Estrogen Receptor α and Bone

Posttranslational modifications and cell-specific deletion

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Department of Internal Medicine and Clinical Nutrition
Institute of Medicine
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UNIVERSITY OF GOTHENBURG

Gothenburg 2019

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ISBN 978-91-7833-590-9 (PRINT)
ISBN 978-91-7833-591-6 (PDF)
http://hdl.handle.net/2077/60795

Printed in Gothenburg, Sweden 2019
Printed by BrandFactory

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ABSTRACT

Estrogen is involved in the regulation and development of reproductive organs. In addition, estrogen regulates several other organs including the skeleton, immune system, and adipose tissue. Estrogen treatment protects against osteoporosis and some other hormone-related diseases, but this treatment is associated with an increased risk of cancer in reproductive organs and venous thrombosis. Because of these side effects it is important to elucidate the mechanisms behind estrogenic effects in different organs, to aid the development of tissue-specific estrogen treatments. The estrogenic effect in the skeleton and several other hormone-sensitive tissues, including adipose tissue, is mainly mediated by estrogen receptor alpha (ERα). ERα is subjected to posttranslational modifications (PTMs) that can affect receptor signaling in a tissue-specific manner. Therefore, the first aim of this thesis was to evaluate whether targeting of three different ERα PTMs – palmitoylation at site C451 – phosphorylation at site S122 – methylation at site R264 –, results in tissue-specific estrogenic effects.

ERα is classically described as a transcription factor that affects the cell via nuclear (genomic) signaling. However, ERα can also be associated to the membrane and exert non-genomic signaling. To study the role of membrane-initiated ERα (mERα) signaling for the estrogenic response, we used mice lacking palmitoylation at site C451, which is crucial for membrane localization. Our study showed that the importance of mERα signaling is tissue-specific, with the trabecular bone in the axial skeleton being strongly dependent on functional mERα signaling, while adipose

tissue is mainly mERα-independent. We also demonstrated that phosphorylation at site S122 in ERα has a tissue-dependent role with an impact specifically on fat mass in female mice. Finally, we found that methylation at site R264 in ERα has no effect on estrogenic regulation of the skeleton or other estrogen-sensitive tissues.

ERα is expressed in several different cell types and ERα expression in bone cells has been shown to affect the skeleton. It is also known that T lymphocytes are involved in the regulation of bone mass. Therefore, the second aim of this thesis was to evaluate whether ERα expression in T lymphocytes is involved in the protective effect of estrogen in the skeleton. We identified that ERα expression in T lymphocytes is dispensable for normal estrogenic regulation of bone mass.

In conclusion, this thesis has increased our knowledge of estrogen signaling mechanisms. Specifically, this thesis shows that mERα is important for estrogen signaling and has a tissue-specific role. In addition, phosphorylation at site S122 modulates the activity of ERα in a tissue-dependent manner. This thesis also shows that methylation at site R264 is dispensable for estrogenic regulation of the skeleton and other estrogen-responsive tissues and that T lymphocytes are not direct target cells for ERα-mediated estrogenic skeletal effects.

Keywords: Estrogen receptor α, bone**,** osteoporosis, adipose tissue, estrogen, posttranslational modifications

ISBN 978-91-7833-590-9 (PRINT)
ISBN 978-91-7833-591-6 (PDF)

Östrogenreceptor α och Ben

Posttranslationella modifieringar och cellspecifik utslagning

Östrogen är inblandat i reglering och utveckling av reproduktionsorganen. Dessutom reglerar östrogen flera andra organ, såsom skelettet, immunsystemet och fettvävnaden. Östrogenbehandling skyddar mot osteoporos och andra hormonrelaterade sjukdomar men är förknippad med ökad risk för bröst- och livmodercancer samt ventrombos. Det är därför viktigt att utreda mekanismerna för östrogens skyddande effekter på ben för att kunna utveckla vävnadsspecifika östrogenbehandlingar. Den östrogena effekten i skelettet och flera andra hormonkänsliga vävnader, inklusive fettvävnad, förmedlas huvudsakligen av östrogenreceptor alfa (ERα). ERα utsätts för posttranslationella modifieringar (PTM) som kan påverka receptorn på ett vävnadspecifikt sätt. Det första syftet med denna avhandling var därför att utvärdera betydelsen av tre olika ERα PTM – palmitoylering av aminosyra C451 – fosforylering av aminosyra S122 – metylering av aminosyra R264 – i vävnadspecifik östrogensignalering.

ERα beskrivs klassiskt som en transkriptionsfaktor som påverkar cellen via signalering i cellkärnan (nukleär signalering). ERα kan också binda till membranet och utöva icke-nukleär signalering. För att studera huruvida membraninitierad ERα (mERα) signalering är viktig för det östrogena svaret använde vi möss som saknade palmitoylering av aminosyra C451, vilket är avgörande för inbindning till membranet. Vår studie visade att mERα-signalering har vävnadspecifik betydelse, där det trabekulära benet i det axiella skelettet var starkt beroende av funktionell mERα-signalering medan fettvävnaden huvudsakligen var oberoende av mERα. Därefter visade vi att fosforylering av aminosyra S122 i ERα har en vävnadsberoende roll med en inverkan specifikt på fettmassa hos kvinnliga möss. Slutligen fann vi att metylering av aminosyra R264 i ERα inte har någon effekt på östrogenreglering av skelettet eller andra östrogenkänsliga vävnader.

ERα uttrycks i flera olika celltyper och ERα-uttryck i benceller har visat sig påverka skelettet. Det är även känt att T-lymfocyter är involverade i

reglering av benmassa. Därför var det andra syftet med denna avhandling att utvärdera om ERα-uttryck i T-lymfocyter är inblandat i den skyddande effekten av östrogen i skelettet. Vi visade att ERα-uttryck i T-lymfocyter inte behövs för östrogenets reglering av benmassan.

Denna avhandling har ökat kunskapen om östrogens signaleringsmekanismer. Avhandlingen visar att mERα är viktig för östrogensignalering och har vävnadsspecifika effekter. Dessutom påverkar fosforylering av aminosyra S122 aktiviteten av ERα på ett vävnadsberoende sätt. Avhandlingen visar också att metylering av aminosyra R264 inte är involverad i ERα-medierad östrogenreglering av skelettet eller andra östrogenkänsliga vävnader och att T-lymfocyter inte är direkta målceller för östrogens ERα-medierade skyddande effekter på skelettet.

LIST OF PAPERS

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

I. Gustafsson KL, Farman H, Henning P, Lionikaite V, Movérare-Skrtic S, Wu J, Ryberg H, Koskela A, Gustafsson JÅ, Tuukanen J, Levin ER, Ohlsson C, Lagerquist MK. **The role of membrane ERα signaling in bone and other major estrogen responsive tissues.** *Scientific Reports,* 2016 Jul 8;6:29473.

II. Ohlsson C*, Gustafsson KL*, Farman HH, Henning P, Lionikaite V, Movérare-Skrtic S, Sjögren K, Andersson A, Islander U, Bernardi AI, Chambon P, Lagerquist MK. **Phosphorylation site S122 in estrogen receptor α has a tissue-dependent role in female mice.**
Manuscript in preparation.

III. Gustafsson KL*, Farman HH*, Nilsson KH, Henning P, Movérare-Skrtic S, Lionikaite V, Lawenius L, Engdahl C, Ohlsson C, Lagerquist MK. **Methylation at site R264 in estrogen receptor alpha is dispensable for the regulation of the skeleton and other estrogen responsive tissues in mice.**
Manuscript in preparation.

IV. Gustafsson KL, Nilsson KH, Farman HH, Andersson A, Lionikaite V, Henning P, Wu J, Windahl SH, Islander U, Movérare-Skrtic S, Sjögren K, Carlsten H, Gustafsson JÅ, Ohlsson C, Lagerquist MK. **ERα expression in T lymphocytes is dispensable for estrogenic effects in bone.** *Journal of Endocrinology* 2018; 20: 121-133.

*Contributed equally

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ABBREVIATIONS

aBMD	Areal bone mineral density
ALP	Alkaline phosphatase
BMD	Bone mineral density
CTX	C-telopeptide of type I collagen
DXA	Dual-energy X-ray absorptiometry
E1	Estrone
E2	17β-estradiol
E3	Estriol
EDC	Estrogen dendrimer conjugate
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
FSH	Follicle-stimulating hormone
GC-MS/MS	Gas chromatography-tandem mass spectrometry
GnRH	Gonadotropin-releasing hormone
HRT	Hormone replacement therapy
IGF-1	Insulin growth factor-1
LH	Luteinizing hormone

M-CSF	Macrophage colony-stimulating factor
mERα	Membrane estrogen receptor alpha
μCT	High-resolution microcomputed tomography
nERα	Nuclear estrogen receptor alpha
OCN	Osteocalcin
OPG	Osteoprotegerin
pQCT	Peripheral quantitative computed tomography
PTM	Posttranslational modification
P1NP	Procollagen type I N-terminal propeptide
RANKL	Receptor activator of nuclear factor kappa-B ligand
RUNX2	Runt-related transcription factor 2
SERM	Selective estrogen receptor modulator
T	Testosterone
TRAP	Tartrate-resistant acid phosphatase
WHI	Women's Health Initiative

1 INTRODUCTION

Estrogen has a large impact on several organs, and this thesis focuses on the skeleton. Estrogen deficiency leads to osteoporosis. Osteoporosis causes great suffering for the patient, lowered quality of life, increased mortality, and high costs for society. Estrogen protects against osteoporosis but is not suitable as treatment due to side effects such as breast cancer and venous thrombosis. In order to find better treatment options for diseases and disorders linked to estrogen deficiency, the mechanism of estrogen signaling needs to be clarified. This thesis increases our knowledge about estrogen signaling mechanisms and the importance of these mechanisms for the skeleton and other estrogen-responsive tissues.

1.1 Estrogens

Estrogens are sex hormones that play a crucial role in the maturation and development of reproductive organs. In addition, estrogens have a significant impact on the central nervous-, immune-, cardiovascular-, adipose-, and skeletal systems (1-10). There are three major endogenous estrogens, estrone (E1), estradiol (E2), and estriol (E3). E2 is the most potent estrogen and the predominant form in non-pregnant females between menarche and menopause. E3 is most important during pregnancy and E1 is the estrogen with the highest levels after menopause.

Estrogens are synthesized from cholesterol. The synthesis starts with the conversion of cholesterol to androgens (androstenedione and testosterone) via several enzymatic steps. The aromatase enzyme then converts the androgens to estrogens (Figure 1, for review see Simpson *et al.*) (11). Since estrogens belong to the steroid hormone family, they are lipophilic and can easily pass over the cell membrane.

