ROLE OF ESTROGEN RECEPTOR BETA IN EPITHELIA LACKING ESTROGEN RECEPTOR ALPHA EXPRESSION

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LIST OF PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals.


   # These authors contributed equally to this study.


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1 THESIS SUMMARY

Estrogens influence the physiology of many organs by activation of the nuclear receptors ERα and ERβ. Traditionally, tissues were designated as estrogen responsive if they expressed ERα but after discovery of ERβ in 1996, to the great surprise of endocrinologists, ERβ was found in many organs previously thought to be “estrogen insensitive tissues”. The epithelium of ventral prostate, ovarian granulosa cells and lung, are ERα-negative but express high levels of ERβ. This thesis focuses on the role played by ERβ in ERα-negative epithelium.

The prostate is of medical interest because it is very frequently the site of malignancy in elderly men. Prostate cancer is the most lethal form of cancer in men after lung cancer. Hormonal changes occurring during aging may be involved in the progression of prostate cancer. We studied the ventral prostate of mice to gain insight into the role of ERβ in prostatic epithelium. We used histochemical, protein profiling and FACS analysis to study the ventral prostate of mice in which ERβ gene has been inactivated (ERβ−/+ mice). We found epithelial hyperplasia, accumulation epithelial cells in the intermediate (proliferating stage) phase of differentiation and dysregulation of several proteins associated to low differentiation level. Among these proteins, serine protease inhibitor Kazal type 3, called Spink3. The human homologous of Spink3, TATI, is positively related to low epithelial differentiation grade (Gleason Score) in human prostate cancer. Analyzing prostate cancer biopsies of patients at different Gleason Scores we observed a negative correlation between TATI and ERβ expression and positive correlation between TATI and Gleason Score. This study showed a role for ERβ in differentiation of the prostatic epithelium in mice and man.

The granulosa cells of the ovarian follicle are epithelial-derived cells which secrete estradiol which is essential for maturation of the ovum, and preparation of the entire body for response to a fertilized ovum. In the ovarian follicles ERβ is abundantly expressed in the nuclei of granulosa cells, while the theca cells are ERα-positive and ERβ-negative. Theca cells are stromal-derived cells which form a highly vascularized layer surrounding the granulosa cells during follicular maturation. Female of ERβ−/−
mice vary rarely ovulate and are considered to be subfertile. In the ovary, there are very few corpra lutea and many atretic follicles. Upon wedge resection of the ovaries maturation of follicles and ovulation were restored, clearly indicating that the hypothalamo-pituitary axis in female ERβ−/− mice is normal. Upon histochemical and immunohistochemical examination, we observed impaired vascularization in the theca cell layer of ovarian follicles in ERβ−/− mice. We hypothesized that ERβ expression in granulosa cells is important for the cross-talk between granulosal and theca cell compartments, in order to allow vascularization of theca layers during follicular maturation. We further conclude that the trauma of ovarian wedge resection can trigger activation of angiogenic signaling and overcome the lack of ERβ functions.

The major function of the lung is to allow gas exchanges between blood stream and the air. In order to perform such a function, the main characteristics of lung are to provide large surface availability and elasticity. These two features depend respectively by the structure of alveoli and the active turnover of the extracellular matrix. The lung alveolar and bronchiolar epithelium are ERβ-positive. ERβ+/− mice are hypertensive and compared to normal mice, their lungs have large alveoli and reduced elastic recoil. Our investigation of the lungs of ERβ+/− mice provide evidence that ERβ is involved in the regulation of key proteins important for the control of extracellular matrix (ECM) deposition in the lung parenchyma. Probably as a result of lung dysfunction, ERβ−/− mice are hypoxic and reluctant to run on the treadmill. We speculate that systemic hypoxia can be one of the causes of systemic hypertension characteristic of ERβ−/− mice. 

The studies in this thesis describe the role of ERβ in some important physiological properties of epithelial tissues, differentiation, stromal-epithelial communication and regulation of ECM organization.
2 INTRODUCTION

2.1 ESTROGEN, HISTORICAL PERSPECTIVE

The term “estrogen” derives from the Latin word “Oestrus” or from the Greek “Oistros”, words which refer to the phase in which females are sexually receptive. Under the influence of gonadotropins produced by the pituitary gland, maturation of ovarian follicles occurs and production of 17β-estradiol increases. It is this follicular estrogen which affects behavior and causes characteristic physiological changes in females. Studies on the uterine action of 17β-estradiol, led Jensen and Jacobson to the conclusion that the biological effects of estrogen occur through the activation of an estrogen receptor (Jensen, Jacobson et al. 1972). For many decades estrogens were considered as “female hormones”, while testosterone was considered as the male hormone. In the 1980s it became clear that estrogens had effects other than those affecting female reproductive organs (uterus, ovaries and mammary gland), indicating more widespread effects of this “reproductive hormone”. The importance of estrogen in bone homeostasis in women was recognized because of the increased risk of osteoporosis in postmenopausal women. However, it was the publication, in 1994, of a case report about a man with a mutation in the ER who presented with abnormal bone density and impaired glucose tolerance, that finally confirmed the importance of estrogens in both males and females (Smith, Boyd et al. 1994).

More than 20 years after its discovery by Jensen, the ER was cloned and characterized as a member of the nuclear receptor super family in 1985 (Greene, Nolan et al. 1980; Walter, Green et al. 1985; Green, Walter et al. 1986). Ten years later, the Gustafsson lab discovered a second ER. The “Jensen” receptor was renamed ERα and the new receptor ERβ (Kuiper, Enmark et al. 1996). ERα and ERβ have distinctive tissue
distributions and to the great surprise of endocrinologists (Kuiper, Carlsson et al. 1997) many tissues previously thought to be "estrogen insensitive tissues" were found to be ERβ-positive and estrogen sensitive. The most notable of the ERα-negative ERβ-abundant tissues were the epithelium of the rodent ventral prostate (Weihua, Makela et al. 2001), the granulosa cells of the ovaries (Cheng, Weihua et al. 2002), and the parenchyma of the lungs (Patrone, Cassel et al. 2003).

The studies in this thesis represent an effort to examine the function of ERβ in these three tissues. In particular my interest was to investigate whether there is a common role-played by ERβ and to try to explain how loss of ERβ (in ERβ−/− mice) results in abnormalities in ventral prostate, ovaries and lung.

