involves suppression of the TNFα/NFκB signaling pathway, resulting in reduced inflammation. The response was especially strong in males.

- There are significant sex differences in the transcriptomic response to colitis, largely related to inflammation and the immune system.

- Loss of intestinal ERβ directly alters the tumor transcriptome in female mice and impacts transcriptional changes during tumor development.

- Intestinal ERβ affects the tumor microenvironment by modulating macrophage, T cell, and NK cell infiltration.

6 Future perspectives

As ERβ is expressed at low levels in tissues outside of the ovary and nearly all cultured cells lack ERβ expression, the *in vivo* molecular mechanism of ERβ has been difficult to study. Therefore, most studies have been performed using ERβ exogenously added to cell lines. While such studies aid in the understanding of ERβ, they will not fully mimic its endogenous function. The findings in **paper I** provide a foundation to better understand the function of ERβ in the ovary and its role in fertility. While there will be tissue and species differences, this work may also give important insight into other areas of ERβ research, including its role in the colon. However, although we provide a map of the cistrome and evaluate transcriptional changes in the whole ovary following ERβ knockout, it would be of interest to investigate the transcriptional changes during different stages of the estrus cycle. During the estrus cycle, the ovary goes through distinct stages, with specific transcriptional activity. Analyzing each stage of the cycle would therefore give a more comprehensive understanding of the role of ERβ during different ovarian processes. Additionally, while bulk RNA-seq provides information regarding the general gene regulation in the ovary, it would be valuable to perform scRNA-seq on the ovary to determine cell type-specific regulation. This would also allow us to capture the ERβ-regulated genes expressed in lowly abundant cell types, which may otherwise be lost in the transcriptional noise of the bulk RNA-seq.

Further, the identification of LRH-1 as a new interaction partner of ERβ provides a new mechanism behind the role of ERβ in regulating female fertility. While we manage to show that the two receptors can bind simultaneously at certain sites and that they are able to repress each other's transcriptional activity, more research is needed to further elucidate the full mechanism behind their crosstalk. First, further mapping of all co-bound sites, potentially during different estrus cycle stages, by sequencing of the ChIP-reChIP could give further insight into how the interaction between ERβ and LRH-1 impacts fertility. To achieve a more comprehensive view of the interaction between ERβ and other DNA-binding proteins, methods such as RIME (Rapid Immunoprecipitation Mass spectrometry of Endogenous protein) should be used to map the complete interactome of ERβ. Lastly, studying the chromatin structure through methods such as Hi-C, and investigating how chromatin accessibility is affected by ERβ loss using for example ATAC (Assay for Transposase-Accessible Chromatin) or FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements), could further enable the understanding

of the role of ERβ within the chromatin landscape during transcription. Altogether, this information would improve the understanding of how ERβ functions as a transcription factor.

With the results from **paper II** demonstrating that intestinal epithelial ERβ protects against colitis and CAC, we have confirmed that ERβ could be a suitable treatment target for the prevention of CRC. Both **paper II** and **IV** indicate that at least part of the protective effects could be mediated through modulation of immune cell infiltration and thereby the tumor microenvironment. This is further supported by another paper from our group demonstrating that ERβ-selective activation attenuated HFD-induced macrophage infiltration (185). However, the immune microenvironment is complex, and it would therefore be valuable to explore immune cell infiltration more extensively through multiplex immunofluorescence or spatial transcriptomics. Further, none of the studies included in this thesis explore effects on the microbiota. We have previously published a paper where we investigated the impact of intestinal ERβ on the composition of the microbiota following AOM/DSS treatment, which showed that ERβ could modulate the gut microbiota (189). Further, we have also shown an impact by estrogen on the microbiota during HFD-induced colon inflammation (190). However, the study on AOM/DSS-treated mice was done using 16S rRNA sequencing, and it would therefore be interesting to perform a more in-depth sequencing using whole genome sequencing and integrate that with the data that we collected in **papers II** and **IV**.

In **papers II-IV,** we also find distinct sex differences, further emphasizing the need to consider and include both sexes when studying colitis and CRC. One of the major sex differences seen throughout the studies was related to immune response, which is known to be impacted by estrogen signaling. To further explore the clinical relevance of sex differences and estrogen on immune infiltration, it would be interesting to profile the immune cells in clinical tumor samples from males and both pre- and post-menopausal females. Another interesting finding was the sex difference related to changes in the expression of genes regulating circadian rhythm. We have previously seen that intestinal ERβ can regulate certain circadian genes following HFD-induced inflammation (185), indicating that ERβ might also play a role also during AOM/DSS-induced colitis. However, both the HFD study and the current AOM/DSS study only include analysis at one time point during the day. To properly characterize the role of ERβ on the circadian rhythm it would be essential to include different zeitgeber times for analysis.

Interestingly, while more research has been done on males in general, the studies on estrogen and ERβ in CRC have largely been performed in female mice, and many question the benefit of including males in studies on estrogen. However, males also produce estrogen and express ERβ, and our research clearly shows a protective effect by estrogen signaling through ERβ also in male mice. Thus, targeted therapy against ERβ also shows clinical potential in males. Still, imbalance between to sexes remains an issue in medical research, with many male-dominated and male-exclusive studies. The underrepresentation of females in research has major implications for female health, affecting the management of diseases which in some cases can have life-threatening consequences. Further, even when both sexes are included, the data is often analyzed without separating males and females, masking potential differences between the sexes.

Finally, the new findings presented in this thesis together with the epidemiological and pre-clinical evidence of estrogenic protection against CRC development, demonstrate an opportunity to screen and develop ERβ-selective treatments. Clinical trials have already demonstrated a good tolerance for treatment with ERβ-selective agonists (191, 192), and an agonist could therefore be a potential treatment option for CRC prevention in risk groups such as IBD patients, to lower inflammation and thereby reduce the risk of developing CAC. While prevention may have the most therapeutic value, there is also potential to use an ERβ-selective agonist after tumors have developed. As we found that loss of ERβ affects immune cell infiltration in tumors, even though tumors in general lack expression of ERβ, it indicates that the surrounding stromal cells that express ERβ could impact the TME. Activating ERβ in the adjacent stroma could therefore have a therapeutic benefit in combination with other treatments such as immunotherapy.

There is also a future potential to use ERβ as a treatment target to improve fertility. Interestingly, tamoxifen has been shown to induce ovulation (193). Although the mechanism is not fully understood, this indicates that targeting the ERs could be of therapeutic significance. Still, the knowledge about ERβ in the human ovary, and reproductive disorders in females overall, is currently lacking. Ovarian research would have to be further advanced toward the pre-clinical stage before a new treatment can be developed. However, our findings can support such research, and could potentially lead to the development of treatment strategies to promote effective follicle development and oocyte maturation.

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