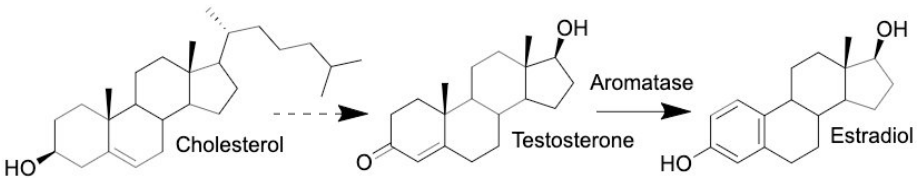

Figure 1. Abbreviated estradiol synthesis.

Estrogen production occurs mainly in the gonads, but small amounts are also produced in the adrenal cortex, adipose tissue and parts of the brain (12). Much of the circulating estrogens in humans are bound to sex hormone-binding globulin (SHBG). However, circulating estrogens in mice are not bound to SHBG since rodents do not express SHBG postnatally (13).

Sex steroid hormone levels fluctuate in females in a cyclic manner (menstrual cycle in women and estrus cycle in mice) and the levels are regulated by feedback systems. Gonadotropin-releasing hormone (GnRH), released from the hypothalamus, stimulates the anterior pituitary to release follicle-stimulating hormone (FSH). Increased FSH levels lead to follicle maturation and increased production of E2. The high circulating E2 levels have a positive feedback effect on the anterior pituitary that drastically increases luteinizing hormone (LH) release. The increased LH level leads to ovulation and formation of a corpus luteum. The corpus luteum produces great amounts of progesterone and estrogen that in turn have a negative feedback effect on the hypothalamus, by inhibiting GnRH release, and on the anterior pituitary, by inhibiting LH/FSH release. If the egg is not fertilized, the corpus luteum degenerates and progesterone and estrogen levels are decreased. The decreased hormone levels are a signal to the hypothalamus to restart the cycle. Thus, E2 levels vary a lot throughout the menstrual/estrus cycle (Figure 2).

The sex hormone levels in males are also regulated by a feedback mechanism. GnRH from the hypothalamus stimulates FSH and LH release from the anterior pituitary that increases the production of testosterone (T) in the testis. The increased levels of T inhibit the release of GnRH, FSH, and LH in a negative feedback loop.

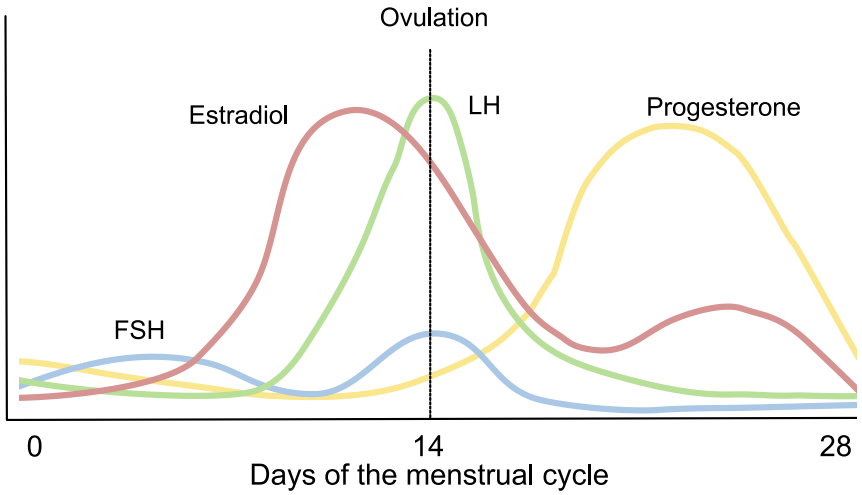

Figure 2. Hormone levels during the menstrual cycle in humans.

1.1.1 Estrogen receptors

Estrogens exert their effects through activation of estrogen receptors (ERs). There are two main receptors, estrogen receptor alpha (ERα) and beta (ERβ), that belong to the nuclear receptor family (14, 15). Starting from the N-terminal, the ER is divided into six main domains (Figure 3). The A/B domains contain the ligand-independent activation function-1 (AF-1) that is involved in the activation of gene transcription (16, 17). The C domain contains the DNA-binding domain (DBD), which binds to estrogen-response elements (EREs) in the promoter regions of target genes. The D domain, a hinge region linking the C and E domain, contains nuclear localization sequences (18). The E/F domains contain the ligand-binding domain (LBD) and ligand-dependent activation function-2 (AF-2) (16, 17).

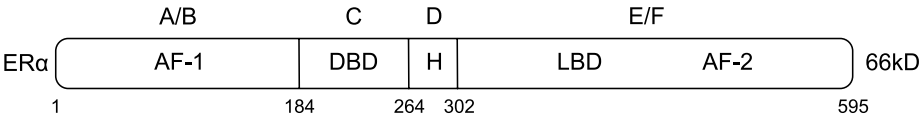

Figure 3. Schematic structure of mouse ERα (19).

ERα and ERβ share some structural components and the most conserved region is the DBD with 95% homology, indicating that the two receptors can bind to the same DNA sequences. The LBD is less homologous (~55%) and this can result in differences in ligand binding affinity between ERα and ERβ (20).

The distribution of the two nuclear ERs has been reported to differ somewhat between tissues, and also within tissues. ERα is suggested to be the main receptor expressed in breast, uterus, prostate, brain, liver, adipose tissue and bone, while ERβ expression is more prominent in the ovaries, prostate, lungs, thymus, spleen, bone marrow and vascular endothelium (21, 22). Regarding bone, it has been reported that ERα is the main ER in cortical bone, while ERβ is more expressed in trabecular bone (23) (for information about cortical versus trabecular bone, please see section 1.2).

In addition to the nuclear receptors, there is also a third estrogen receptor, the G protein-coupled estrogen receptor 1 (GPER), which is attached to the cell membrane (24, 25). Studies from our lab have shown that GPER is not required for the bone protective effects of E2 treatment or the estrogenic effects in other estrogen-responsive tissues such as fat, uterus, or thymus (26, 27). In contrast, another study demonstrated bone-protective effects when ovariectomized rats were treated with the GPER-agonist G1 (28). Thus, the physiological importance of this membrane-bound receptor is not fully understood (29).

ERα is the main mediator of estrogenic effects in the skeleton and also in other tissues discussed in this thesis, including adipose tissue and the immune system (30-35). Therefore, this thesis work is focused on ERα.

1.1.2 Estrogen receptor α signaling pathways

1.1.2.1 Genomic pathways

Classical ERα activation starts with ligand binding in the cytosol. This leads to conformational changes of the receptor, which releases the receptor from heat shock proteins followed by dimerization of the receptor. The ligand-receptor complex enters the nucleus and binds to estrogen response elements where it regulates gene transcription by acting as a transcription factor (36, 37). This is the classical genomic pathway (Figure 4A).

The ligand-receptor complex can also bind to and activate other transcription factors, e.g. specificity protein 1 (SP-1), activator protein 1 (AP-1), and nuclear factor-kB, which bind to other response elements (RE) in the promoter regions of target genes (38, 39). This is called the non-classical genomic pathway (Figure 4B). There are several co-regulator proteins that can bind to the ligand-receptor complex and modify the effect on gene transcription (40).

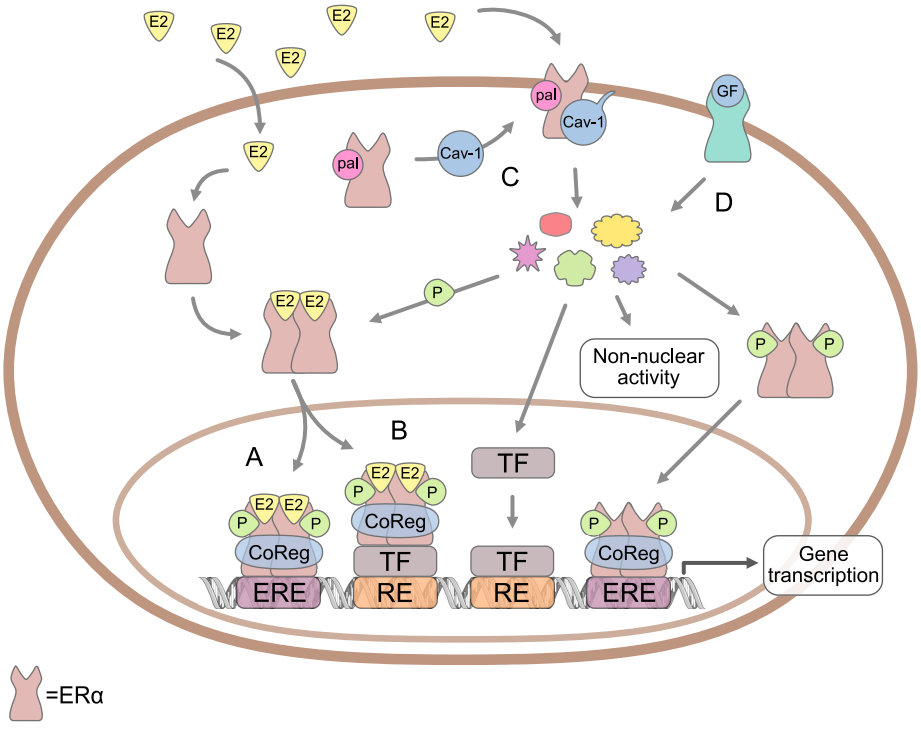

Figure 4: Estrogen receptor alpha signaling pathways. A) classical genomic pathway B) non-classical genomic pathway C) non-genomic pathway D) ligand-independent pathway. ERα= Estrogen receptor alpha, E2= Estradiol, GF= Growth factor, TF= Transcription factor, CoReg= Co-regulator proteins, ERE= Estrogen response element, RE= Response element, pal= Palmitoylation, Cav-1= Caveolin-1, P=Phosphorylation.

1.1.2.2 Non-genomic pathways

Estrogen receptors are members of the nuclear receptor superfamily and were for long thought only to have nuclear effects and to mainly function as transcription factors. However, some estrogenic effects were found to be too rapid for the genomic pathways (41-43). These rapid effects demonstrate the existence of non-genomic estrogen signaling, where the ERα-mediated effect occurs outside the nucleus and does not involve the direct influence of the ERα on gene transcription.

In non-genomic estrogen signaling, activation of ERs located in the plasma membrane, or in the cytoplasm, start signaling cascades that can directly affect the cell function and survival (Figure 4C). Non-genomic ERα signaling often involves protein-kinase cascades, including the mitogen-activated protein kinase (MAPK) signaling pathway and the phospatidylinositol 3-kinase (PI3K) signaling pathway, and may indirectly lead to changes in gene transcription due to phosphorylation of transcription factors (44, 45). Activation of ERs located at the cell membrane can also cause mobilization of intracellular calcium and stimulation of cyclic adenosine monophosphate (cAMP) production (46).

1.1.2.3 Ligand-independent pathways

In addition to the ligand-dependent genomic and non-genomic pathways, ERs can also be activated in the absence of a ligand. Phosphorylation of the receptor or interaction with various co-regulators can activate the ER (Figure 4D). Epidermal growth factor (EGF) and insulin growth factor-1 (IGF-1) are shown to be involved in ligand-independent activation of ERα (47, 48).