2.2 ESTROGEN RECEPTOR ALPHA (ERα) AND ESTROGEN RECEPTOR BETA (ERβ): NUCLEAR RECEPTOR FAMILY MEMBERS

Estrogen receptors are transcription factors. As all other members of the NR family, ERs have a typical structural architecture composed of: an N-terminal region (A/B domain), highly variable in sequence and length; a highly conserved DNA-binding domain (C); a hinge domain (D), a ligand binding domain (E) and a C-terminal domain (F). The A/B domain is involved in protein-protein interactions with cofactors or the proteins of the transcriptional machinery (Giguere, Hollenberg et al. 1986; Kumar, Green et al. 1987; Hollenberg and Evans 1988). There is less than 18% homology between ERα and ERβ in the A/B domain suggesting that these two receptors may interact differently with proteins. The C domain contains a short motif responsible for DNA-binding specificity called the P-box, which is involved also in dimerization of nuclear receptors (Luisi, Xu et al. 1991). The most conserved region is the DNA-binding domain, with 97% homology between ERα and ERβ. The D domain harbors a
nuclear localization signal (Picard and Yamamoto 1987) and is believed to provide flexibility of the three-dimensional structure between the E and the C domains. The ligand binding domain, E, is approximately 60% conserved between the two estrogen receptors. It is composed of 12 α-helixes whose folding creates the ligand binding pocket. Domain E is also involved in heat-shock protein association, receptor dimerization, nuclear localization and interaction with cofactors (Pratt, Jolly et al. 1988; Webster, Green et al. 1988; Graupner, Wills et al. 1989). The F domain, whose sequence is extremely variable, seems to contribute to the transactivation capability of the receptor (Vegeto, Allan et al. 1992).

Early after the discovery of ERβ, structural considerations suggested that ERα and ERβ would bind to similar sites on DNA and potentially activate the same target genes, but also that they could be activated by distinct ligands and by differential interactions with co-regulator proteins, and therefore have distinct effects on the transcription of genes.

![Figure 1](image.png)

**Figure 1.** Comparison of human ERα and ERβ.

### 2.3 MECHANISM OF ERS ACTIVATION

There is much emerging evidence suggesting that there are multiple distinct pathways through which ERs activate their biological responses (Nilsson, Makela et al. 2001). After ligand binding, ERs form hetero- or homodimers and bind directly to estrogen
response elements (EREs) through the DNA binding domain. Alternatively, ER dimers do not bind directly the DNA, but activate non-ERE target genes by binding to other transcription factor complexes like Fos/Jun, called also AP-1 responsive elements, or SP-1 (Kushner, Agard et al. 2000; Saville, Wormke et al. 2000). ER is also involved in an inhibition pathway: after binding with ER, NFkB does not activate its responsive elements NFkBRE (Ray, Prefontaine et al. 1994; Galien and Garcia 1997). In yet another pathway, ERs are involved in a non-genomic process through which signal cascades are triggered by protein-protein interaction between ligand-bound ERs and various second messengers (SM). This mechanism is thought to explain the rapid effects that estrogens elicit in many tissues, but this process is still not well understood (Heldring, Pike et al. 2007). In addition to these mechanisms, ligand independent mechanisms of ER activation have been described: activation of kinases by growth factor signaling leads to phosphorylation and thus activation of ERs (Kato, Endoh et al. 1995). This mechanism seems to explain the hormone-independent growth of some tumors (Coutts and Murphy 1998; Shim, Conaway et al. 2000).
Figure 2. Model representing the mechanistically distinct molecular pathways used in the regulatory actions of ERs.

2.4 ER COREGULATORS

Other important players involved in activation mechanisms of ERs are the coregulators (or cofactors). These factors are proteins recruited by ERs to activate (coactivators) or to repress (corepressors) ER transcription. Many coregulators are enzymes, such as acetylases/deacetylases, kinases/phosphatases, and methylases/demethylases, which may influence ER signaling by modification of proteins in the vicinity of the sites were ERs are tethered. Coactivators and corepressors are believed to be recruited in protein complexes whose components vary according to the type of tissue. The capacity of ERs to recruit different coregulators in different tissues has been proposed as an explanation for the differential tissue effects of estrogen and Selective Estrogen Receptor Modulators (SERMs) (Smith, Nawaz et al. 1997; Webb, Nguyen et al. 2003).
2.5 STEROID HORMONE METABOLISM: BIOSYNTHESIS OF SEX HORMONES

The biosynthesis of steroid hormones occurs in the gonads (testis, ovaries) and the adrenal gland through hydroxylations, oxidations and reductions catalyzed by specific enzymes. Cholesterol is the precursor of all steroid hormones. Circulating cholesterol, carried by low-density lipoprotein (LDL), can either be used immediately for hormone synthesis or be stored as cholesterol esters. In both gonads and adrenal glands, the first and rate limiting step in steroid biosynthesis is the cleavage of its side chain to produce pregnenolone, a reaction catalysed by the cytochrome P450 side-chain-cleavage enzyme (P450\textsubscript{sc}})(Payne and Hales 2004; Ghayee and Auchus 2007).

2.5.1 Formation of Androgens and Estrogens

About half of the pregnenolone formed in human adrenal gland is metabolized to DHEA.

Pregnenolone is converted to 17α-OH pregnenolone by P450\textsubscript{17} enzyme or in progesterone by 3β-hydroxysteroid oxidoreductase-\(\Delta^{4,5}\)-isomerase enzyme complex.

Cytochrome P450\textsubscript{17} (17-20 desmolase) is the enzyme which catalyzes the removal of the C20-C21 side chain of 17α-OH pregnenolone converting it into dehydroepiandrosterone (DHEA). Through the 3β-hydroxysteroid oxidoreductase-\(\Delta^{4,5}\)-isomerase enzyme complex, DHEA is converted to androstenedione, which is then converted to testosterone by 17-oxo-reductase. Testosterone is then aromatized by aromatase to estradiol. Alternatively, DHEA can be converted to androstenediol (5-androstene-3β, 17β-diol) by 17-oxo-reductase and afterwards be converted to testosterone by 3β-hydroxysteroid oxidoreductase-\(\Delta^{4,5}\)-isomerase enzyme complex.
In primates but not in rodents, the adrenal gland secretes large amounts of DHEA and its sulfated metabolite (DHEA-S). These steroids can serve as precursors of androgens and estrogens in peripheral tissues.

The principal hormones produced in the testis are testosterone and androstenedione although production of estradiol has been also found in germ cells and Leydig cells (Hess 2003). The ovary also synthesizes both androgens and estrogens. Upon activation by Luteinizing Hormone (LH) in theca cells, which express high levels of cytochrome P450_{17}, the ovary converts cholesterol to testosterone. Testosterone is then aromatised to form estradiol by aromatase in granulosa cells of growing follicles, after stimulation by Follicle Stimulating Hormone (FSH) (Sasano, Okamoto et al. 1989; Havelock, Rainey et al. 2004). Most of the estradiol production in granulosa cells derives from aromatization of testosterone produced by theca cells. After expulsion of the oocyte from the ovary, the follicle becomes a corpus luteum and produces progesterone.
Figure 3. Biosynthesis of sex hormones in adrenal and gonadal glands.

