The role of estrogen receptor α in the regulation of bone mass

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In memory of my father

Science and knowledge bring peace and calmness, so choose to live in a place with knowledgeable people.

Ferdowsi, the Persian poet (c. 940- 1020)

به زنده یاد پدرم

نگه کن به جایی که دانش بود

ز داننده کشور به رامش بود

"فردوسی"

The role of estrogen receptor α in the regulation of bone mass

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ABSTRACT

Estrogens are major regulators of skeletal growth and maintenance in both females and males. Estrogen receptor α (ERα) is the main mediator of estrogenic effects in bone. Thus, estrogen signaling via ERα is a target for treatment of estrogen-related bone diseases including osteoporosis. However, treatment with estrogen leads to side effects in both genders. The aim of this thesis was to characterize different ERα signaling pathways in order to increase the knowledge regarding the mechanisms behind the protective effects of estrogen on bone mass versus adverse effects in other organs.

We have evaluated the role of ERα expression in two distinct hypothalamic nuclei. Female mice lacking ERα expression in proopiomelanocortin (POMC) neurons, mainly found in the arcuate nucleus, displayed substantially enhanced estrogenic response on cortical bone mass while lack of ERα in the ventromedial nucleus revealed no effects on bone mass. We therefore propose that the balance between inhibitory effects of central ERα activity in hypothalamic POMC neurons and stimulatory peripheral ERα-mediated effects in bone determines cortical bone mass in female mice.

We have also evaluated the role of ERα signaling pathways in males. We found that the ERα activation function (AF)-2 was required for the estrogenic effects on all evaluated parameters. In contrast, the role of ERαAF-1 was tissue specific, where trabecular bone was dependent on ERαAF-1, while effects on cortical bone did not require ERαAF-1. In addition, all evaluated effects of the selective estrogen receptor modulators (SERMs) were dependent on a functional ERαAF-1.

In addition to nucleus, ERα is also located at the plasma membrane, where it can initiate extra-nuclear signaling. We found that extra-nuclear ERα signaling affects cortical bone mass in males and that this effect is dependent on a functional ERαAF-1. To further determine the role of membrane-

initiated ERα signaling, we used a mouse model lacking an ERα palmitoylation site, which is crucial for membrane localization of ERα. We showed that membrane ERα signaling is essential for normal development and maintenance of trabecular and cortical bone, and is crucial for normal estrogen response in both trabecular and cortical bone in male mice.

The studies presented in this thesis have increased our knowledge regarding estrogen signaling pathways in both females and males and may contribute to the design of new, bone-specific treatment strategies that maintain the protective effects of estrogen but minimize the adverse effects.

Keywords: estrogen receptor α, bone, estrogen

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Östrogen receptor alphas betydelse for reglering av benmassa

Benskörhet (osteoporos) är en av de stora folksjukdomarna i Sverige och orsakar mycket lidande och stora kostnader för samhället. Östrogener tillhör de viktigaste hormonerna som reglerar tillväxt och upprätthållande av skelettet både hos kvinnor och män, och behandling med östrogener minskar risken för osteoporos. Östrogenernas effekter medieras främst via östrogenreceptor alfa (ERα), vilket gör östrogensignalering via ERα ett mål för behandling av östrogenrelaterade sjukdomar såsom osteoporos. Behandling med östrogener kan ge biverkningar i båda könen. Syftet med denna avhandling var därför att studera ERαs signalering och öka kunskapen om mekanismerna bakom de benskyddande effekterna av östrogener. Detta för att underlätta framtagande av benspecifika östrogenlika behandlingsalternativ mot benskörhet med mindre biverkningar.

Vi har utvärderat betydelsen av ERα i två distinkta hypotalamuskärnor hos honmöss. Honmöss som saknar ERα i POMC-neuron, som huvudsakligen finns i arkuatuskärnan, visade kraftigt ökad östrogenrespons i kortikalt ben. Honmöss som saknar ERα i ventromediala kärnan visade däremot ingen påverkan på benmassan. Utifrån dessa studier föreslår vi att balansen mellan de hämmande effekterna av central ERα-aktivitet i POMC-neuronen i hypotalamus och de stimulerande effekterna av perifer (lokal) ERα-aktivitet i ben är viktig för regleringen av kortikal benmassa hos honmöss.

Vi har också utvärderat betydelsen av ERα-signalering hos hanmöss. Vi visar att ERαs aktiveringsfunktion (AF)-2 är nödvändig för östrogenernas effekter i alla vävnader vi utvärderat hos hanar. Däremot är betydelsen av ERαAF-1 vävnadspecifik, där den krävs för östrogenernas effekter på trabekulärt ben, men inte på kortikalt ben. Vi har även visat att AF-1-delen av ERα krävs för att de selektiva östrogenreceptormodulerare (SERMs) som utvärderats (Raloxifene, Lasofoxifene, Bazedoxifene) ska ha effekter på skelettet.