1.1.3 Posttranslational modifications of ERα

ERα is widely subjected to posttranslational modifications (PTMs) such as phosphorylation, methylation, and palmitoylation (49-51), which can modify the function of the receptor in a cell-specific manner.

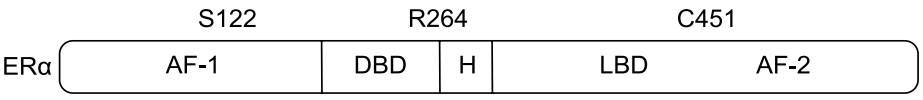

Figure 5. Three posttranslational modification sites in ERα. Phosphorylation site S122, methylation site R264, and palmitoylation site C451.

1.1.3.1 Palmitoylation

A small pool of ERα, 5-10% depending on cell type, is located in the cell membrane (52, 53). Palmitoylation at site C451 in the murine ERα (corresponding to C447 in humans) enables caveolin-1 to bind the receptor and this binding is essential for the membrane association of ERα (Figure 4,5) (49, 54). Palmitoylation site C451 has been mutated to create mice lacking the possibility to attach ERα to the membrane. Female mice lacking membrane-associated ERα (mERα) are shown to have disturbed sex hormone levels and abnormal ovarian function, leading to infertility and underdeveloped mammary glands (55, 56). The role of mERα for the skeleton is evaluated in paper I.

1.1.3.2 Phosphorylation

There are several sites for phosphorylation in ERα. Phosphorylation is a reversible addition of a phosphoryl group to the amino acids serine, threonine, or tyrosine (19, 57). Phosphorylation of ERα is involved in both the ligand-dependent and the ligand-independent activation of ERα and has been shown to modulate the DNA binding, protein stability, nuclear localization, hormone sensitivity, and interaction with other proteins (e.g. co-regulatory factors) (58-64). Phosphorylation site S118 (corresponding to S122 in mice) is the most studied phosphorylation site in ERα and this site is located in the N-terminal AF-1 region (Figure 5) (19, 57). The importance of this phosphorylation site has been evaluated *in vitro* in several cell lines such as COS-1 cells (a monkey kidney cell line), HeLa cells (a human cervical cancer cell line), and MCF-7 cells (a human breast cancer cell line) (60, 62, 65, 66). The shift of serine to alanine at site S118 leads to changed estradiol-induced gene transcription in some but not all of the *in vitro* studies, indicating that phosphorylation site S118 might have tissue-dependent effects (60, 62, 65, 66). Despite that the importance of phosphorylation site S118 in ERα has been known for a long time, there are until now no *in vivo* studies confirming the *in vitro* data.

1.1.3.3 Methylation

Methylation of proteins, mainly occurring on the amino acids arginine and lysine, can affect signaling transduction, protein-protein interactions, and subcellular localization of proteins (67-69). Human ERα can be methylated at arginine site R260 (corresponding to R264 in mice) and this site is located in the DBD domain of ERα (Figure 5) (19,

51). Methylation of R260 by the arginine methyltransferase PRMT1 is associated with cytoplasmic translocation of the receptor (51). Methylation is a reversible process and the enzyme JMJD6 can demethylate ERα (70). *In vitro* studies, in which the arginine (R) at site 260 has been switched to alanine (A) (R260A), shows that methylation at this site is important for ERα-mediated activation of the PI3K signaling pathway (51).

1.2 The skeleton

The skeleton has several functions in addition to giving structural support. It permits movement, provides protection of organs, provides the space for hematopoiesis, and acts as a storage for minerals (calcium and phosphate). The skeleton can be divided into the axial skeleton (vertebrae, ribs, and skull) and the appendicular skeleton (limbs and linked bones), and in total the human skeleton contains 206 bones.

The bones consist of two structurally different compartments; the cortical bone (compact bone) and the trabecular bone (spongy bone), also called cancellous bone. The cortical bone, found mainly in the shafts of the long bones, constitutes 80% of the total bone mass, and the remaining 20% is the trabecular bone, most commonly found in vertebrae and at the end of the long bones. In addition to the inorganic mineral components (e.g. hydroxyapatite, calcium carbonate, and phosphate), bone also consists of an organic bone matrix, containing protein components such as collagen, and cells. The collagen fibers provide the skeleton with elasticity and the ability to absorb energy (71).

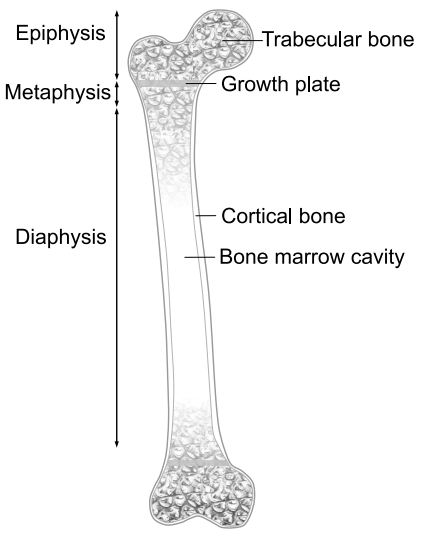

Figure 6: Schematic picture of a femur.

The long bones are divided into three parts: the diaphysis (the shaft), the epiphyses (the ends of the long bones) and the metaphysis (found between the epiphysis and the diaphysis). The

diaphysis consists mainly of cortical bone, while the epiphysis and metaphysis are mostly trabecular bone surrounded by a layer of cortical bone (Figure 6).

1.2.1 Bone cells

Osteoclasts are multinucleated bone-resorbing cells (Figure 7). They are formed by the fusion of mononucleated progenitor cells that are derived from hematopoietic stem cells (72, 73). The cytokines macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) are crucial for osteoclast differentiation and proliferation via binding to their respective receptors, c-fms and RANK, on preosteoclasts (74-76). RANKL is mainly produced by osteoblasts, osteocytes, and lymphocytes (77-80). RANKL can be inhibited by osteoprotegerin (OPG), a decoy receptor also produced by osteoblasts, as well as epithelial cells, and B lymphocytes (81, 82). Osteoclastogenesis is determined by the RANKL/OPG ratio.

Activated osteoclasts adhere to the bone surface and form a ruffled border. The osteoclasts then produce an acidic resorption lacuna and release enzymes that degrade the bone, e.g. cathepsin K. Osteoclasts also release tartrate-resistant acid phosphatase (TRAP), which is correlated to the activity of the osteoclasts (83, 84). C-telopeptide of type I collagen (CTX) is a product released in the bone degradation process, and can be measured in serum as a marker for bone resorption (85).

Osteoblasts, the bone formation cells, originate from mesenchymal stem cells (Figure 7). Runt-related transcription factor 2 (RUNX2) and osterix are important transcription factors required for osteoblast differentiation (86-88). Activated osteoblasts secrete several bone matrix proteins, e.g. collagen type I and osteocalcin (OCN). They also produce the enzyme alkaline phosphatase (ALP), which is involved in the mineralization process of the newly formed bone matrix. ALP, OCN, and procollagen type I N-terminal propeptide (P1NP) can be measured in serum as markers for bone formation (89, 90).

After bone formation, about 2/3 of the osteoblasts go into apoptosis (91). Some osteoblasts are trapped in the bone matrix and become osteocytes. The osteoblasts left at the bone surface become flat bone lining cells (Figure 7).

Osteocytes are the most common bone cell type in the skeleton (95%) and act as mechanosensors in the bone. Osteocytes are located in cavities (lacunas) and have extensions that form a network (canaliculi) that connect them to each other and to the bone surface (Figure 7). This network is sensitive to mechanical forces and sends signals that regulate both osteoclast and osteoblast activity (77).

Estrogen signaling has effects in all bone cells. Estrogen deficiency leads to increased osteocyte apoptosis, and an increased number of osteoclasts and a subsequent increase in bone resorption (92-95). Estrogen treatment inhibits osteoclast development, promotes osteoclast apoptosis and inhibits osteoblast apoptosis, leading to decreased bone resorption and increased bone formation (96-99).

1.2.2 Bone modeling and remodeling

Bone is a dynamic tissue that changes continuously throughout life. During development and growth, skeletal bone formation often occurs without prior bone resorption. This process is called bone modeling. Bone modeling is also responsible for changes in bone shape due to mechanical forces in the adult skeleton.

The skeleton needs to be repaired to remain strong and healthy. During life, the bone receives microcracks and old bone, therefore, needs to be replaced by new bone. This process is called bone remodeling. Bone remodeling in adults results in a completely new skeleton every 10 years (91). Bone remodeling takes place in a basic multicellular unit (BMU) that includes osteoclasts, osteoblasts, and osteocytes (Figure 7). The remodeling cycle includes four stages, starting with the activation of osteoclasts that are recruited to the bone that needs to be replaced (activation stage). The osteoclasts begin to resorb the bone (resorption stage), after the bone resorption the bone pit is cleaned (reversal stage). Then the osteoblasts start the formation of new bone (formation stage). It takes approximately 2–4 weeks for the activated osteoclasts to resorb the bone and 4–6 months for the osteoblasts to form new bone. Bone remodeling requires a tight collaboration between osteoclast resorption and osteoblast formation. This collaboration is called “coupling” and ensures that the same amount of bone is formed as has been resorbed. Osteoclasts can regulate osteoblast activity in several ways. Osteoclast resorption leads to the release of the transforming growth factor beta (TGF-β), that increases osteoblast proliferation, differentiation, and

activity (100). Mature osteoclasts can also release other osteoblast stimulatory factors that stimulate osteoblast number and activity, and there is also evidence for cell-cell interaction between osteoclasts and osteoblasts (101, 102). The coupling between resorption and formation explains why decreased bone resorption also can lead to decreased bone formation (103).

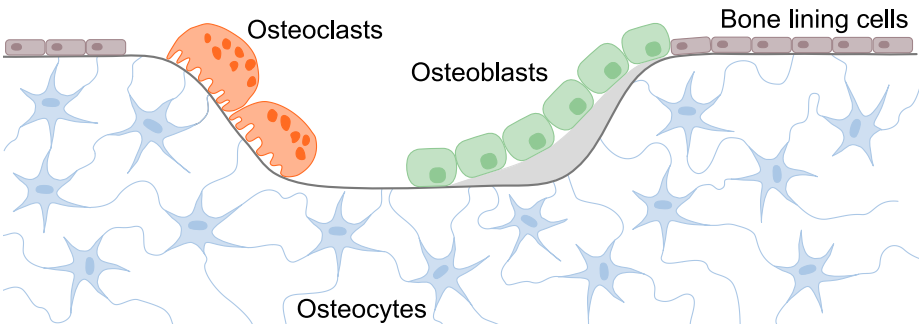

Figure 7: Schematic picture of a bone remodeling site.