2.6 PHYSIOLOGICAL SIGNIFICANCE OF ERβ: ROLE OF ERβ IN EPITHELIA LACKING ERα EXPRESSION

Ventral prostate epithelium, granulosa cells of the ovary and epithelium of the lung represent three functionally different classes of epithelial tissues (glandular exocrine, glandular endocrine and lining epithelium). These tissues express high level of ERβ but ERα is undetectable.
2.6.1 The epithelium

The epithelium is a various type of tissue, which lines the surface of the body, body cavities and forms the structure of glands. The epithelium mediates several activities such as absorption, secretion, selective diffusion and physical protection. A characteristic feature of epithelial tissues is their polarization. There is a basal surface which adheres to the basement membrane, and an apical surface, with secretory functions, which faces the lumen of glands. All epithelial cells are interconnected between each other by a variety of membrane specializations called cell junctions, through which they exchange metabolites and “information”. Intercellular communication is necessary to regulate the patterns of growth and differentiation of all epithelial tissues. Errors in this process can lead to diseases such as cancer.

The basement membrane (BM) is a more condensed modification of the extracellular matrix (ECM), organized in a sheet-like structure, which binds and provides support to the basal side of the epithelium, separating it from the underlying tissue (stroma). The main components of the BM are glycoproteins (laminin, fibronectin, entactin), glycosaminoglycans and collagen type IV. In the network formed by the components of ECM, as well as BM, transient molecules such as growth factors are “trapped”. These are produced by cells of the stroma and epithelium, and are involved in the cross-talk between the two tissues. It has been demonstrated that ECM components can directly affect the activity of several growth factors during important processes such as angiogenesis (Sasisekharan, Moses et al. 1994; Schlessinger, Lax et al. 1995).

There are two distinct functional types of epithelium:

- **lining epithelium**, a tissue specialized in protection or absorption;
- **glandular epithelium (glands)**, a tissue specialized in secretion. Glands that
secrete molecules or proteins inside tubular structures (ducts) are called exocrine glands. Glands in which the ducts disappear during development and end up forming isolated structures, which release their secretions into the bloodstream, are called endocrine glands.

2.6.2 ERβ in exocrine glands: the prostate

The prostate is an exocrine gland found mostly in males. Its function is to secrete proteins which form the optimal environment for spermatozoa to be active. Growth, development and physiology functions of the prostate depend on androgens, mainly dihydrotestosterone (DHT) and testosterone. Prostate cancer (PC) as well as a benign form of prostate tumor called benign prostatic hyperplasia (BPH) are diseases which commonly occur during aging. In western countries, after cardiovascular diseases and lung cancer, prostate cancer is a major cause of natural death in men. Development of BPH is thought to be associated with a hormonal imbalance that occurs in elderly men, when plasma levels of testosterone decrease and estrogen levels increase together with the body fat mass. The causes of PC are still unknown. The risk of developing PC seems to be related to the diet: people from Asian countries (such as Singapore, Japan, China and Hong Kong), who are very high consumers of phytoestrogen-containing food but eat little red meat, have low incidence of PC (Goetzl, Van Veldhuizen et al. 2007).

The prostatic epithelium is a stratified columnar epithelium, in which three layers of cells corresponding to three phases of differentiation lineage are distinguishable:

1) Basal cells which contain slowly proliferating epithelial stem cells and neuroendocrine cells. These cells are androgen sensitive, but independent of androgens for survival;
(2) Intermediary cells, which have secretory capacity and proliferate rapidly in response to androgen.

(3) Luminal cells, which are dependent upon androgen for survival and for their secretory activity but do not proliferate in response to androgen.

Figure 4. Prostatic epithelium differentiation continuum.

Morphologically the mouse prostate looks very different from the human prostate. In rat and mouse, the prostate is divided into anatomically distinct lobes, the anterior prostate, the ventral prostate and the dorsolateral prostate. Each lobe is composed of secreting ducts and tubes. One of the most striking morphological differences between the rodent and the human prostate is absence in rodents of the abundant dense fibromuscular layer surrounding the ducts and the profuse stroma located between the lobules in human prostate.

ERα and ERβ are not uniformly distributed throughout the prostatic lobes. In contrast to the anterior and lateral lobes, the ventral prostate of an adult mouse/rat expresses
high levels of ERβ in both stroma and epithelium, while ERα is very low or undetectable. Thus the ventral prostate is a good tissue to study the physiological role of ERβ. The expression of the androgen receptor (AR) is very high in almost all the epithelial cells and in the stroma in the rodent prostate. In the human prostate, ERβ is expressed in the basal epithelial cells and AR in the luminal epithelium.

Analysis of ERβ<sup>−/−</sup> mice has revealed presence of hyperplastic foci in the ventral prostates when mice are older than 5 months of age (Krege, Hodgin et al. 1998; Weihua, Makela et al. 2001; Imamov, Morani et al. 2004). This finding was taken to mean that ERβ plays a role in the homeostasis of prostatic epithelium. With several in vivo techniques the expression pattern of specific proteins expressed by epithelial cells in different phases of differentiation were analysed. We have demonstrated that prostatic epithelium of ERβ<sup>−/−</sup> mice is hyperproliferative and that it has an altered cellular differentiation, with accumulation of basal-intermediary cells (Imamov, Morani et al. 2004). These results indicate that ERβ may have an important role for the differentiation of the epithelium in the adult prostate of rodents. In another study, our group demonstrated that ERα is expressed in newborn ventral prostate epithelium and that it is important for branching morphogenesis of the prostatic ducts. After the first two weeks of life, epithelial expression of ERα is reduced and ERβ becomes the predominant ER (Omoto, Imamov et al. 2005).

ERβ has been shown to have a role in human prostate. During development of PC, ERβ is downregulated (Chang and Prins 1999; Horvath, Henshall et al. 2001; Leav, Lau et al. 2001; Nojima, Li et al. 2001; Signoretti and Loda 2001; Bardin, Boule et al. 2004; Zhu, Leav et al. 2004).
Testicular testosterone is converted into the more potent AR agonist, dihydrotestosterone (DHT), by the \(5\alpha\)-reductase type 1 and 2 enzymes (Bruchovsky and Wilson 1968) present in both prostatic stroma and epithelium. For many years, DHT was considered to be the main hormone guiding prostate development and function. However, the idea was challenged when in 2001 Mahendro et al. showed that mice in which both forms of \(5\alpha\)-reductase had been inactivated, have a normal functional prostate (Mahendroo, Cala et al. 2001). The question was then raised as to what is the real function of DHT in the prostate. In 1989 we hypothesized that DHT is a precursor of an estrogen, \(5\alpha\)-androstane-3\(\beta\),17\(\beta\)-diol (3\(\beta\)-Adiol) and that physiological levels of an estrogen could be produced in the total absence of aromatase (Warner, Stromstedt et al. 1989). We demonstrated that 3\(\beta\)-Adiol is abundant in the prostate and is a good natural ligand for ER\(\beta\) (Weihua, Lathe et al. 2002).
As described in the figure 5, DHT can be metabolized to two stereoisomers, 3α-Adiol and 3β-Adiol, by the reversible reactions catalyzed by 3α- and 3β-hydroxysteroid oxidoreductases (3α-HSD and 3β-HSD). Because of the high concentration of 3β-Adiol found in the prostate, actually 100-fold higher than estradiol (Voigt and Bartsch 1986), and the exclusive expression of ERβ in prostatic epithelium, 3β-Adiol appears to be a natural ligand for ERβ in the prostate (Weihua, Lathe et al. 2002; Guerini, Sau et al. 2005; Oliveira, Coelho et al. 2007).
2.6.3 ERβ as prodifferentiating factor in prostate cancer