ERα finns inte bara i kärnan och cytosolen utan också i plasmamembranet, där receptorn kan initiera extranukleär signalering. Vi har visat att extranukleär ERα-signalering påverkar kortikal benmassa i hanar och att denna effekt är beroende av AF-1 delen av ERα. För att ytterligare utvärdera betydelsen av membraninitierad ERα-signalering har vi använt en musmodell som saknar ett palmitoyleringssite i ERα. Palmitoyleringen är nödvändig för att ERα ska kunna lokalisera sig till plasmamembranet. Vi visar att

membraninitierad ERα-signalering är viktig for normal utveckling och upprätthållande av både trabekulär och kortikal benmassa, och också för en normal östrogenrespons i både trabekulärt och kortikalt ben hos hanar.

Resultaten som presenteras i denna avhandling har ökat vår kunskap om östrogensignalering hos både hanar och honor. Detta kan bidra till design av nya benspecifika behandlingsalternativ som har benskyddande östrogena effekter, men som ger färre biverkningar.

LIST OF PAPERS

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

I. **Farman HH**, Windahl SH, Westberg L, Isaksson H, Egecioglu E, Schele E, Ryberg H, Jansson JO, Tuukkanen J, Koskela A, Xie SK, Hahner L, Zehr J, Clegg DJ, Lagerquist MK, and Ohlsson C.

   Female mice lacking estrogen receptor-α in hypothalamic proopiomelanocortin (POMC) neurons display enhanced estrogenic response on cortical bone mass

   Endocrinology, 2016. 157(8): p. 3242-52.

II. Börjesson AE, **Farman HH**, Engdahl C, Koskela A, Sjögren K, Kindblom JM, Stubelius A, Islander U, Carlsten H, Antal MC, Krust A, Chambon P, Tuukkanen J, Lagerquist MK, Windahl SH, and Ohlsson C.

   The role of activation functions 1 and 2 of estrogen receptor-α for the effects of estradiol and selective estrogen receptor modulators (SERMs) in male mice

   Journal of Bone and Mineral Research, 2013. 28(5): p. 1117-26.

III. **Farman HH**, Wu J, Gustafsson KL, Windahl SH, Kim SH, Katzenellenbogen JA, Ohlsson C, and Lagerquist MK.

   Extra-nuclear effects of estrogen on cortical bone in males require ERαAF-1

   Journal of Molecular Endocrinology, 2017. 58(2): p. 105-111.

IV. **Farman HH**, Gustafsson KL, Henning P, Grahnemo L, Lionikaite V, Movérare-Skrtic S, Wu J, Ryberg H, Koskela A, Tuukkanen J, Levin ER, Ohlsson C, and Lagerquist MK.

   Membrane estrogen receptor-α is essential for estrogen signaling in the male skeleton

   Journal of Endocrinology, 2018. 239(3): p. 303-312.

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ABBREVIATIONS

The frequently (more than three times) used abbreviations are listed below.

AF-1	Activation function-1
AF-2	Activation function-2
AAV	Adeno-associated virus
aBMD	Areal bone mineral density
Bza	Bazedoxifene
BMD	Bone mineral density
CNS	Central nervous system
CTx	Collagen c-telopeptides
CHD	Coronary heart diseases
DHT	Dihydrotestosterone
DEXA	Dual energy Xray absorptiometry
ELISA	Enzyme-linked immunosorbent assay
EDC	Estrogen dendrimer conjugate
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
ERs	Estrogen receptors
FTIR	Fourier transform infrared microspectroscopy
GC-MS/MS	Gas chromatography-tandem mass spectrometry
HRT	Hormone replacement therapy

KO	Knockout
Las	Lasofoxifene
MC4R	Melanocortin 4 receptor
mERα	Membrane-initiated ERα
MISS	Membrane-initiated steroid signaling
μCT	Micro computed tomography
NOER	Nuclear only ER
orx	Orchidectomized
OPG	Osteoprotegerin
ovx	Ovariectomized
pQCT	Peripheral quantitative computed tomography
Ral	Raloxifene
RANKL	Receptor activator of nuclear factor- κB ligand
SERM	Selective estrogen receptor modulator
shRNA	Short hairpin RNA
VMN	Ventromedial nucleus
vBMD	Volumetric bone mineral desity
WHI	Women’s Health Initiative
WT	Wild type