1.3 Estrogen and the skeleton

Estrogen is important for the skeleton throughout life with involvement in bone development, growth, and maintenance. During puberty, E2 levels increase and this stimulates rapid growth and induces the closing of the growth plate (104). In women, E2 levels decrease dramatically after menopause, which leads to bone loss (105), and a similar bone loss is seen after ovariectomy in mice (106). Treatment with estrogen has been shown, both in humans and in mice, to increase both trabecular and cortical bone mass (106, 107).

1.3.1 ERα deletion

In order to study the importance of ERα signaling for the regulation of the skeleton, mice with deletion of ERα (both global and cell-specific) have been developed and studied.

Mice with global deletion (deletion in all cells of the body) of ERα show decreased bone growth and increased bone mass in both female and

male mice (108-111). This skeletal phenotype is mainly caused by disturbed sex steroid feedback regulation. To avoid these confounding effects, several of the phenotypes have been investigated in gonadectomized mice treated with E2 to assess the importance of ERα for the E2 treatment response. Female ovariectomized mice with deleted ERα have a reduced response to E2 treatment in both trabecular and cortical bone, demonstrating the essential role of ERα signaling for the female skeleton (32, 106). The same phenotype is seen after E2 treatment of castrated male mice (112, 113).

The importance of human ERα has been shown in two case reports describing a male and a female patient with impaired ERα function (114, 115). Both these reports describe disturbed sex steroid feedback regulation, similar as seen in the experimental mouse studies. Furthermore, both the male and the female patient display delayed skeletal maturation and decreased bone mass demonstrating an important role of ERα for the regulation of the human skeleton.

To investigate how ERα expression in different bone cells affects the estrogenic effects in bone, several experimental studies with cell-specific ERα deletion have been performed. These studies demonstrate a complex relationship between ERα signaling in bone cells at different developmental stages and effects on the two bone compartments found in bone; trabecular and cortical bone.

In females, cortical bone is reported to be dependent on ERα in preosteoblasts and osteoblasts, while the trabecular bone is dependent on ERα in mature osteoblasts and probably osteocytes (conflicting data) (116-119). In males, ERα in preosteoblasts have been shown to have an impact on cortical bone, while ERα in mature osteoblasts and osteocytes have a role in the regulation of trabecular bone (116, 120). Furthermore, only trabecular bone in female mice seems to be affected by ERα inactivation in osteoclasts, whereas cortical bone in females and both trabecular and cortical bone in male mice are reported to be normal (121, 122).

In addition to bone cells, other target cells have been implicated in ERα-mediated effects on bone. Studies from our research group and others have shown that ERα signaling in nerve cells affects the skeleton (123-125). Our research group has also shown that the estrogen response in bone is attenuated in female mice lacking ERα in hematopoietic cells

(126). However, it is not completely known which hematopoietic cell is responsible for this attenuation of the E2 treatment response. In addition to osteoclasts, other cells with hematopoietic origin have been shown to be involved in the regulation of bone mass such as B and T lymphocytes (127-129). Mice with B lymphocyte-specific inactivation of ERα was recently shown to display a normal response to estrogen, suggesting that B lymphocytes are not direct target cells for ERα-mediated effects on the skeleton (130). The research group of Pacifici has shown that mice lacking T cells are protected from ovariectomy-induced bone loss (131). Furthermore, estrogen deficiency caused by ovariectomy increases T lymphopoiesis and the production of tumor necrosis factor-alpha (TNFα) from T lymphocytes, leading to increased bone loss (131, 132). These studies show that T lymphocytes are important for the estrogenic regulation of bone. In order to evaluate whether these estrogen effects are direct effects on T lymphocytes, or indirect via estrogen signaling in other cells, we have developed a mouse model with T lymphocyte-specific deletion of ERα (Paper IV).

1.4 Osteoporosis

Osteoporosis is caused by an imbalance in the bone remodeling process and characterized by low bone mass and deteriorated skeletal microarchitecture, leading to an increased risk of fracture. Osteoporosis is defined as bone density < –2.5 standard deviation (SD) of the mean of a young adult female population. Primary osteoporosis is a progressive bone loss caused by aging and declining sex steroids (Figure 8). Secondary osteoporosis is caused by disease (e.g. inflammatory disease, malabsorption) or medications (e.g. glucocorticoids). Every second women and every fifth man will suffer from an osteoporotic fracture during their lifespan (133). Typical sites for osteoporosis fractures are the hip, vertebrae, and forearm (134). The most common osteoporosis treatment is bisphosphonates, which is reported to decrease osteoclast activity (135). Other drugs approved for osteoporosis treatment include monoclonal antibodies, e.g. the RANKL inhibitor Denosumab (136), synthetic parathyroid hormone (PTH) Teriparatide, and selective estrogen receptor modulators, e.g. Raloxifene (137).

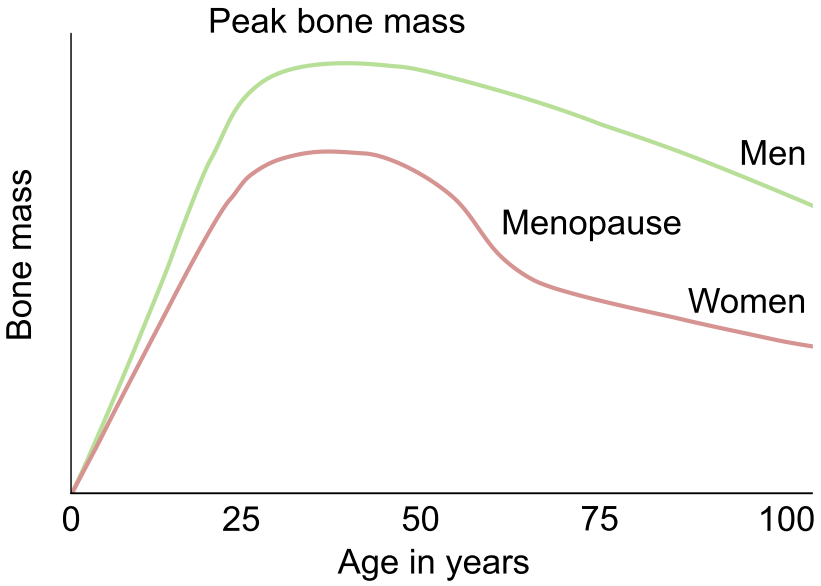

Figure 8: Change in bone mass with age.

1.4.1 Post-menopausal osteoporosis

Postmenopausal osteoporosis was first described by Albright in 1940. He showed that postmenopausal women suffer from declining bone mass (Figure 8) (105). Estrogen deficiency following menopause leads to both increased bone resorption and bone formation but with a higher resorption in relation to formation resulting in bone loss. Ovariectomy of mice leads to abolished endogenous estrogen production that mimics the estrogen deficiency in women after menopause.

1.4.2 Estrogen and osteoporosis in men

Estrogen is not only beneficial for the female, but also for the male skeleton. For long it was believed that testosterone was the main regulator of the male skeleton but this belief was challenged by case reports in the 1990th. In addition to the ERα-deficient male (described in 1.3.1), two males with a deficient aromatase enzyme, leading to estrogen deficiency, showed the same skeletal phenotype as the male with a mutated ERα gene (138, 139). Estrogen treatment restored the low bone mass in these aromatase deficient males (138, 140), demonstrating that estrogen signaling is important for the male skeleton.

These observations in humans are confirmed by studies in male mice lacking ERα and the aromatase enzyme (108, 141). Furthermore, castrated men, as well as orchiectomized male mice, have decreased bone mass, which is restored by E2 treatment (4, 142). In addition, low estrogen levels have been shown to be associated with low bone mass and increased fracture risk in older men (4, 143-145). Taken together, estrogen signaling is important for the male skeleton during development and growth and for skeletal maintenance in the elderly. Despite this, there is currently no recommendation for treatment with estrogen or estrogen-like products against osteoporosis in men.

1.5 Estrogen effects in other tissues

The estrogen deficiency following menopause increases visceral fat mass and body weight, and this increase can be prevented by hormone replacement treatment (146). Increased body fat, especially visceral fat, augments the risk to develop the metabolic syndrome. Similar to postmenopausal women, ovariectomized mice have increased fat mass, which is decreased by E2 treatment (147). Studies have shown that estrogen deficiency increases both the area and the number of adipocytes (34). In adult mice, global ERα deletion leads to increased body weight and increased percent fat mass and also increased serum leptin levels (32, 34, 148, 149). Furthermore, ovariectomized mice with a global ERα deletion have a reduced response to E2 treatment in adipose tissue as compared to control mice, demonstrating the essential role of ERα signaling in fat (32, 106).

Besides bone and fat, estrogen is also highly involved in the development and regulation of the immune system, especially the adaptive immune system involving B and T lymphocytes (150). High estrogen levels cause thymic atrophy and block early T lymphocyte development in thymus (151-153). In addition, studies have shown that ERα is necessary for normal thymic development and estrogen-induced alteration of thymus (31, 153). Ovariectomy-induced estrogen deficiency increase B lymphopoiesis in the bone marrow, while E2 treatment decrease B lymphopoiesis (154-156). Despite the decreased B lymphopoiesis, E2 treatment increase antibody production probably due to increased cell survival of mature B lymphocytes (157, 158).

The uterus is another estrogen-responsive tissue and estrogen signaling via ERα is crucial for normal cyclical uterine growth during the menstrual cycle. However, E2 stimulates proliferation of cells in the uterus, thereby increasing the risk of tumor progression in endometrial cancer (159). Mice with a global deletion of ERα have reduced uterus size since their uterus cannot respond to E2, either endogenous or exogenous, demonstrating a crucial role for ERα in the regulation of the uterus (106).

1.6 Hormone replacement therapy

There are several symptoms and diseases associated with the reduced E2 levels in postmenopausal women including hot flushes, weight gain, different metabolic disorders, mood and sleep disturbances, and osteoporosis. Hormone replacement therapy (HRT) reduces several of these postmenopausal symptoms and prevents the development of diseases associated with low levels of E2, such as osteoporosis. HRT, containing estrogen and progestin, was commonly used in the 1990s. The Women's Health Initiative (WHI) study, launched in the early '90s, was designed to assess the risks and benefits of long-term use of HRT. Although HRT was shown to reduce fracture risk, it was also associated with severe adverse effects, such as the increased risk of venous thrombosis, heart disease, and breast cancer (160, 161). The WHI study drastically reduced the use of HRT and nowadays HRT is only recommended for short-term use to reduce severe postmenopausal symptoms.