In the 2003 was published the result of the Prostate Cancer Prevention Trial (PCPT). In this study, more than 18000 healthy volunteers aged 55 or above were divided in two groups and treated with placebo and Finasteride (Proscar MSD), a 5α-redactase inhibitor. By reducing the intraprostatic level of DHT, Finasteride is considered to be a preventive agent for prostate cancer (Rubin and Kantoff 2003; Thompson, Goodman et al. 2003; Pitts 2004). The analysis of the results done 7 years later showed a reduction of the incidence of PC in the Finasteride treated group (24.8% placebo vs. 18.4% Finasteride). However, the histological analysis of the PC samples showed more aggressive tumors in the Finasteride treated group: Gleason score 7-10 tumors were higher in Finasteride group (placebo 237/1068, 22.2% vs. Finasteride 280/757, 37%; p<0.001). From the medical point of view, tumors with Gleason score above 7 are indication of poor survival. It is hypothesis from our group that higher incidence of poorly differentiatated tumors in Finasteride treated group is due by the fact that, inhibiting 5α-redactase (see Figure 5), Finasteride reduces the level of the natural ligand for ERβ in the prostate, 3β-Adiol (Imamov, Lopatkin et al. 2004). Our published and unpublished data showed that ERβ is a prodifferentiative factor (Weihua et al. 2002, Imamov et al. 2004, Schwend et al. 2007 manuscript) and that, upon Finasteride treatment, ventral prostate of 7-month-old mice result in less differentiated epithelium.

From the clinical point of view, the reduction of DHT by inhibiting 5α-redactase, lead to beneficial effects: DHT is a potent ligand for AR and its inhibition results in reduced of epithelial proliferation of the prostate. Nevertheless, loss of ERβ signaling can be a potential danger. We think that Finasteride should not be given alone, but in combination with an ERβ agonist. Phytoestrogens seem to be very good candidates to be combined with Finasteride. Genistein, a phytoestrogen much present in asian soy-bean food, is shown to have much higher affinity to ERβ than to ERα (Miller, Collini et
al. 2003; Kolonel, Altshuler et al. 2004) and, the beneficial effect of asian food against many forms of cancer is well known. Soybean isoflavons are shown to be protective against invasive carcinoma of the rat prostate/seminal vesicles induced by injection of DMAB (3,2'-dimethyl-4-aminobiphenyl) and testosterone (Onozawa, Kawamori et al. 1999; Kato, Takahashi et al. 2000; Jarred, Keikha et al. 2002).

2.6.4 **ERβ in endocrine glands: the ovarian follicle**

Besides being the female organ which produces ova, the ovaries are also the principal producers of the two most important hormones, estrogen and progesterone, responsible for developing and maintaining female sexual traits, as well as maintaining a pregnancy.

Histologically, the ovary appears as a bulb outlined by a single layer of cuboidal cells called surface epithelium. The surface epithelium is an undifferentiated and proliferative tissue subjected to continuous remodelling in order to repair the periodic wounds that occur during the expulsion of the mature egg (ovulation). Because of its features, the surface epithelium is believed to have a tumorigenic potential (Ahmed, Thompson et al. 2007).

Ovarian follicles are the endocrine structures of the ovary and their function depends on signals by gonadotropic hormones, FSH and LH, released by pituitary gland. The cells of the follicles are the oocyte, the granulosa cells and the thecal cells. The thecal cells are fibromuscular-like cells packed in a network of extracellular matrix around the follicles. During folliculogenesis, the thecal layer grows and forms a highly vascularized thick layer surrounding the granulosa cells, providing them with nutrients and “information”. Thecal cells express LH receptor (LHR) and, upon its activation by LH from the pituitary gland, the theca cells convert cholesterol to testosterone. Then, the granulosa cells, after activation by FSH receptor (FSHR), are
able to aromatize testosterone to estradiol and to produce other hormones, such as progesterone and inhibin, necessary for the oocyte development and for the preparation of the body to the potential pregnancy. At the end of folliculogenesis, after the mature oocyte is secreted from the ovary to the fallopian tube, the follicle changes morphology and the granulosa cells start producing the progesterone in high amounts. The new endocrine structure formed is the corpus luteum. If fertilization occurs, the embryo delivers a hormone, human chorionic gonadotropin (hCG), which in turn keeps the corpora lutea functioning. If the ova is not fertilized the corpus luteum degenerates.

Soon after discovery of ERβ (Kuiper, Enmark et al. 1996) it was shown that the only ER expressed in granulosa cells is ERβ and that ERα is only present in the theca cells (Sar and Welsch 1999). Studies on aromatase knock-out mice (Arko), demonstrated that estrogen is essential for the ovulation (Findlay, Britt et al. 2001) but not for the granulosa cells to proliferate. In ERβ⁺ mice, there is arrest of follicular growth and very rarely are corpora lutea formed (Cheng, Weihua et al. 2002). In the few cases of successful gestation, ERβ⁺ mice have small litters consisting of not more than three pups per litter (Krege, Hodgin et al. 1998; Weihua, Saji et al. 2000; Pettersson and Gustafsson 2001). Recently we have reported that growing follicles of ERβ⁺ ovaries have a defect in the remodeling of theca layer, which results in poor vascularization. This phenomenon is accompanied by imbalanced expression in granulosa cells of two factors involved in the angiogenesis process, PDGFRα and PDGFRβ (Inzunza, Morani et al. 2007). A similar defect in PDGFRs pattern has been reported in FSHR knock-out mice (FSHR⁺ mice), another infertile mutant strain (Danilovich, Roy et al. 2001; Yang, Balla et al. 2003). A synergism between ERβ and FSHR signaling seems to be needed in order to complete follicular maturation (Couse, Yates et al. 2005).
Wedge resection is a technique used to treat infertile women affected by polycystic ovarian syndrome (PCOS). In all ERβ−/− mice wedge resection temporarily restored fertility. There were two complete pregnancies with full size litters after wedge resection. We found that after wedge resection there was a marked increase in vascularization of the theca layer of growing follicles and we postulated that wounding of the ovary led to increased vascularization as part of the wound healing (Inzunza, Morani et al. 2007). The mechanism through which wedge resection reestablishes fertility in PCOS patients is not known. Nevertheless, it has been suggested that other signals triggered by the trauma of the operation itself can result in activation of the follicular growth (Farquhar 2004).

2.6.5 ERβ in lining epithelium: the lung alveoli

The terminal airways of the lung are composed of three kinds of epithelial tissue:

- the bronchiolar epithelial cells, which cover the surface of the bronchi/bronchioles;
- the alveolar type I, which form the wall of the alveoli and are tightly interconnected with the capillary net through which the gas exchanges occur between blood and air;
- the alveolar type II, which are secretory cells producing surfactants necessary for the maintenance of surface tension during breathing.