1 INTRODUCTION

1.1 GENERAL INTRODUCTION

Osteoporosis is a condition characterized by low bone mass and microarchitectural deterioration of bone tissue leading to increased risk of fracture (1, 2). Estrogens are the major hormonal regulators of skeletal growth and maintenance in both females and males (3). Estrogen receptor α (ERα) mediates estrogen effects in bone and other tissues (4-11). Thus, estrogen signaling via ERα is a target for treatment of bone diseases including osteoporosis. Estrogen treatment results in positive estrogenic effects in bone, but also adverse effects in other organs of both genders (5, 12-20). Thus, it would be beneficial to develop bone-specific estrogen treatments, which mimic the positive effects in bone and avoid the side effects. To achieve this, we need to increase our knowledge about the mechanisms behind estrogen effects in bone and other organs. In this thesis, we characterize different ERα signaling pathways in bone versus other tissues *in vivo*.

1.2 BONE

The skeleton protects internal organs and supports body movement. Moreover, bone stores minerals such as calcium and phosphates and is the location for hematopoiesis. The human skeleton contains over 200 bones. Bone tissue consists of 70% inorganic components (i.e. mineral crystals), 20% organic components (i.g. type I collagen), and 5-8% water. The skeleton is commonly divided into two major categories, the axial and the appendicular skeleton. The axial skeleton consists mainly of flat bones (ribs, skull, and sternum) and vertebrae, while the appendicular skeleton consists mainly of long bones (e.g., tibia, femur, and humerus).

1.3 CORTICAL AND TRABECULAR BONE

The skeleton consists of two types of bone tissue: the cortical or compact bone and the trabecular or spongy bone, also called cancellous bone. Cortical bone, the harder outer shell of bone, is stiffer and more compact than trabecular bone. Cortical bone makes up 80% of the bone tissue and is mainly found in the shaft of long bones (diaphysis). The spongy-like trabecular bone comprises the remaining 20% of the bone tissue and is predominantly found

in the vertebrae, pelvis, and in the metaphysis and epiphysis of the long bones (Figure 1).

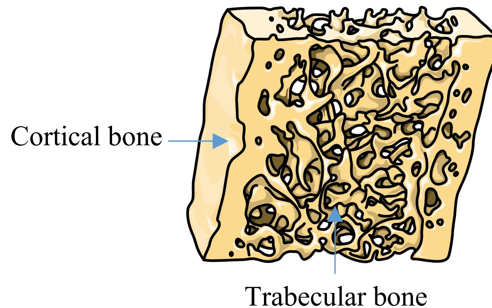

Figure 1. Cortical and trabecular bone.

1.4 STRUCTURE OF LONG BONES

The diaphysis – the shaft of long bones – is composed of cortical bone surrounding the marrow cavity. The very ends of long bones are called epiphyses and the region between the epiphyses and diaphysis are called metaphyses. These two regions consist of trabecular bone surrounded by cortical bone. The growth plate separates the epiphysis and metaphysis (Figure 2).

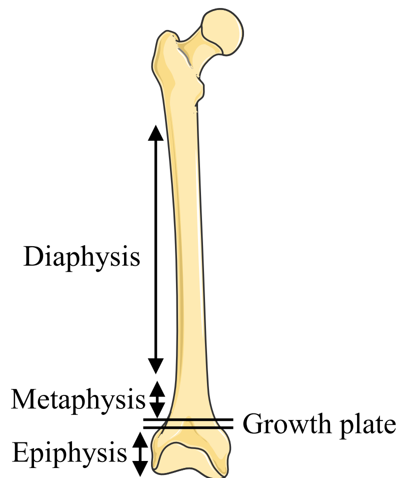

Figure 2. Schematic drawing of a femur.

1.5 BONE CELLS

There are three types of cells found in bone: osteocytes, osteoblasts, and osteoclasts.

1.5.1 OSTEOCYTES

The most abundant bone cells in the adult skeleton are osteocytes (90-95%) that are generated from osteoblasts (21). Osteocytes are long-lived and do not divide (22). Osteocytes are located in lacunae and form a network with each other via dendritic extensions called canaliculi, a place for nutrition and signaling molecule exchange. This network makes osteocytes able to detect mechanical pressure and load and they thereby regulate bone remodeling through different mechanisms including regulation of osteoblast and osteoclasts differentiation and function (22, 23).

1.5.2 OSTEOBLASTS

Osteoblasts account for approximately 4–6% of the cells in the adult human skeleton (22). Osteoblasts differentiate from mesenchymal stem cells and they are responsible for bone formation. Bone morphogenetic proteins (BMPs), transforming growth factor β (TGFβ), and wingless-type MMTV integration site family (WNT) are important growth factors involved in osteoblasts differentiation (24-26). The Runt-related transcription factor 2 (Runx2) and Osterix (Osx1) are two key transcription factors that are essential for osteoblast differentiation (27).