The risk of adverse effects with HRT treatment has prompted the development of selective estrogen receptor modulator (SERM). SERMs can have tissue-specific agonistic (stimulatory) and antagonistic (inhibitory) effects and many of the SERMs available today lack some, but not all, of the side effects of estrogen. Tamoxifen is a first-generation SERM, with antagonistic effects in breast tissue, and today, this drug is widely used as a treatment of breast cancer. Tamoxifen has agonistic effects in bone but unluckily also in the uterus (162). In order to be able to treat postmenopausal osteoporosis, the search for a substance with agonistic effects in bone and antagonistic effects in breast and uterus continued and the second generation of SERM was developed. One of these substances is Raloxifene, the first SERM accepted for the treatment of postmenopausal osteoporosis and also for the treatment of

breast cancer, and this drug is still used in the clinic (163-165). There are also the third-generation SERMs, including Lasofoxifene, that prevents vertebral fractures (166). Another third-generation SERM is Bazedoxifene which prevents vertebral as well as non-vertebral fractures in high-risk osteoporotic patients (167). Furthermore, the combination of conjugated estrogen and bazedoxifene (tissue-selective estrogen complex- TSEC) has been successful in studies with agonistic effects on bone mass in vertebrae and hip and antagonistic effects in the uterus (168).

2 AIM

This thesis intends to increase the knowledge of the mechanism of estrogen receptor α signaling. The overall aim is to identify mechanisms behind the protective effects of estrogen in bone in order to aid the development of new tissue-specific treatments.

Specific aims of the four papers included in this thesis:

I. To evaluate the importance of membrane localization of ERα.

II. To investigate the role of phosphorylation site S122 in ERα in estrogen-responsive tissues.

III. To evaluate the involvement of methylation site R264 in ERα for the regulation of bone and other estrogen-responsive organs.

IV. To determine whether T lymphocytes are direct target cells for the ERα-mediated bone-protective effects of estrogen.

3 METHODS

3.1 Animal models

Estrogens are hormones and they affect various tissues in the body. It is therefore important to study estrogen signaling *in vivo* to have the interactions between different tissues represented. Mice are a commonly used animal model to study human diseases and the effects of treatments. There are several advantages of using mice as a model. For example, the mouse and human genomes are similar and the mouse genome is quite easy to manipulate. The mice have a short lifespan and are reproduced easily. Therefore, it is relatively easy to develop and study mice with a modified genome. In addition, mice and humans have several similarities in anatomy and physiology. There are, however, some differences to have in mind. The human growth plate closes due to high E2 levels at puberty, while the growth plate in mice never fully closes and, therefore, the mice have a longitudinal growth throughout life. Next, mice do not have a distinct menopause. However, the estrogen deficiency at menopause can be mimicked by ovariectomy of the mice.

The C57BL/6 mouse strain, used throughout this thesis, is the most commonly used strain for genetic manipulation. The mean lifespan for this strain is two years. These mice are sexually mature at 6–8 weeks and their peak bone mass is reached at 4–6 months of age (169).

In paper I-III we have used mice with point mutations in the gene for ERα (encoded by Esr1) (Table 1). The point mutations in our three models lead to an amino acid shift to alanine (A), an amino acid that cannot be posttranslationally modified.

Table 1. Overview of point mutations in ERα evaluated in this thesis.

Paper	Site	Amino acid change	PTM
I	C451	Cysteine – Alanine	Palmitoylation
II	S122	Serine – Alanine	Phosphorylation
III	R264	Arginine – Alanine	Methylation

In paper IV, we have used a mouse model with a T lymphocyte-specific ERα inactivation. To obtain these mice we have used the established

Cre-loxP system (170-172). The Cre recombinase recognizes loxP sites and cuts out the part of the genome found between the loxP sites. We used mice in which the Cre recombinase is driven by an Lck promoter. This promoter is specifically expressed in the earliest step of T lymphocyte maturation and in all subsequent T lymphocyte lineage cells (173). These Cre-mice were bred with mice with exon 3 of the ERα gene flanked by loxP sequences, leading to specific inactivation of ERα in all T lymphocyte lineage cells.

3.1.1 Ovariectomy and estradiol treatment

In females, the sex steroids are mainly produced in the ovaries. The levels of sex steroids are drastically decreased following ovariectomy. Ovariectomy leads to bone loss similar to the bone loss seen at menopause. In addition, the drastic decrease in estrogen levels after ovariectomy leads to a decreased uterus size, increased thymic atrophy, and increased adipose tissue. Our mouse model that lacks mERα, used in paper I, have a disturbed negative feedback regulation of sex steroids, which leads to confounding elevated serum levels of e.g. estradiol and testosterone. To avoid this confounding factor we ovariectomized and treated the mice with E2 and evaluated the estrogenic response in different organs in our model.

E2 (17β-estradiol) is administrated subcutaneously, either by slow-release pellets or by daily injections (Table 2). In paper IV, we used a slow-release pellet releasing a supraphysiological dose of E2. In paper I, which was performed after the study resulting in paper IV, we used a pellet resulting in a more physiological E2 dose. There is evidence showing that slow-release pellets give a very high supraphysiological dose at the beginning of the treatment period (174). Therefore, we decided to do subcutaneous injections in our last treatment study (paper III) to be able to give an E2 dose in a more physiological range.

Table 2. E2 doses and administration routes in our studies.

Paper	E2 dose	Administration
I	16.7 ng/mouse/day	Slow-release pellet
III	0.6 μg/mouse/day	Subcutaneous injection
IV	167 ng/mouse/day	Slow-release pellet

3.2 Bone analyses

3.2.1 Dual-energy X-ray absorptiometry

Dual-energy X-ray absorptiometry (DXA) is a frequently used method for measuring bone mineral density (BMD), both in clinical practice and in preclinical animal research. In the clinic, DXA BMD in the hip and vertebrae is used as a diagnostic tool and to investigate if an osteoporosis treatment has been successful. In animal research, DXA is a quick and non-invasive method that is very useful in longitudinal studies. The DXA has two X-ray beams with different energy levels. The soft tissue and bone tissue absorb the energy differently and it is, therefore, possible to separate soft tissue from bone tissue. DXA gives a two-dimensional image and therefore provides us with the areal bone mineral density (aBMD). However, due to low resolution, DXA does not allow us to separate cortical bone from trabecular bone. For DXA analyses in paper I-IV, we have used a Lunar PIXImus densitometer (Wipro GE Healthcare), and, in paper III, we have also used Faxitron UltraFocus dual-energy x-ray absorptiometry (Faxitron Bioptics).

3.2.2 Peripheral Quantitative Computed Tomography

Peripheral Quantitative Computed Tomography (pQCT) is used both in clinical and animal research. Compared to DXA, pQCT provides the volumetric BMD and due to its higher resolution also a separation of the trabecular and cortical bone. The pQCT has an X-ray source rotating around the bone that gives a three-dimensional measurement of the bone. In paper III, the trabecular bone is measured with a metaphyseal scan and the trabecular bone region is defined as the inner 45 % of the cross-sectional area. Cortical bone is determined with a mid-diaphyseal scan and we used a pQCT XCT Research M (version 4.5B; Norland) at a resolution of 70µm.

3.2.3 High-resolution microcomputed tomography

High-resolution microcomputed tomography (µCT) has a higher resolution compared to the pQCT, which gives us the possibility to determine the microstructure of the bone, e.g. cortical porosity and trabecular number, thickness, and separation. The bone is placed on a rotating plate between the x-ray source and the charge-coupled detector (CCD) array. The plate rotates slowly 180 degrees and then the scan is

completed. This gives us several 2D images which are then reconstructed into a 3D image. The trabecular bone in the vertebrae is measured in the vertebral body caudal of the pedicles. In long bones, the trabecular bone is measured in the metaphyseal region of the distal femur and the proximal tibia. The cortical measurements are performed in the mid-diaphyseal region of the long bones. We have used an 1172 model μCT (Bruker MicroCT) with a voxel size of 4.5 μm.

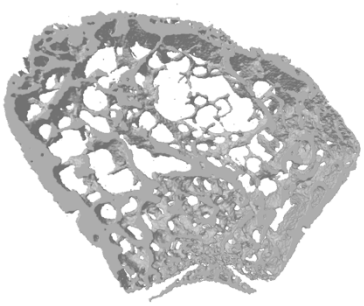

Figure 9: Three-dimensional μCT picture showing cortical and trabecular bone in the distal metaphysis of a femur.

3.2.4 Three-point bending

Calculations from pQCT and μCT analyses can predict the strength of the bone, but this calculation is not taking other important components for bone strength into account e.g. the quality of collagen. To measure the actual mechanical bone strength, we used the three-point bending test. The bone is placed in the mechanical testing machine (Instron 3366, Instron) on two supporting points with a loading point placed over the mid-diaphysis part of the bone. The loading point moves downward with increasing load until the bone breaks, and load at failure is the maximal load for the bone. Three-point bending is mainly an assessment of cortical bone strength, since the mid-diaphysis, where the breaking of the bone occurs, mainly consists of cortical bone.

3.2.5 Histomorphometric analyses

Histomorphometry is a histological assessment of bone phenotypes. In addition to the bone parameters we can receive from the μCT analyses, histomorphometry adds information about bone turnover and bone cells.

Static bone histomorphometry gives us information at a single time point, while dynamic histomorphometry gives information about bone formation over time.

In static bone histomorphometry, the bone sections are stained in order to determine the cortical and trabecular bone areas as well as information about bone cells in relation to structural features. In addition, cell numbers and the tissue-area covered by the cells can be analyzed.

Dynamic bone histomorphometry gives us information about the bone formation rate and mineralization rate. Injection with fluorescent agents at least twice before the sacrifice is needed. The most common fluorescent agents are tetracycline, calcein, and alizarin red. These agents are incorporated into newly formed bone where they can be visualized using a fluorescence microscope. Thereafter, the distance between the labels is used to calculate bone formation rate and mineralization rate.

In paper I, we injected calcein day 1 and 8 prior to sacrifice and we used the OsteoMeasure histomorphometry system (OsteoMetrics) for analysis.

3.3 Serum analyses

3.3.1 ELISA

In addition to histomorphometric analyses, bone turnover can be assessed by measurements of bone formation and degradation products in serum. We have used enzyme-linked immunosorbent assay (ELISA) kits to measure C-telopeptide of type I collagen (CTX-I), a bone resorption marker, and osteocalcin and procollagen type I N-terminal propeptide (P1NP), which are bone formation markers. We have also used ELISA kits to measure serum levels of leptin and insulin.

3.3.2 GC-MS/MS

Immunoassay kits, including radioimmunoassays (RIA) and ELISAs, can be used to measure sex steroid levels in serum. However, these methods are not able to accurately measure very low levels of hormones (175, 176). Mouse serum contains low levels of sex steroids, especially estradiol, and we have, therefore, used the sensitive and specific gas

chromatography coupled to tandem mass spectrometry (GC-MS/MS) method to measure sex hormone levels in our projects (177).

3.4 Gene expression analyses

Quantitative polymerase chain reaction (qPCR) is a sensitive method to measure gene expression in different cells or tissues. RNA is extracted from the tissue and reversed transcribed to complementary DNA (cDNA). The cDNA is then mixed with primers and fluorescently labeled oligonucleotide probes. During replication, the fluorescence is emitted proportional to the amount of amplified cDNA. The amplification can be followed over time and in each cycle, the cDNA is doubled, which makes the amplification exponential. The gene of interest and an internal standard are labeled with two different probes emitting light at different wavelengths, and can therefore be analyzed at the same time. Expression of the gene of interest is, in our studies, quantified relative to the housekeeping gene 18S. We used the ABI Prism 7000 sequence Detection System (PE, Applied Biosystem) for our gene expression analyses.