An important characteristic of the lung is elasticity which this organ must maintain in order to allow regular inflation/deflation during the breathing. Lung elasticity is very dependent on bronchio-alveolar structure, correct surfactant production and ECM composition. The importance of estrogens in physiology and development of the lung has been known for a long time. Estrogen influences surfactant production and
alveologenesis. In fact, postnatal maturation of lung in mammals differs between male and female: adult virgin female rats and mice have smaller alveoli, higher numbers of alveoli and bigger alveolar surface area compared to aged-matched males (Massaro and Massaro 2006); (Massaro, Mortola et al. 1996). ERβ is the only ER detectable in adult lung (Patrone, Cassel et al. 2003). Nevertheless, adult ERα+/− mice have reduced alveolar septation (Massaro and Massaro 2004). ERβ+/− mice have reduced number of alveoli (Patrone, Cassel et al. 2003; Massaro and Massaro 2004) and reduced lung volume at a transpulmonary pressure of 20 cm of H2O and reduced elastic recoil (Massaro and Massaro 2004). Massaro’s lab attributed the phenotype of ERβ+/− mice to a defect in ECM maintenance. From experiments on ovariectomized WT, ERβ+/− and ERα+/− mice, Massaro et al. concluded that, in absence of estrogens, lung loses normal alveolar septation and that ERα is necessary for the alveolar regeneration upon estradiol replacement. According to the authors, developmental alveologenesis requires both ERs and is a process distinct from regeneration. The conclusion of Massaro’s lab was that ERα signalling is important to activate the process of alveolar regeneration in adult lungs, while ERβ seems to be important only for the alveolar formation during the development. Although with immunohistochemical and Western blotting techniques, ERα is not detectable in the adult lung (Patrone, Cassel et al. 2003), it may be expressed in a limited number intra alveolar stem cells or some other estrogen sensing cell-type involved in paracrine or endocrine stimulation of alveolar regeneration (Massaro, Clerch et al. 2007).

We have shown that in ERβ+/− mice there is accumulation of collagen in the alveolar septa and dysregulation of caveolin-1 and metalloproteinases (MMP2, MT1-MMP, TMIP2), all indications of defective regulation of ECM deposition (Morani, Barros et al. 2006).
Furthermore, ERβ⁻/⁻ mice are hypoxic and reluctant to run on treadmill. It is our hypothesis that systemic hypertension (Zhu, Bian et al. 2002) and left ventricular hypertrophy in the heart (Forster, Kietz et al. 2004) observed in adult ERβ⁻/⁻ mice can be a result of systemic hypoxia due to defective lung function(Forster, Kietz et al. 2004; Morani, Barros et al. 2006).

Summarizing results from our and other labs, we can conclude that both ERs are necessary for the alveolar formation during development. In the adult lung, ERα is necessary for alveolar regeneration while ERβ seems to be involved in the control of ECM deposition.

2.6.6 Menopause and the lung

Clinical studies have revealed an important correlation between decrease in plasma estrogen level and lung dysfunction in women. Pulmonary diffusion capacity, an index of alveolar surface area and gas exchange capacity (Powell and Hopkins 2004), decreases by 6% per decade in men while in women it decreases by 2% until menopause. At postmenopause, pulmonary diffusion capacity decreases by 6% per decade (Neas and Schwartz 1996). About 75% of patients with Chronic Obstructive Pulmonary Disease (COPD) who never smoked are women aged above 55 years (Massaro, Clerch et al. 2007). Pulmonary function is often measured by spirometry tests on patients. This technique permits measurement of forced expiratory vital capacity (FVC) and forced expiratory volume per second (FEV₁). FVC indicates the total volume of air that lungs can exhale during forceful exhalation and FEV₁ the volume of air exhaled during the first second. In a study performed on 1298 women 45 to 56 years of age (Real, Svanes et al. 2007; Real, Svanes et al. 2007), with irregular menstrual cycles and amenorrhea for 6 months, FVC and FEV₁ were lower than in women menstruating regularly. A previous study showed that after 3 months of
Hormonal Replacement Treatment (HRT) using Estrogen and Estrogen+ Progesterone or Tibolone (Carlson, Cushman et al. 2001; Pata, Atis et al. 2003) the same parameters increased in postmenopausal women, and in women who had undergone hysterectomy/ovariectomy. In order to improve pulmonary function and/or to prevent pulmonary dysfunction caused by estrogen depletion in elderly patients, SERMs might be a good choice of treatment.

2.7 COUNTERACTING ROLE ON ERA PLAYED BY ERβ IN EPITHELIA EXPRESSING BOTH OF THE RECEPTORS

2.7.1 ERβ in mammary epithelium

Mammary gland is an exocrine organ, in which milk secretions produced by alveolar secreting epithelial cells are drained into lobules and then ejected out through the nipple.

The development and physiology of mammary gland depends on the hormonal signaling of estrogen and progesterone. ERα plays a key role in developing mammary ducts and the ductal tree is very rudimentary in ERα⁻/⁻ mice (Lubahn, Moyer et al. 1993). The exact role of E2 in mammary epithelial proliferation has been unclear, because ERα was never found colocalized with proliferation markers in ductal epithelial cells (Shyamala, Singh et al. 1986; Saji, Jensen et al. 2000). For a long time, E2-induced proliferation was thought to be indirect, via growth factors secreted from the stromal compartment (Zhang, Bennett et al. 2002). However, in mice ERα expression is confined to the epithelium while ERβ is localized in both stroma and epithelium. In the ERβ⁻/⁻ mouse mammary gland, there is normal ductal branching (Forster, Makela et al. 2002). One possible clarification regarding ERα signaling and proliferation was suggested by Cheng et al (Cheng, Weihua et al. 2004). In this study it
was shown that ERα in the epithelial cells is the receptor that receives the proliferation signal from E2, but also that ERα is rapidly lost very early in G₁ phase. When cellular DNA was labeled with bromodeoxyuridine (BrdU), ERα could never be found colocalized with S phase markers like Cyclin A and PCNA, but it was found to be re-expressed in BrdU-labeled daughter cells (Cheng, Weihua et al. 2004). A conclusion of these studies was that the ERα signal initiates the proliferation of the epithelial cells. Although there is normal branching in the mammary gland of ERβ⁻/⁻ mice, the epithelium appeared to be incompletely differentiated with a decreased expression of several proteins characteristic of differentiated epithelial cells like adhesion molecules (E-cadherin and integrin α2), gap junction and tight junction proteins (connexin 32 and occludin) in lactating mammary gland (Forster, Makela et al. 2002). It is possible that ERβ plays a pro-differentiative role in mammary epithelium as it does in the prostate.

Studies of MCF7 cells, a breast cancer cell line expressing ERα and not ERβ, showed that ERα-positive cells respond to E2 with increased proliferation. When ERβ was artificially introduced into these cells, E2-induced proliferation was inhibited (Hartman, Muller et al. 2004). Ductal cells in the mammary gland appear to be one example of cells where ERα and ERβ counteract each other on estrogen-stimulated proliferation. The proliferative response to E2 seems to be determined by the ratio of ERα/ ERβ. The functions of ERβ in the breast are probably related to its antiproliferative as well as its prodifferentiative functions (Strom, Hartman et al. 2004).