Osteoblasts secrete different bone proteins, including collagen, that are main components of the unmineralized bone matrix (osteoid). Proteins produced by osteoblasts, including collagen type I, alkaline phosphatase (ALP), and osteocalcin (OC) can be analyzed in serum or urine as a measurement of osteoblast activity (28-30).

The life-time of osteoblasts is approximately three months (31). When osteoblasts age, they face three possible destinies: 1) undergo programmed cell death (apoptosis), 2) become embedded in the bone as osteocytes, or 3) become lining cells (21). The lining cells are flat and cover the surface of the bone.

Osteoblasts secrete both receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) (32). RANKL induces osteoclast activation, while OPG binds to RANKL and thereby inhibits osteoclasts activation (Figure 3). Estrogen interferes with RANK signaling and

upregulates the expression of OPG (33, 34). Thus, estrogen deficiency leads to increased bone resorption via increased RANKL-signaling (35).

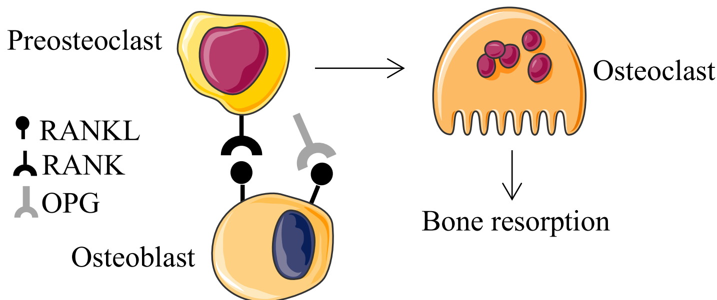

Figure 3. The RANKL/OPG system. RANKL binds to RANK and induces osteoclast differentiation. OPG can inhibit this interaction by binding to RANKL and thereby prevents osteoclast differentiation. *This illustration was adapted with permission from Associated Professor Marie Lagerquist.*

1.5.3 OSTEOCLASTS

Osteoclasts, the least abundant bone cell type (1-2%), are responsible for resorbing bone. When an osteoclast attaches to the bone surface, it forms a ruffled border and creates an acidic microenvironment that leads to bone resorption. Osteoclasts undergo apoptosis and are removed by phagocytes after their two weeks life-span (36). Osteoclasts originate from hematopoietic stem cells through fusion of several mononucleated cells, which results in large multinucleated osteoclasts. Osteoclast differentiation depends on macrophage colony stimulating factor (M-CSF) and RANKL (37). M-CSF stimulates the proliferation of osteoclasts by binding to c-fms receptors on preosteoclasts (38). RANKL binds to its receptor RANK on preosteoclasts and osteoclasts and this binding is essential for proliferation, survival and activation of osteoclasts (39).

1.6 BONE REMODELING

Bone remodeling is a constantly ongoing process in which osteoclasts resorb bone and osteoblasts form new bone. Through bone remodeling, the skeleton repairs micro-cracks and other damages, responds and adapts to mechanical loading, and maintains calcium homeostasis (40, 41). The bone resorption and formation processes in bone remodeling occurs as a cyclic event in both cortical and trabecular bone. This process occurs in basic multicellular units

(BMUs), consisting of all bone cell types (osteoclasts, osteoblasts, and osteocytes), lining cells, and blood supply (42).

The bone remodeling cycle begins with recruitment of preosteoclasts to the bone surface, where they fuse and become mature osteoclasts (Figure 4). The osteoclasts resorb the bone by digesting the bone matrix at the resorption site. When the resorption-phase ends, preosteoblasts migrate to the resorption site and differentiate into mature osteoblasts. The osteoblasts form new bone by first producing bone matrix and then mineralizing it (43, 44). Osteocytes are differentiated osteoblasts that are surrounded by bone matrix. Osteocytes form a network for communication between cells. Osteoblasts and lining cells are also connected to this network. When a mechanical load applies to bone, the network would sense it and signals to the BMUs to start the bone remodeling process. Thus, the network of bone cells is important for adapting the bone to mechanical load (45).

In humans, bone resorption takes 4–6 weeks, bone formation takes 4–6 months, and the whole bone remodeling cycle takes approximately half a year. The adult skeleton is completely regenerated every 10 years (31).