3.5 Protein analyses

3.5.1 Western blot

Western blot is used to separate and identify proteins in a sample, e.g. homogenized tissue. Briefly, the homogenized sample is exposed to heat and the proteins are denatured. The proteins are then separated by molecular weight with gel electrophoresis. The samples are transferred to a membrane, which is incubated with a primary antibody specific to the protein of interest, followed by a secondary antibody labeled with a detection reagent. The secondary antibody can be visualized with various methods, and in our case, we have used UV-light (ChemiDoc System, Bio-Rad). The thickness of the band corresponds to the amount of protein present. In order to know that a similar amount of protein was loaded onto the gel for every sample, we have also analyzed glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a protein that should be expressed similarly in all cell types. The protein of interest is correlated to GAPDH to give a relative quantification of the amount of

protein. We have used western blot to investigate ERα protein levels in paper I.

3.5.2 Simple western

Simple Western is a relatively new automated western blot method that is used to separate and identify proteins in tissues. The tissue is homogenized and mixed with a fluorescent master mix and exposed to heat to denature the proteins. The samples, blocking buffer, primary and secondary antibodies, chemiluminescent reagents, and wash buffer are loaded on a plate. The plate is then loaded in the WES machine (ProteinSimple) as well as capillaries where the separation and detection process occur. The proteins are separated by molecular weight, fixed in the capillary wall with UV-light, marked with a primary antibody, followed by a secondary antibody and chemiluminescent reagents, the chemiluminescent reaction is detected with a charge-coupled-device (CCD) camera, in a fully automated process. The data are analyzed using the Compass Software (ProteinSimple). The amount of protein of interest is quantified by correlation to β-actin, a protein that is similarly expressed in all tissues. We used simple western to investigate the ERα protein levels in paper II and III.

3.6 Flow cytometry

Flow cytometry is frequently used to identify cells and determine the frequency of different cell populations. A single-cell suspension is stained with antibody-conjugated fluorochromes. We used antibodies specific to cell surface epitopes. After staining, the cells are injected to a flow cytometer where the cells are exposed to lasers and the fluorochromes attached to the cells emit light in different wavelengths that are detected and presented as a plot.

We stained single-cell suspensions from bone marrow and thymus to determine the frequency of T and B lymphocytes. We used a FACSVerse for cell analysis (Becton Dickinson) and FlowJo software version 10.4.2 (Tree Star) for data analysis.

4 RESULTS

4.1 Paper I

The role of membrane ERα signaling in bone and other major estrogen responsive tissues

In this study, we have evaluated the importance of membrane ERα (mERα) signaling in bone and other estrogen-responsive tissues. To this end, we have used mice with a point mutation that leads to a lack of ERα in the plasma membrane. These mice have disturbed feedback regulation of sex steroids and to eliminate this confounding factor we have studied these mice after ovariectomy and estradiol treatment.

Main results

- The estrogenic response in the trabecular bone in the axial skeleton was abolished in mice lacking mERα compared to controls.
- The response to estrogen treatment was partly reduced in trabecular and cortical bone in the appendicular skeleton in mice lacking mERα as compared to control mice.
- Mice lacking mERα had a partly reduced estrogenic response in uterus compared to control mice.
- The liver and adipose tissue responses to estrogen treatment were similar in mice lacking mERα and controls.

Conclusion

We demonstrate that mERα is important for the estrogenic response in female mice in a tissue-dependent manner. The trabecular bone in the appendicular skeleton is strongly dependent on mERα, while both the trabecular and cortical bone in the appendicular skeleton, as well as the uterus, are partly dependent on mERα. Adipose tissue and liver are independent of mERα. Our novel findings state that ERα signaling mechanisms outside the nucleus have a large impact on the estrogen response.

4.2 Paper II

Phosphorylation site S122 in estrogen receptor α has a tissue-dependent role in female mice

In vitro studies have shown that phosphorylation at site S122 in ERα is involved in both ligand-dependent and ligand-independent ERα signaling pathways. In this study, we have investigated the role of phosphorylation site S122 in ERα in various estrogen-responsive tissues *in vivo*.

Main results

- All measured bone parameters (e.g. total body aBMD, cortical thickness, and bone volume per tissue volume) were similar between mice lacking phosphorylation site S122 in ERα and control mice.
- Mice lacking phosphorylation site S122 in ERα had increased body weight compared to control mice.
- Percent fat, dissected fat depots, and serum leptin levels were increased in mice lacking phosphorylation site S122 in ERα compared to control mice.
- Lymphopoiesis in bone marrow and thymus, as well as uterus and liver weights, did not differ between mice lacking phosphorylation site S122 in ERα and control mice.

Conclusion

The present study demonstrates that phosphorylation at site S122 in ERα is essential for the normal regulation of adipose tissue, while it is dispensable for the regulation of the skeleton, lymphopoiesis, liver, and uterus. This is the first *in vivo* study demonstrating that phosphorylation in a transactivation domain in a nuclear steroid receptor has a tissue-dependent role.

4.3 Paper III

Methylation at site R264 in estrogen receptor alpha is dispensable for the regulation of the skeleton and other estrogen responsive tissues in mice

In vitro studies have shown that methylation at site R260 (corresponding to R264 in mice) in ERα is required for the ERα-mediated activation of the PI3K signaling pathway and this pathway has been shown to be involved in the regulation of osteoblasts. In this study, we evaluated the role of methylation site R264 in ERα for bone and other estrogen-responsive tissues *in vivo*.

Main results

- DXA measurements of gonadal intact mice showed that total body and lumbar spine aBMD were similar between mice lacking methylation site R264 in ERα and controls.
- Neither trabecular nor cortical bone was altered by the lack of methylation site R264 in ERα.
- Lack of methylation site R264 in ERα had no impact on the weights of estrogen-regulated soft tissues (e.g. gonadal and peritoneal fat, liver, uterus, or thymus).
- Mice lacking methylation site R264 in ERα had a normal estrogen treatment response on all measured bone parameters.
- The increase in uterus weight, as well as the decrease in thymus weight and percent fat, were similar in mice lacking methylation site R264 in ERα and control mice after estrogen treatment.

Conclusion

This *in vivo* study shows that mice lacking methylation site R264 in ERα have a normal physiological regulation as well as a normal estrogen-response in the skeleton and other estrogen-regulated tissues. Thus, in mice, methylation site R264 in ERα is dispensable for the regulation of the skeleton and several other estrogen-responsive tissues.

4.4 Paper IV

ERα expression in T lymphocytes is dispensable for estrogenic effects in bone

T lymphocytes are implicated in the estrogenic regulation of the skeleton. In this study, we evaluated whether T lymphocytes are direct target cells for the ERα-mediated bone-protective effects of estrogen. For this purpose, we used mice with T lymphocyte-specific deletion of ERα and analyzed properties of the bone.

Main results

- Deletion of ERα in T lymphocytes did not affect any of the bone parameters investigated in gonadal intact mice.
- Uterus and thymus weight did not differ between gonadal intact mice with deletion of ERα in T lymphocytes and controls.
- Deletion of ERα in T lymphocytes did not alter the bone loss after ovariectomy.
- The estrogen response was not altered by deletion of ERα in T lymphocytes in any of the evaluated bone parameters.
- The increase in uterus weight and decrease in thymus weight after estrogen treatment were similar between mice with deletion of ERα in T lymphocytes and controls.

Conclusion

Deletion of ERα expression in T lymphocytes has no impact on the skeleton or the other estrogen-responsive organs investigated. Furthermore, ERα in T lymphocytes is not required for ovariectomy-induced bone loss or for the estrogenic response in bone after ovariectomy. Thus, our data indicate that T lymphocytes are not direct target cells for the ERα-mediated bone-protective effects of estrogen.

5 DISCUSSION

Estrogen is involved in the regulation of numerous tissues including the reproductive organs, the immune system, the adipose tissue, and the skeleton (1, 2, 6, 7). Estrogen deficiency following menopause increases the risk of developing osteoporosis and is associated with increased fracture risk (2, 178). Estrogen treatment protects from bone loss but it is associated with adverse effects such as the increased risk of cancer in reproductive organs and venous thrombosis (107, 160, 178-180). Therefore, it is of importance to elucidate the mechanism behind the positive effects of estrogen versus side effects in order to aid the development of tissue-specific treatments. ERα is the main mediator of the protective effects of estrogen in bone and other estrogen-responsive organs (30-35). Therefore, the focus of this thesis was on ERα signaling pathways in bone versus other estrogen-responsive tissues.

The mechanisms behind ERα signaling are diverse and may depend on a number of conditions, such as the availability of signal transduction molecules and downstream targets, suggesting tissue-specific or cell type-specific mechanisms.

It has been shown, by our research group and others, that targeting specific domains in ERα results in tissue-specific effects. Deletion of the AF-1 domain in ERα results in a normal estrogen response in cortical bone, while trabecular bone and uterus need a functional AF-1 domain for the full effect of estrogen treatment (106, 181). ERα is widely subjected to posttranslational modifications such as phosphorylation, methylation, and palmitoylation (49-51), which affect different intracellular estrogen signaling pathways, and we have evaluated if it is possible that targeting of specific posttranslational sites also can result in tissue-specific effects. The posttranslational modifications mentioned above have been shown to modify ERα signaling mechanisms *in vitro* (49-51), and in this thesis, we have studied the importance of these posttranslational modifications *in vivo* (papers I-III).

A second approach to achieve tissue-specific estrogenic effects is to identify the target cells for the protective effects of estrogen in the bone. In paper IV we have evaluated one potential target cell, the T lymphocyte.

5.1 Membrane ERα

ERα is classically described as a nuclear receptor acting as a transcription factor and exerting effects in the cell via modulation of gene transcription. However, this nuclear signaling cannot be responsible for all functions of ERα in cells. The first evidence of a "non-nuclear" signaling pathway was already discovered 50 years ago with rapid responses to estrogen treatment in rat uterus (41).

The first demonstration *in vivo* that non-nuclear estrogen signaling can have tissue-dependent effects came from studies using estrogen dendrimer conjugate (EDC), which is a large molecule with several estrogens attached (182-184). EDC cannot enter the nucleus and thereby enables studies of non-nuclear estrogenic effects. Bartell *et al.* demonstrated that EDC treatment prevents cortical bone resorption caused by estrogen deficiency in female mice, while it has no effect on uterine growth (185). Similarly, our research group recently showed that EDC treatment increases cortical but not trabecular bone mass in male mice (186). Thus, several studies using EDC have shown that non-nuclear ERα signaling is important for the skeleton and other tissues (183, 185-188). However, studies using EDC cannot answer the question whether the estrogen receptors mediating these non-nuclear effects are situated in the cytosol or in the plasma membrane. Studies in the 1970s showed that estrogen can bind to the plasma membrane, suggesting a membrane-associated estrogen receptor, and later studies have confirmed that ERα can bind to the plasma membrane and exert non-genomic signaling (43, 189, 190).