2.7.2 ERβ in the uterine epithelium

The epithelium of the uterus, or endometrium, is usually simple columnar and it contains simple or branched tubular glands.
ERα and ERβ are both highly expressed in the stroma and epithelium of immature uterus in mice. In the uterus of immature ERβ−/− mice there is overexpression of progesterone receptor (PR) and an exaggerated response of the epithelium to E2, as indicated by hyperproliferation, enlargement of the lumen, increase in protein content of uterine secretion and increase in expression of growth factors (Weihua, Saji et al. 2000). It seems that ERβ keeps the uterus quiescent before the ovary begins to secrete estradiol. Our group suggested that the main ligand for ERβ in immature uterus in absence of estradiol is 3βAdiol (Weihua, Saji et al. 2000), which is secreted by prepubertal ovary (Eckstein 1983).

In the mature uterus, ERβ is involved in the cervical ripening, which is essential for parturition (Wu, Geimonen et al. 2000), and in decidualization, which is critical for implantation of the fetus (Tessier, Deb et al. 2000).

2.8  ERβ IN THE IMMUNE SYSTEM

Autoimmunity is the cause of more than 100 serious chronic illnesses (Alarcon-Segovia, Alarcon-Riquelme et al. 2005; Peter, Johnson et al. 2005). Autoimmune diseases such as Sjogren’s syndrome, systemic lupus erythematosus (SLE), and rheumatoid arthritis, are more common in women than in men. The role of estrogen in the immune system is complex with apparently contradictory actions. Estrogens seem to offer protection against end-stage renal disease during the development of SLE, but it promotes autoimmunity by inhibiting the destruction of immature autoreactive B cells in the bone marrow of mice (Bynoe, Grimaldi et al. 2000). Furthermore, treatment with estradiol of SLE-prone mice increases while tamoxifen reduces the incidence of SLE (Wu, Lin et al. 2000). Moreover estrogen deficiency causes the development of
the autoimmune exocrinopathy Sjögren syndrome in ovariectomized mice (Ishimaru, Saegusa et al. 1999). The overall effect of estrogens in the immune system is determined by a balance between ERα and ERβ signaling. ERα⁻/⁻ mice develop autoimmune nephritis (Shim, Kis et al. 2004), whereas female ERβ⁻/⁻ mice develop myeloid like-leukemia disease (Shim, Wang et al. 2003). In mice in which the synthesis of estradiol has been blocked by deletion of the aromatase enzyme (Ar⁻/⁻ mice) there are signs of Sjogren’s syndrome (Shim, Warner et al. 2004). Based on these results it seems clear that ERα and ERβ play distinct roles in the immune system. The hypothesis of our group is that ERβ plays an important role in regulating the differentiation of pluripotent hematopoietic progenitor cells whereas ERα induces proliferation.

2.9 SELECTIVE ESTROGEN RECEPTOR MODULATORS (SERMS)

There are many indications that ERα and ERβ have opposite functions in the tissues expressing both of the receptors. In tissues and cell lines of mammary epithelium for example, it has been noticed that E₂ in the presence of ERα elicits proliferation, but in the presence of ERβ it inhibits proliferation (Strom, Hartman et al. 2004; Forster, Makela et al 2002). The relatively low level of homology between the ligand binding domains of the two ERs (59%) very early suggested that it might be possible to activate selectively ERα or ERβ. This fitted nicely with the idea of selective estrogen receptor modulators (SERMS). The first SERM produced was an antiestrogen, Tamoxifen, used in the treatment of ERα-positive breast cancer (Cole, Jones et al. 1971; Lippman and Bolan 1975; Jordan 1976; Jordan and Dowse 1976; Jordan and Jaspan 1976). Despite its blocking of ERα in the mammary epithelium, Tamoxifen was found to maintain bone density in ovariectomized rats, i.e it behaved as an ER agonist in bone
(Jordan, Phelps et al. 1987; Turner, Wakley et al. 1987; Turner, Wakley et al. 1988). In athymic mice, tamoxifen prevented estrogen-stimulated human breast tumor growth, but also it stimulated the growth of endometrial carcinoma (Jordan and Robinson 1987; Gottardis, Robinson et al. 1988). In more recent years different kind of SERMs have been produced: molecules that work as agonists or antagonists (Tamoxifen, Toremifene, Droloxifene, Idoxifene) and molecules that act only as antagonists, called SERDs (Fulvestrant, TAS 108, ZK 703, ZK 253). Estrogens and SERDs have a steroidal structure, whereas other SERMs are different even if they can bind ERs (Baumann and Castiglione-Gertsch 2007). The binding of SERMs to ERs causes a modification of the ER structure, which results in dimerization of the ER and protein-protein interactions with other factors, the coregulators. A further observation is that the ER configuration changes in a unique fashion for every different ligand which binds to ER (Brzozowski, Pike et al. 1997; Shiau, Barstad et al. 1998; Paige, Christensen et al. 1999). According to the type of coregulators involved, coactivators or corepressors, the SERMs can respectively activate or repress transcription of their target genes. The concept today is that SERMs act as ER agonists or antagonists according to the pool of coactivators/corepressors recruited by the ERs in any single cell-type. Nowadays there are some commercially available SERMs that act as selective agonists for ERα or ERβ:

- propyl pyrazole triol (PPT), which has 400 times more affinity for ERα than ERβ;
- 2,3-bis(4-hydroxyphenyl)propionitrile (DPN), which has 70 times more affinity for ERβ than ERα (Harrington, Sheng et al. 2003);
- WAY-202041 (ERB-041), which has 200 times more affinity for ERβ than ERα (Harris, Albert et al. 2003).

Because of their typical structure, SERDs bind to ERs and cause its rapid degradation, as it has been described with the use of Fulvestrant (Robertson, Nicholson et al. 2001).
The possibility to create ER isoform specific agonist/antagonist is a great opportunity to treat hormonal diseases and also the effects of menopause. Because of the differences in tissue expression of ERα and ERβ and the tissue specificity of coregulators, the challenge of the future will be to create SERMs which will be tissue or even cell specific.
There are several reports indicating that ERα and ERβ play opposite roles in tissues expressing both receptors, often directly or indirectly affecting each other’s function (Matthews and Gustafsson 2003).

The aim of this thesis is to describe the physiological unique role of estrogen signaling in ERβ dominant epithelial tissues, such as ventral prostate, lung and the granulosa cells, where ERα is absent.
4 NOTES IN METHODOLOGY

4.1 LABORATORY ANIMALS

Mice were managed in accordance with the guidelines for care and use of experimental animals by Stockholm’s Södra Djurförsöksetiska Nämnd. Mice were fed with standard diet with water *ad libitum*. Knock-out and wt mice were obtained by breeding heterozygous pairs. Genotyping was performed by PCR on DNA isolated from tails of 2 weeks old mice as described elsewhere.