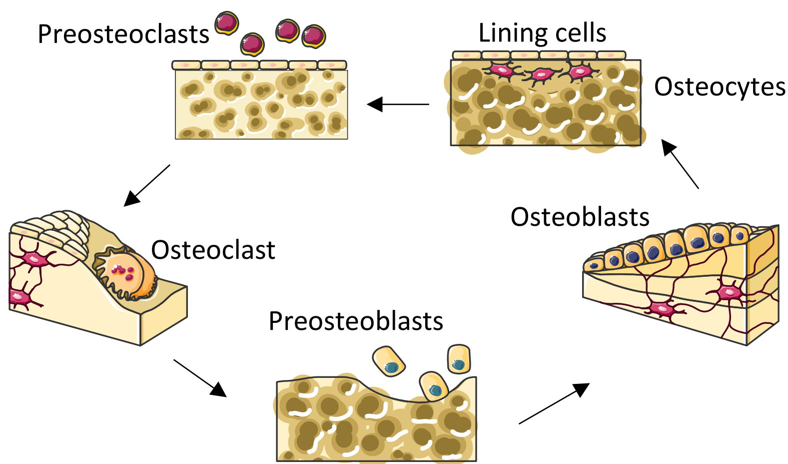

Figure 4. The bone remodeling cycle starts when preosteoclasts are recruited and then they differentiate into multinucleated osteoclasts. The osteoclasts resorb bone by digesting the mineral matrix. When the resorption-phase ends, preosteoblasts migrate to the bone resorption site and differentiate into mature osteoblasts. The osteoblasts produce bone matrix which is subsequently mineralized. Some osteoblasts are trapped in the bone matrix and differentiate into osteocytes.

1.7 ESTROGENS

Estrogens, the female sex hormones, belong to the sex steroid hormone family and they are produced both in women and men. Estrogens are pivotal hormones for survival and health in both genders. Among many crucial functions, such as glucose homeostasis, cardiovascular health, immune robustness, fertility, and neuronal function, estrogens are also essential for a healthy bone development and maintenance. Estrone, estriol, and 17β-estradiol (E2) are three different estrogens found in the physiological system. Estrone is mainly produced at extragonadal sites (e.g., adipose tissue and liver) and is present at low levels in fertile women and high levels after menopause. Estriol is produced by the placenta during pregnancy. In this thesis, we mainly focus on E2, which is the most potent estrogen (Figure 5).

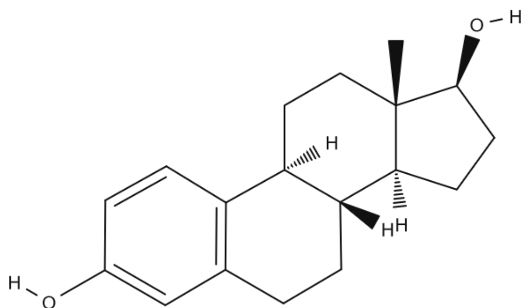

Figure 5. The molecular structure of 17β-estradiol (E2). *The image is from the chemistry database Pubchem.*

E2 is mainly produced in granulosa cells in the ovaries, but is also produced by adrenal cortex (only in humans, not in mice), adipose tissue, and testicles (via aromatization of testosterone [T]). The majority of E2 in serum is bound to sex hormone binding globulin (SHBG) in humans and is thereby unable to enter cells. However, rodents lack SHBG (46). Only 1–3% of the circulating E2 is free in the solution and biologically active. The free serum E2 levels in fertile, ovariectomized (ovx) or orchidectomized (orx), and old mice are listed below in the Table 1 (47).

E2 is a key regulator in bone metabolism via different mechanisms. It regulates the bone remodeling process by affecting bone formation and bone resorption via direct or indirect effects on osteoblasts, osteoclasts, and osteocytes (48-51).

Table 1 Serum E2 levels in pg/mL (47)

	Mouse	
	Female	**Male**
Fertile	2.7 ± 1.0	< 0.3
ovx/orx	< 0.3	< 0.3
Old age	3.6 ± 0.7	< 0.3

1.8 STRUCTURE OF ESTROGEN RECEPTORS

Estrogens, as other steroids, are lipophilic and pass over the cell membrane. E2 mainly exerts its effects via binding to nuclear estrogen receptors (ERs), ERα and ERβ. A membrane-bound G protein-coupled estrogen receptor-1 (GPER-1) has been suggested by some studies (52-54), but not all (55-58), to be a membrane-associated ER.

ERα and ERβ belong to the nuclear receptor superfamily and act as ligand-activated transcription factors. ERα and ERβ overlap in structure and have high sequence homology (Figure 6). The primary structure of these receptors consists of six different functional domains A-F (59-62). The first domain in the N-terminus is the A/B domain. This is the least conserved region (<20% homology between ERα and ERβ) and it contains the ligand independent activation function-1 (AF-1) (62). The C domain is the best conserved region between ERα and ERβ with more than 95% homology. This region contains the DNA binding domain (DBD) that is involved in dimerization of the receptor (60, 61, 63). Both ERα and ERβ can dimerize and form homo- or heterodimers. They can also bind to the same DNA sequences (53). The D domain comprises the nuclear localization signal and this domain increases the flexibility between the C- and E/F domains (60, 62).The E and F domains, found in the C terminus, contain the ligand binding domain (LBD) and the ligand dependent AF-2 (59-61). The high homology in the DNA binding domain and low homology in the ligand binding domain suggests that ERs can bind the same DNA sequences but respond differently to different ligands. ERα and ERβ have similar affinity for E2 (64).