In vitro studies have shown that cysteine at site 451 (C451) in ERα is a site for palmitoylation. Palmitoylation is the attachment of a palmitic acid to an amino acid, and in ERα this posttranslational modification enables binding of the receptor to caveolin-1 and subsequent membrane localization (49, 191, 192). In order to study the effects of membrane-localized ERα *in vivo*, we have used a mouse model with a point mutation leading to an amino acid shift at site 451 from cysteine to alanine. Alanine cannot be palmitoylated and membrane localization of ERα is thereby blocked (56).

The first study using mice that lack mERα showed that the absence of mERa leads to infertility and disturbed sex steroid levels in female mice (56). The disturbed sex steroid levels were confirmed in our study (paper

I) (193), and these data demonstrate that mERα signaling is required for normal sex steroid feedback regulation. Increased sex steroid levels can have an impact on several organs via activation of the androgen receptor and/or ERβ. To eliminate this confounding factor we have chosen to evaluate the role of mERα in ovariectomized mice treated with E2.

Skeletal analysis showed that mERα signaling is involved in the estrogenic regulation of both trabecular and cortical bone in ovariectomized female mice treated with E2 (paper I) (193). These data were confirmed by another study using a separate mouse model with the same point mutation (194). The fact that EDC treatment only affects cortical bone, while the loss of mERα affects both the trabecular and the cortical bone compartment, suggests that trabecular bone effects require nuclear estrogen receptor signaling and that this signaling is dependent on a functional mERα. Interestingly, we found that mERα signaling is crucial for the effects of E2 in trabecular bone in the axial skeleton, while trabecular bone in the appendicular skeleton is only partly dependent on mERα. Thus, ERα signaling may differ depending on the skeletal site.

Estrogen decreases fat mass through ERα signaling (34, 148). In paper I, we have shown that E2 effects in adipose tissue are mainly mediated via nuclear ERα (nERα) signaling, since there were no significant differences in E2 response between female mice lacking mERα and controls. In contrast, Pedram *et al.* suggested, in a study using intact mice lacking mERα and mice with an ERα variant only situated in the membrane, that both the membrane and nuclear ERα are important for suppression of adipogenesis (195). Due to this conflicting results, the relative role of mERα and nERα signaling for effects on adipose tissue needs further investigation. Another estrogen-responsive organ is the liver, and we found that the increase in liver weight was similar between mice lacking mERα and wild type controls after E2-treatment, demonstrating that the E2 response in this tissue is mERα independent.

The role of mERα signaling for the response to E2 treatment in different estrogen-responsive tissues has also been evaluated in male mice (196). In male mice, both cortical and trabecular bone were shown to be partly dependent on mERα (196). Furthermore, we found the response to E2 treatment in thymus to be partly dependent on mERα in both males and females (193, 196). In contrast, the estrogenic response in adipose tissue was dependent on mERα in males, while it was mERα independent in

females. Thus, the estrogenic regulation of fat mass shows gender differences.

It is clear that nuclear ERα signaling interacts with non-nuclear ERα signaling and we show for the first time *in vivo* that mERα is important for several different estrogen-responsive tissues. mERα shows a tissue-dependent pattern in female mice with estrogen-responsive tissues that are i) strongly mERα dependent (trabecular bone in the axial skeleton), ii) partly mERα dependent (e.g. thymus, uterus, and bone in the appendicular skeleton), and iii) mERα-independent (adipose tissue and liver) (Figure 10). These findings may have clinical relevance since targeting mERα signaling will result in estrogenic responses in some, but not all, tissues.

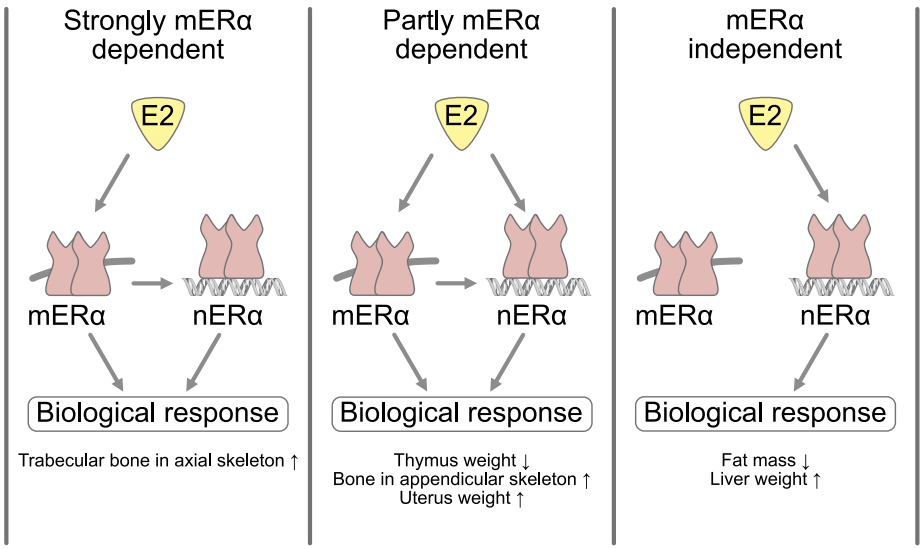

Figure 10: Overview of the tissue dependent role of mERα. ↑= estrogen increases, ↓ = estrogen decreases, nERα = nuclear ERα, mERα = membrane ERα, E2 = estradiol.

5.2 Phosphorylation of ERα

Phosphorylation site S122 is located in the AF-1 domain. Therefore, we evaluated if the phosphorylation site S122 in ERα is involved in the tissue-specific effects mediated by AF-1 (paper II). Furthermore,

phosphorylation of ERα has been shown to modulate both ligand and ligand-independent activation of the receptor and to affect gene transcription (59, 62). In addition, protein stability, hormone sensitivity, and nuclear localization are affected by phosphorylation of ERα (57-61, 63, 64). *In vitro* studies have shown that phosphorylation site S118 in ERα (corresponding to S122 in mice) affects estradiol-induced gene transcription in some but not all cell types, indicating a tissue-specific role of the phosphorylation (60, 62, 65, 66).

In paper II, we investigated whether the phosphorylation at site S122 in ERα has an impact on estrogen-regulated tissues *in vivo*. We clearly demonstrate that phosphorylation site S122 in ERα is not involved in the normal regulation of bone mass in mice. We also show that the regulation of evaluated immunological parameters, including thymus weight, thymus cellularity, frequency of B and T lymphocytes in bone marrow as well as T lymphocyte development in the thymus, were unaffected by lack of phosphorylation at site S122 in ERα.

Interestingly, when analyzing the body weight, we observed that mice incapable of being phosphorylated at site S122 in ERα had increased body weight. The increased body weight was due to increased fat mass, while lean mass was unaffected, and we also observed increased levels of leptin in serum. Leptin is secreted by adipocytes and fat mass correlates directly with leptin levels (197). Furthermore, insulin levels were increased in mice lacking phosphorylation site S122 in ERα as compared to control mice. Thus, we show that phosphorylation of site S122 in ERα has a tissue-dependent role and is required for normal regulation of fat mass and glucose metabolism in female mice, while other estrogen-responsive tissues do not require phosphorylation at this site. Importantly, we demonstrate for the first time *in vivo,* the importance of a specific phosphorylation site in a transactivation domain in a nuclear steroid receptor.

Increased knowledge regarding the importance of phosphorylation site S122 *in vivo* may contribute to the development of tissue-specific estrogenic drugs that can be used in various estrogen-associated diseases.

5.3 Methylation of ERα

Methylation of proteins has been shown to affect apoptosis, cell differentiation, signaling transduction, and subcellular localization of proteins (67-69). *In vitro* studies of human ERα have shown that methylation of site R260 is associated with cytoplasmic localization of the receptor (51). Furthermore, *in vitro* studies have also shown that methylation of site R260 in ERα is required for activation of the PI3K signaling pathway and this pathway has been shown to be involved in the regulation of osteoblasts (51, 198, 199). Thus, these *in vitro* data suggest that methylation of site R260 might affect the regulation of the skeleton. However, our *in vivo* results (paper III) show that methylation site R264 in the murine ERα is not involved in the regulation of the skeleton or any of the other evaluated estrogen-responsive tissues; uterus, thymus, and adipose tissue. The negative data was unexpected, considering the effects earlier demonstrated on intracellular signaling in the *in vitro* studies. However, this result still gives us one new piece in the large puzzle of estrogen signaling mechanisms. Furthermore, paper III demonstrates the importance of *in vivo* studies to validate the findings from *in vitro* studies.

5.4 Cell-specific deletion of ERα

In addition to the studies on estrogen signaling pathways, we have also evaluated a possible target cell for the protective effects of estrogens in bone.

There is a close relationship between the immune system and bone, e.g. osteoclasts develop from hematopoietic stem cells, which also give rise to the immune cells, and the immune cells are mainly produced in the bone marrow in close contact to the trabecular bone (200). In addition, several of the cytokines enhancing or suppressing osteoclastogenesis and osteoblastogenesis are secreted by immune cells (129, 131, 173, 201-203). Multiple studies have shown that mice lacking T lymphocytes are protected from ovariectomy-induced bone loss (131, 132, 204, 205). Furthermore, estrogen deficiency caused by ovariectomy leads to increased proliferation of T lymphocyte and enhanced T lymphocyte production of tumor necrosis factor (TNF) (131, 132). The high levels of TNF increase the osteoclastogenesis that leads to increased bone resorption (206, 207). Furthermore, studies have shown that T

lymphocyte activation and T lymphocyte production are increased in postmenopausal women (208). All these studies show that T lymphocytes are involved in the estrogenic regulation of bone. Therefore, we investigated whether estrogen has a direct effect on ERα in T lymphocytes or if the involvement of T lymphocytes is indirectly mediated by estrogen signaling in other cells. In paper IV, we have shown that ERα in T lymphocytes is dispensable for the estrogenic regulation of bone mass, using a mouse model in which ERα is inactivated in all T lymphocytes. These data suggest that T lymphocytes are regulated by estrogenic effects mediated via ERα in other cell types (Figure 11).