4.2 CREATION OF ER$\beta$-/- MICE

By homologous recombination in embryonic stem cells, ER$\beta$-/- mice were originally created by insertion of a Neomycin resistance gene cassette into exon 3, which is located in the region coding for the second zinc finger of the DNA binding domain. Nevertheless, some distinct splice variants of ER$\beta$ have been found in ER$\beta$-/- mice, with lack of DNA binding domain and/or with frameshift resulting in shorten forms of ER$\beta$ protein (Krege, Hodgin et al. 1998). Whether these variants of ER$\beta$ protein are active or not is not really known yet. We are aware of the possible implications of this problem in the interpretation of our animal studies using this model.

In order to minimize genetic instability issues, for our experiments we used backcross 10$^{th}$ generations.

Several abnormalities have been found in male and female ER$\beta$-/- mice. In summary, ER$\beta$-/- female mice are subfertile due to accelerated follicular atresia (Cheng, Weihua et al. 2002), and exhibit an increased sensitivity to estrogen in uterus (Weihua, Saji et al.
ERβ−/− males develop prostatic epithelial hyperplasia (Krege, Hodgin et al. 1998; Weihua, Makela et al. 2001; Imamov, Morani et al. 2004) and increase aggressive behavior (Ogawa, Chester et al. 2000). Both male and female ERβ−/− mice are hypertensive (Zhu, Bian et al. 2002) with cardiac ventricular hypertrophy (Forster, Kietz et al. 2004), reduced alveologenesis (Patrone, Cassel et al. 2003) and defects in elastic recoil in the lung (Massaro and Massaro 2004) and hypoxia (Morani, Barros et al. 2006). Development of the central nervous system is severely compromised in ERβ−/− mice. This results in hypocellularity in the cortex (Wang, Andersson et al. 2001), increased sensitivity to neurotoxins and anxiety (Krezel, Dupont et al. 2001).

4.3 IMMUNOHISTOCHEMISTRY

This technique is a good qualitative method to detect which kind of cells express or do not express a certain marker. We used tissues fixed in 4% paraformaldehyde buffered solution and embedded in paraffin.

4.4 WESTERN BLOTTING

By differential centrifugation we separated the tissue homogenates into membrane, nuclear and cytosolic fractions. Protein content was measured by the Bradford. Equal amounts of protein were loaded in each lane in the SDS-PAGE gels and the blotted membrane was later stained with Comassie for confirmation.
This technique is a semi-quantitative method to measure the proteins extracted from two different sources. One sample is labeled with $^{12}$C-$N$-nicotinoyloxy-succinimide (ICPL-light), while the other one with $^{13}$C-$N$-nicotinoyloxy-succinimide (ICPL-heavy). These two labels are different isotopes from the same molecule; therefore they have the same chemical properties but they differ in molecular weight by 6 Dalton. The two samples are then mixed and subsequently separated by electrophoresis. The ratio of $^{12}$C/$^{13}$C isotopes is then calculated by mass spectrometry after trypsination in order to measure the protein ration between the two labeled samples.
5 RESULTS AND DISCUSSION

5.1 PAPER I

Previous studies reported the presence of hyperplastic foci and upregulation of AR in ventral prostate of ERβ−/− mice. With this study we examined the role of ERβ in epithelial homeostasis of ventral prostate of mice, by analysis of differentiation, proliferation and apoptosis markers. The number of BrdU positive cells in the ventral prostates of adult ERβ−/− mice is about 3 fold higher than that in WT mice. Immunostaining and western blotting with antibodies specific for the anti-apoptotic marker, Bcl-2, showed marked increase of this marker in the prostatic epithelium of ERβ−/− mice. More interestingly, Bcl-2 seems to be highly expressed all luminal epithelial cells of ERβ−/− prostate, whereas in WT mice it is expressed only in the basal epithelium, which contains slowly proliferating epithelial stem cells. TUNNEL assay confirmed the reduced apoptosis signal in ventral prostate of ERβ−/− mice. Increased incorporation of BrdU and reduced apoptosis are indications that overall confirm the hypercellularity previously observed in the ventral prostates ERβ−/− mice.

Our next question was then whether or not ERβ affects the epithelial differentiation. By immunohistochemistry we observed that the number of p63-positive cells was threefold higher in the epithelium of ERβ−/− than in WT prostates. Normally P63 is a marker expressed only by cells in the basal epithelium and, a striking finding, was that p63-expressing cells were found also in the luminal epithelium of ERβ−/− prostates. Cytokeratin 8 (CK8), a marker of fully-differentiated luminal cells, was downregulated in prostates of ERβ−/− mice, while CK19, a marker of basal and intermediary cells, was
markedly increased. By cytofluorimetric analysis (FACS) performed on isolated prostatic epithelial cells, we could confirm the increase number of p63 and also CK19 positive cells in ERβ⁻/⁻ prostates. All these data indicate that there is an accumulation of epithelial cells with basal-intermediary stage of differentiation in ventral prostates of ERβ⁻/⁻ mice. This means that ERβ may have a role in the regulation of the cellular differentiation process in prostate of mice.

5.2 PAPER II

There are several studies in literature showing a correlation between ERβ downregulation and progression of prostate cancer in men. The issue addressed in Paper 2 was whether the prodifferentiating role of ERβ which we demonstrated in prostate epithelium of mice also could be demonstrated in the human prostate. Progression of cancer is usually associated with lost of differentiation. As described in the paper I, in ERb⁻/- mouse prostates there are more AR positive cells, more proliferating and more undifferentiated epithelial cells. With the present study we wanted to identify proteins which are up or downregulated in prostates of ERβ⁻/⁻ mice, and to see whether there is homology between progression of human prostate cancer and the phenotypical changes in prostates of ERβ⁻/⁻ mice.

We used isotope-coded protein labeling (ICPL) in combination with LC-ESI-MS/MS to make a direct semi-quantitative comparison of the proteins expressed in WT and ERβ⁻/⁻ mice prostates. By this technique we found upregulation of several known AR regulated proteins (serine protease inhibitor Kazal type 3, called Spink3; semenoclotin, seminal vesicle secretion protein 3, glycogen phosphorylase, major prostatic secretory glycoprotein) and downregulation of cytoskeletal proteins (cytokeratin 8, α- and β-actin as well as villin-2) in prostates of ERβ⁻/⁻ comparing with WT mice. These data
confirmed the increased AR signaling associated with low differentiation in ventral prostates of ERβ<sup>-/-</sup> mice reported in the paper I.

More interestingly, the main upregulated protein in ERβ<sup>-/-</sup> prostates was spink3 (about 5 fold higher in ERβ<sup>-/-</sup> than WT prostates). This result was also confirmed by western blotting. The human homologue of spink3 is tumor associated trypsin inhibitor (TATI) well known as molecular marker of prostate cancer progression.

By immunohystochemical analysis of 15 human prostate biopsies with various differentiation grades (Gleason Score), we observed a negative correlation between TATI and ERβ expression and positive correlation between TATI and Gleason Score. To conclude, with this study we confirm that ERβ is downregulated in poorly differentiated epithelium of human prostate cancer and we provide evidence that the ERβ also has a prodifferentiating function in the human prostate.