Figure 6. Schematic picture of the different domains of ERα and ERβ, A-F. The sequence homology between the receptor is given in percent. *This illustration was adapted with permission from PhD Anna Törnqvist.*

The distribution of ERs varies, not only in different tissues but also in different bone compartments (i.e. cortical versus trabecular bone). Both receptors are expressed in trabecular and cortical bone, while ERα predominates in cortical bone (65, 66). Studies have shown that ERβ antagonizes ERα in bone and other tissues (67, 68). Higher levels of estrogen is required to affect bone remodeling in trabecular bone compared to cortical bone; a suggestion that is supported by both mouse and human studies (4, 69).

Upon E2 binding to an ER, the ER undergoes conformational changes that allows helix 12 in the LBD to fold in an agonistic orientation (70). This specific folding attracts cofactors important for gene regulation. Helix 12 is also important for ERαAF-2; hence AF-2 is ligand dependent (70-72). In contrast, AF-1 can interact with cofactors independently of ligand binding; therefore AF-1 is ligand-independent (73, 74). The ER subtype, the cell type, and the promotor context determine which cofactors that bind to the AFs and thereby regulate the gene transcription (70, 75). For full transcriptional activity, a synergism between both AFs is required (73, 75-78).

1.9 ERα-THE MAIN MEDIATOR IN BONE

The estrogen effects on bone are mediated by the two related, but distinct, receptors, ERα and ERβ (79). Studies by us and others have demonstrated that ERα is the main mediator of estrogen effects in bone (5, 8, 11). Global deletion of ERα ($ER\alpha^{-/-}$) in both genders leads to disturbed serum levels of sex hormones; high serum levels of both E2 and T in females and high serum T levels in males (80, 81). Both genders of $ER\alpha^{-/-}$ mice display decreased bone turnover, decreased cortical thickness, but increased trabecular bone mass (82). Removal of gonads (gonadectomy) leads to bone loss in wild type (WT) mice and also in both genders of $ER\alpha^{-/-}$ mice. However, E2 treatment restores neither cortical nor trabecular bone in gonadectomized $ER\alpha^{-/-}$ females or males (5, 7, 8, 83). Thus, ERα has a crucial role in mediating E2 effects in cortical and trabecular bone in both genders. In contrast to $ER\alpha^{-/-}$, $ER\beta^{-/-}$ mice of either sex have no changes in serum sex hormone levels (81). In addition, male $ER\beta^{-/-}$ mice display a normal bone phenotype, demonstrating that ERβ is not involved in regulation of bone mass in males. In contrast, $ER\beta^{-/-}$ females have been demonstrated to have increased bone mass (72, 74-75), possible due to a repressive role of ERβ on ERα-regulated gene transcription (84).

1.10 ERα TARGET CELLS

ERα is expressed both in bone cells as well as in other tissues and to determine the target cell for the effects of estrogen on the skeleton, several mouse models with cell-specific inactivation of ERα have been developed.

1.10.1 DELETION OF ERα IN BONE CELLS

Expression of ERα in different bone cells indicates that estrogen effects may be mediated locally in the skeleton. Deletion of ERα in osteoclasts leads to decreased trabecular bone due to higher osteoclast numbers and increased bone resorption in female mice (85, 86). Thus, ERα in osteoclasts is of importance for trabecular but not cortical bone in female mice, whereas it has no effect in male mice.

A number of studies have used different Cre models to delete ERα in different stages of osteoblast/osteocytes differentiation. ERα in osteoblast precursors regulate cortical bone, while ERα in mature osteoblasts/osteocytes has a moderate effect on the regulation of trabecular bone in male mice (19, 20, 87, 88). In female mice, ERα in the osteoblast lineage is crucial for cortical bone (87-89), while a role in the regulation of trabecular bone is supported by some studies but not others (87-90). Collectively, it has been suggested that ERα in mature osteoblasts contributes to the regulation of trabecular bone in female mice (20).

ERα in osteocytes is not required for cortical bone but seems to regulate trabecular bone in both genders (20, 91, 92). Thus, local ERα signaling in the skeleton has a stimulatory effect on the skeleton.