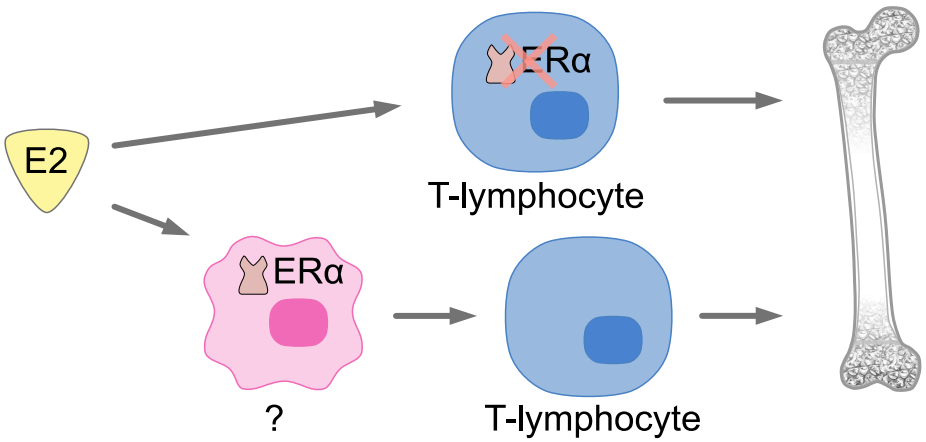

Figure 11: T lymphocytes' effects in bone are regulated by ERα in other cell types.

Henning *et al.* showed that the deletion of ERα specifically in hematopoietic cells attenuates the response to E2 treatment in both trabecular and cortical bone (126), demonstrating that ERα expression in a cell of hematopoietic origin is involved in the estrogenic regulation of bone mass. Deletion of ERα in osteoclasts affects trabecular, but not cortical, bone in females (121), and we, therefore, suggest that there is another cell type of hematopoietic origin that is involved in the effect of E2 in cortical bone. Paper IV in this thesis suggests that T lymphocytes are not direct target cells for ERα-mediated bone-protective effects of estrogen treatment, and Fujiwara *et al.* showed similar results for B

lymphocytes (130, 209). Thus, the hematopoietic target cell for the effects of E2 on cortical bone still remains to be identified.

6 CONCLUDING REMARKS

Estrogens play a critical role in the regulation of several tissues, including the immune system, adipose tissue, and the skeleton. Declining estrogen levels following menopause are associated with an increased risk of osteoporosis. Estrogen replacement treatment protects the bone but this treatment is associated with adverse effects. Thus, a better understanding of how estrogen regulates bone metabolism is important not only from a biological perspective but also in search of new tissue- or cell-specific treatment strategies.

The studies included in this thesis show that posttranslational modifications of ERα modulate estrogen signaling in a tissue-dependent manner. Importantly, we demonstrate that mERα signaling is exerting tissue-dependent effects. mERα is crucial for the trabecular bone in the axial skeleton, while uterus and bone in the appendicular skeleton are partly dependent on mERα. In addition, we show for the first time *in vivo* that phosphorylation site S122 in ERα has a tissue-specific role, crucial for the adipose tissue but dispensable for the skeleton. Methylation of site R264 is of no significance in any of the evaluated tissues. We also show that T lymphocytes is not direct target cells for the ERα-mediated bone-protective effects of estrogen.

Several approaches and avenues remain unexplored in developing targeted compounds for effective protection of bone without side effects in other tissues. However, our studies have added valuable information, since we show that certain posttranslational modifications of ERα have tissue-specific effects. Modulation of posttranslational modifications of ERα might be a possible strategy in the future to develop tissue-specific estrogen treatments.

7 FUTURE PERSPECTIVES

During the last decades, we have increased our knowledge about estrogen signaling mechanisms, however, there is still work remaining to fill the knowledge gaps in the complex process of estrogen signaling. Our finding that mERα signaling is of great importance for estrogenic effects has added a new level of complexity to the estrogen signaling mechanisms. Furthermore, by using mice lacking mERα, we have gained knowledge that is important for the development of tissue-specific estrogen treatments. Further studies evaluating the importance of mERα signaling is of interest, and there are several questions to be answered. What is the role of mERα signaling in specific cell types? Is mERα signaling involved in the tissue-specific effects of current SERMs? What is the relative importance of mERα signaling and nERα signaling in different tissues? If we can answer these questions we will gain knowledge useful in the development of tissue-specific estrogen treatments with positive effects in the skeleton as well as other tissues.

Our novel finding that phosphorylation site S122 in ERα modulates estrogen signaling in a tissue-dependent manner suggests that this site might be a possible treatment target. We have shown that normal regulation of adipose tissue and insulin levels in mice needs functional phosphorylation at site S122. Thus, targeting this site might be important for the development of future treatments of metabolic disorders related to estrogen signaling. Furthermore, *ex vivo* studies in human breast cancer tissues have shown that phosphorylation at site S118 (corresponding to mice S122) of ERα can be an indicator of response to endocrine therapy e.g. tamoxifen. It would, therefore, be of great interest to use our model to study the significance of lack of this phosphorylation site for cancer development in the mammary gland *in vivo*.

In this thesis, we show that T lymphocytes are not direct target cells for the ERα-mediated estrogenic response in bone. Therefore, further studies evaluating which other cell types of hematopoietic origin that is responsible for the estrogenic effects in the bone are needed. A possible candidate cell to evaluate might be the megakaryocyte since megakaryocytes have been shown to be involved in the regulation of bone metabolism and are regulated by estrogen (210-213).

8 RELATED PUBLICATIONS NOT INCLUDED IN THE THESIS

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9 ACKNOWLEDGEMENTS

När jag sökte min tjänst för att doktorera fick jag tips om att Marie var toppen som handledare och att Claes Ohlssons grupp var väldigt duktiga, inspirerande och roliga att arbeta med. Så rätt det var! Arbetet fram till denna bok har varit så roligt och lärorikt.

Marie, min roliga, underbara och bästa handledare! Tänk vad du har lärt mig mycket. Du har från dag ett gett mig förtroende att lösa saker på egen hand samtidigt som du alltid har funnits där och svarat på alla mina frågor i tid och otid. När jag vimsar räddar du upp och när du vimsar räddar jag upp, så bra team vi varit!

Min bihandledare och gruppledare, **Professor Claes**, så mycket kunskap du har och sån inspiration du ger med alla dina idéer. Du hittar alltid den röda tråden som leder projekten framåt till det bästa. Sen att du är lika tävlingstokig som jag gör ju bara att alla utmaningar blir ännu roligare. Kanske att jag kan tänka mig att göra ett till Vasalopp?

Min sprudlande bihandledare **Cecilia**, du sprider så mycket glädje omkring dig. Du är en sån inspiration och bevis på att man aldrig ska ge upp.

Alla fantastiska kollegor i korridoren på Vita stråket och även ni utanför korridoren. Det är så härligt att ha så kloka, snälla och inspirerande människor runt mig hela dagarna. **Maria B**, du tog emot mig som ny doktorand med öppna armar, trygg och stöttande. Så glad att ha dig som vän. **Karin**, så roligt att du började doktorera i samma grupp som jag. Att få ha min fina vän även som kollega och rumskamrat har betytt obeskrivligt mycket för mig. **Petra, Sofia** och **Anna W**, våra lunchträningar gör oss starka i kropp å själ. Till våren springer vi upp till vår utsiktsplats i solen igen! **Anna W** vill även passa på att tacka dig för din grymma matchmake! Du besitter många kvalitéer utöver spjutspetsforskningen. Alla doktorandkollegor, ni som redan har er doktorstitel, **Jianyao, Vikte** och **Joel** och ni som snart är där, **Lina, Hannah, Jimmy, Daniel C, Maria N** och **Maria N**. Det har varit ett sant nöje att dela denna tid med er. Ett extra tack till **Helen** min närmsta doktorandkollega. Tack till **Louise, Klara, Jenny, Liesbeth, Anna T, Malin, Edina**, **Johan, Sara, Anna-Karin, Henrik, Maha, Daniel H, Anna E, Thomas, Matti,** och **Ulf** för goda råd och trevligt sällskap. Ni som jobbar och har jobbat på labbet, **Anna W, Ulrika B, Andreas, Ava,**

Biljana och **Lotta**, hur skulle det sett ut här utan er?! **Lotta**, jag sa att du inte fick gå i pension innan jag var klar, jag var för långsam… Tack för allt du lärt mig på labbet! Tack till **Anette**, för alla ben du snurrat i CT:n. Önskar att du hade fått vara med oss fler jordsnurr, du är saknad. För att rodda runt allt som vi ställer till med och inte ställer till med krävs det en bra administration. När jag började var det **Anna-Lena** som styrde med hela handen, sen kom **Veronica** och **Maria U**. Nu har vi **Lisa** och **Mirela** på plats, så bra ni är.

Tack till alla medförfattare och till er som korrekturläst min avhandling.

E2-gruppen, tack för att ni delat med er av expertisen ni besitter inom immunsystemet, reumatiska sjukdomar och FACS.

Det första jag gjorde innan jag ens börjat min tjänst var att jag fick följa med på kurs i Hemavan, så fantastiskt att få starta mitt doktorerande på det viset. De välorganiserade **CORS**-kurserna och möten fortsatte, Åre, Barcelona, Stockholm och Marstrand. Förutom all vetenskap jag fått med mig under dessa möten har jag också lärt känna ben-forskare runt om i landet, inte minst er på Mölndals sjukhus. **Daniel**, vågar du utmana mig i en löptävling snart? Inte nog med att jag fick åka till Hemavan innan jag börjat, första veckan på jobbet var det **CEROSS**-möte ute på Marstrand. Lyx-doktorand!

Djurskötarna, **Marcus** och **Elin**, tack för att ni tagit så väl hand om mina möss och för alla roliga pratstunder i djurrummet.

Alla fantastiska vänner som förgyller mitt liv utanför jobbet. **Apotekar-gänget**, det är så roligt att vi fortfarande umgås, luncherna i korridoren på Medicinareberget har bytts ut mot lite (eller mycket) godare mat i mysigare miljöer. **Hästgänget**, ni kanske inte har förstått vad jag pysslat med på jobbet, men ändå lyssnat tålmodigt på mitt pladder. Nu ska jag bli doktor och det ska firas med fest. Det är ni bäst på! **Onsdagsklubben**, lill-lördag och vad är väl bättre än att träffa er, mina fantastiska vänner. Ni är så kloka, snälla och bra på att laga mat är ni också.

Mamma och **pappa**, jag hade inte klarat mig utan er! Ni är de bästa föräldrarna ever! Ni har alltid trott på mig och pushat när jag tvekat. Städat upp i oredan som jag ibland lämnar efter mig. Att få ha ett så kärleksfullt hem där jag alltid kan landa betyder så mycket. **Elin** och **Moa** mina älskade systrar! Vad vore livet utan er? Ni finns alltid där och det känns så tryggt att veta. Tack för alla gånger ni följt med mig på

diverse upptåg, tagit hand om mina hästar och hejat på mig när jag sprungit tokiga lopp. Mosters busungar, **Adam** och **Oliver**, så tråkigt det hade blivit utan era små hyss…

Simon, för ett år sedan sprang du in i mitt liv, tänk så bra det blev. Tack för all hjälp och pepp den sista tiden när jag bara skrivit. Snart ska du få en springtur i skogen med din Doktor Karin. Du är den finaste människa jag mött, jag älskar dig! **Alva** och **Axel**, de sötaste små barnen som jag fick på köpet. Så roligt att få dela livet med er.

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