5.3 PAPER III

Previous in vitro analysis showed that ERβ seems to be important for the FSH-stimulated follicular maturation. Females of ERβ<sup>-/-</sup> mice result sub-fertile, and ovaries are characterized by premature follicular atresia and very few corpora lutea. With this study we wanted to obtain a deeper insight into the role of ERβ in the ovarian follicular maturation.

The first big question was whether ERβ<sup>-/-</sup> female mice are subfertile because of some defect in the hypothalamo-pituitary axis or intrinsic dysfunction in the follicular structures. To answer this question, we approached by using two known methodologies, ovarian transplantation, and ovarian wedge resection, a technique used to treat infertile women affected by PCOS.
Transplantation of ovaries from WT mice into ERβ\(^+\) recipient mice and vice versa was not effective, because there was a reduction (60%) in fertility when normal ovaries were transplanted into normal mice. A resection of ¼ of the ovary (wedge resection) of ERβ\(^+\) mice definitely improved the fertility: all of 5 ERβ\(^+\) mice subjected to ovarian wedge resection became pregnant at the first mating period and, as it happens in case of treated PCOS patients, such fertility decreased again at each further mating period. Only 1 in 5 intact ERβ\(^+\) mice became pregnant at each mating period. This result provided us the evidence that ovaries of ERβ\(^+\) mice are functional and that the hypothalamo-pituitary axis is not affected by lack of ERβ signaling.

The question of what the role of ERβ is in the development of the follicles still remained. Upon histological analysis of the ERβ\(^+\) ovaries, we observed a more condensed theca layer, with poor vascularization, surrounding the granulosa cells of growing follicles. Azan staining indicated accumulation of collagen overall the stroma and the theca layers in the ERβ\(^+\) ovaries. Immunohistochemical studies revealed dysregulation of PDGFRs and reduced signal of smooth muscle actin), markers of angiogenesis, in the ovarian follicle of ERβ\(^+\) mice.

These data indicate a probable defect in the remodeling of the theca layer which results in poor vascularization, a critical process during follicular development, when the ERβ signaling is missing. It seems that ERβ, which is expressed in granulosa and not in theca cells, modulates a cross-talk between the two cellular compartments, which is necessary for the vascularization of theca layer during follicular development.

The mechanisms through which wedge resection is able to overcome the lack of ERβ signaling in ovaries of ERβ\(^+\) mice could involve powerful angiogenic signals released by the trauma of the resection itself.
After 5 months of age, ER$\beta$<sup>−/−</sup> mice are hypertensive and develop progressive consequent cardiac ventricular hypertrophy. The importance of estrogens in physiology and development of the lung has been known for long time. Recent physiological studies reported that ER$\beta$<sup>−/−</sup> mice have reduced and enlarged alveoli, with reduced elastic recoil (a physiologic property dependent on lung elasticity and so on the ECM organization). ER$\beta$ has been hypothesized to be involved in the regulation of ECM in lung.

In paper 4 we analyzed the involvement of ER$\beta$ in the control of the ECM deposition in lung and tested whether ER$\beta$<sup>−/−</sup> mice are hypoxic or not. Hypoxia could be one cause of systemic hypertension observed in these mice.

By histochemical analysis, we observed thicker alveoli and areas of collapsed alveoli in lungs of ER$\beta$<sup>−/−</sup> mice after 5 months of age. By azan staining and electron microscopy, the lungs of ER$\beta$<sup>−/−</sup> mice showed accumulation of collagen fibers in the alveolar parenchima. Dysregulation of metalloproteases (MT1-MMP, MMP2) and Caveoli-1 evidenced by western blotting and immunofluorescence, confirmed the fibrotic-like phenotype observed in lungs of ER$\beta$<sup>−/−</sup> mice.

Utilizing endogenous (HIF1-α) and exogenous (hypoxyprobe) markers to detect tissue hypoxia by immunohistochemistry, we observed that liver, brain, kidney, periovarian sac, prostate and heart are hypoxic in adult ER$\beta$<sup>−/−</sup> mice and that, after exercise on treadmill, such hypoxia worsened. Furthermore, is important to mention that ER$\beta$<sup>−/−</sup> mice were reluctant to run.

By this study we could draw three conclusions:

- ER$\beta$ regulates proteins involved in the regulation of ECM deposition in lung;
- ER$\beta$<sup>−/−</sup> mice result hypoxic, which could be the cause of systemic hypertension previously observed in these mice;
- The ERβ−/− mouse is not a good model to study \textit{in vivo} the role of ERβ in cardiovascular system, since there are secondary effects (such as hypoxia) which can affect the interpretation of the data.


7 CONCLUSIONS

Overall, the articles included in this thesis show the involvement of ERβ in the regulation of three important physiological features of the epithelial tissues: differentiation, stroma-epithelium communication and organization of ECM.

Our data obtained upon comparison of the phenotype of ventral prostates of WT and ERβ−/− mice, revealed a pro-differentiating role played by ERβ in the mouse prostatic epithelium. By analysis of biopsies of human prostate cancers in different grades of differentiation (Gleason Scores) we could extrapolate our data obtained in mice to human. These studies suggest that activation of ERβ with a specific agonist would be a potential cure “to keep” the differentiation status of the epithelium in the prostate and so to prevent the progression of prostate cancer. Obviously, such treatment would give benefits only in the first phases of carcinogenesis, when ERβ is still expressed.

With our studies on the phenotype of the ovaries of ERβ−/− mice, we provided some insights into the estrogen signaling in the maturation of ovarian follicles. Based on our observations, we speculate that ERβ may play a role in the reorganization and so vascularization of the thecal layer by promoting the communication between granulosa (ERβ positive epithelial-derived tissue) and theca (ERβ negative stromal-like tissue) cells. The fact that a simple wedge resection restored fertility of ERβ−/− female mice, suggests also that lack ERβ signaling does not affect the hypothalamo-pituitary axis. The mechanisms through which wedge resection is able to overcome the lack of ERβ signaling in ovaries of ERβ−/− mice could involve powerful angiogenic signals released by the trauma of the resection itself.
ERβ−/− mice have systemic hypertension and cardiac ventricular hypertrophy. All the evidence provided by us and other groups about ERβ in the lung indicate a role of this receptor in the maintenance of the ECM composition and lung alveolar structure. We have observed a reduced capacity of ERβ−/− mice to run on a treadmill and found an increase in hypoxia in many tissues after exercise. According to these data we speculate that systemic hypertension in ERβ−/− mice is a result of lung dysfunction.

Many more studies need to be done. For example we are still questioning whether and to what extent the immune system of ERβ−/− mice is involved in the phenotype observed in several organs, like prostate, lung and ovary. It is already known that ERβ−/− mice have myeloid-like leukemia with hyperproliferation and extensive peripheral invasion of immune cells. One approach to this question would be to perform bone marrow transplantation from WT to ERβ−/− mice and vice versa.
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