1.10.2 ERα IN THE CENTRAL NERVOUS SYSTEM

Bone is traditionally considered to be regulated by the local environment, including mechanical loading and hormones. However, it is now recognized that the central nervous system (CNS) also is involved in the regulation of bone. It has been known for long that bone is an innervated tissue containing both efferent and afferent fibers (93). The first clear evidence to define a central pathway to bone was found when studying leptin-deficient mice. These mice, despite its hypogonadism, had a high bone mass phenotype that was restored by intracerebroventricular injections of leptin, demonstrating that central leptin signaling decreases bone mass (94). In contrast, peripheral leptin treatment increases bone mass, suggesting that leptin has opposite peripheral vs. central effects on bone mass (94-96). Furthermore, the neurotransmitter serotonin has also been suggested to have opposite

peripheral and central effects on bone mass; central serotonin signaling enhances bone mass, while peripheral serotonin reduces bone mass. However, this finding has been confirmed by some but not others (97-99).

In addition to leptin and serotonin, a number of other molecules have been identified to regulate bone mass via signaling in the hypothalamus and brainstem, including neuromedin U (NMU), cocaine and amphetamine-regulated transcript (CART) (17), and neuropeptide Y (NPY) (100).

The CNS is a target for estrogen and ERα is widely distributed in the brain (17). In a previous study, using Nestin-Cre mice, our group deleted ERα in nervous tissue, which resulted in increased cortical and trabecular bone mass (101). This indicates that estrogen signaling in neuronal cells may have a negative impact on bone mass in contrast to the positive, stimulatory effects of peripheral (local) estrogen signaling. However, the primary target cell for this central inhibitory effect of estrogen on bone mass was not determined in this study. This question was addressed in paper I in this thesis (102).

1.11 ERα INTRACELLULAR SIGNALING

The ERs are transcription factors and they can bind to DNA and affect gene transcription in target cells. The ERs have four main signaling pathways, three of them are classified as ligand dependent and one is classified as ligand independent (103). The three ligand dependent pathways are: the classical (direct) genomic pathway, the non-classical (indirect) genomic pathway, and the non-genomic pathway (Figure 7).

In the classical (direct) genomic pathway, ligand and receptor binding results in ER dimerization and the ER-ligand complex then translocates to the nucleus, binds to estrogen-response elements (ERE) in the DNA and regulates gene transcription (63, 103, 104). In the non-classical (indirect) genomic pathway, the dimerized ER-ligand complex binds other transcription factors (such as activator protein 1 [AP-1], specificity protein 1 [SP-1], Fos/Jun, or nuclear factor kappa-light-chain-enhancer of activated B cells [NF-κB]) which can bind to other, non-ERE, sites (response elements [RE]) in DNA (105-107)

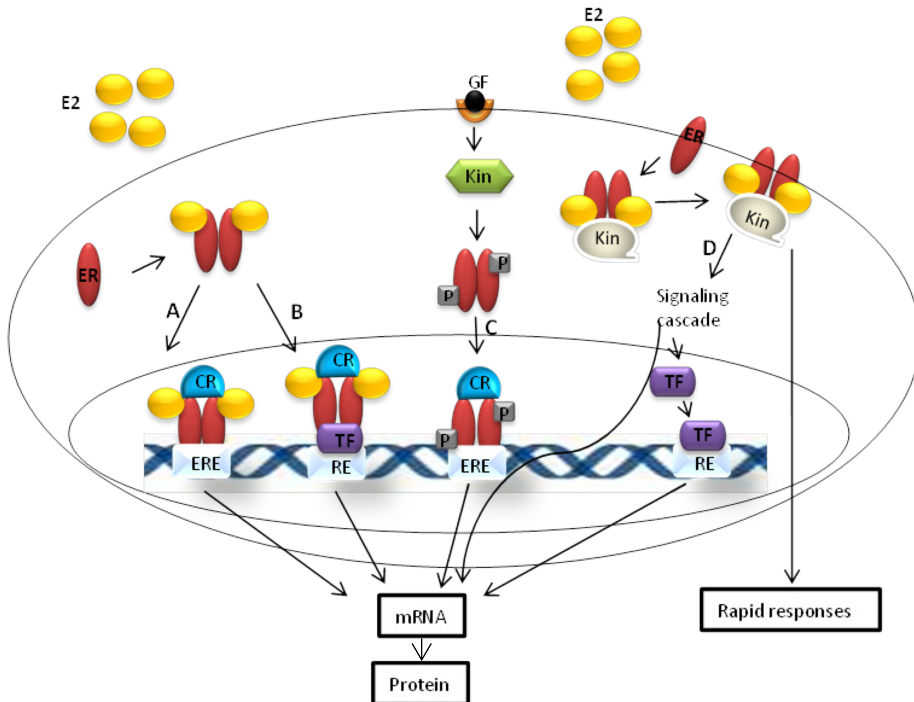

Figure 7. *Schematic picture of estrogen receptors (ER) signaling pathways. A) classical genomic pathway, B) non-classical genomic pathway, C) ligand-independent pathway, D) membrane-initiated signaling pathway. ERE; estrogen response element, RE; response element, TR; transcription factors, CR; coregulators, P; phosphorylation, Kin; kinase.*

In addition to genomic pathways, ERs can elicit non-genomic (also called extra-nuclear) signaling. In contrast to the genomic pathways, non-genomic signaling responses occur rapidly (second to minutes). Examples of E2-induced rapid cell responses include rapid mobilization of intracellular calcium, generation of cyclic adenosine monophosphate (cAMP), modulation of potassium currents, phospholipase C activation, and stimulation of protein kinase pathways (e.g., phosphatidylinositol-4,5-bisphosphate 3-kinase [PI3K]/protein kinase B [PKB or Akt], and extracellular signal-regulated kinase [Erk]) (108-114). ERα has been shown to be the primary endogenous mediator of these rapid E2 actions (115) and a subpopulation of ERα that is present at or near the plasma membrane has been shown to be important for these rapid non-genomic effects (115). This subpopulation of ERα can start membrane-initiated steroid signaling (MISS), which influences intracellular signaling cascades. This pathway can either have direct cellular effects without affecting gene transcription or it can lead to recruitment of

transcription factors to the nucleus and thereby alter gene transcription (110). Cross-talk between genomic pathways and MISS can also occur, where a signal from one pathway can modulate the signal from another pathway (116) and this cross-talk has been suggested to be of importance in some contexts (117).

Palmitoylation, a post-translational modification, is the attachment of a palmitic acid to a cystein residue (118). The palmitoylation site Cys447 in the human ERα promotes plasma membrane association of the receptor (119). Mutation of this palmitoylation site in ERα in mice inhibits the membrane localization of ERα and thus provides a tool to evaluate the importance of MISS (120, 121). In paper IV of this thesis, we have used this tool to determine the role of MISS for bone mass in male mice.

ERs can also signal via a ligand-independent pathway, where other factors (such as growth factors [GFs] like epidermal growth factors [EGF] and insulin-like growth factor-1 [IGF-1]), bind to their receptors and activate various kinases including mitogen-activated protein kinase (MAPK) that in turn activates the ERs by phosphorylation. The phosphorylated ERs can translocate to the nucleus where they recruit other coactivators and bind to DNA and thereby regulate gene transcription (103, 122-126). However, ER phosphorylation does not only occur in the absence of ligand, E2 treatment can also stimulate phosphorylation of ERα. ERα has several phosphorylation sites which are mainly located in the AF-1 domain (103, 125).

1.12 OSTEOPOROSIS

Osteoporosis is characterized by low bone mineral density (BMD) and structural deterioration of bone, which results in increased risk of fractures (1). Osteoporosis is defined as being primary or secondary. Primary osteoporosis is a progressive bone loss due to aging and is influenced by decline in sex hormone levels or genetic factors. Secondary osteoporosis represents the bone loss due to a primary disease, such as rheumatoid arthritis or as a side effect due to medication, e.g. glucocorticoid therapy (127). Osteoporosis is clinically diagnosed by BMD measurements at lumbar spine, femoral neck, and total hip with dual energy Xray absorptiometry (DEXA). In 1994, the World health organization (WHO) defined the diagnosis osteoporosis as BMD less than minus 2.5 standard deviations of the mean of a population of young adult women (128, 129).

The term “post-menopausal osteoporosis” was first used by Fuller Albright in 1940. He noted the association between decline of estrogen levels following

ovariectomy or menopause and the development of osteoporosis (130). Osteoporosis is a significant public health problem in women. An observational study by Caudy et al. showed that the number of women who will experience a fracture in one year exceeds the combined number of women who will experience incident breast cancer, myocardial infarction, or stroke (131).

Osteoporosis is a great health problem also in men. In 1989, Stepan and colleagues showed that, similar to ovx women, castrated men also experience rapid bone loss (132). The general belief was that the major sex steroid regulator of bone mass was estrogen in women and T in men. This traditional view on the role of sex steroids in women and men was challenged in 1994 by a case report. A 28-year-old male, with a homozygous mutation in the ERα gene, had unfused epiphyses and suffered from osteopenia despite normal T and elevated estrogen levels (9). In addition, two other males with aromatase deficiency were described to have a similar skeletal phenotype as the ERα-mutated man and estrogen treatment increased bone mass in these aromatase deficient males (133, 134). Since then, extensive observational and interventional human studies, together with studies using gene-manipulated mouse models, have confirmed a key role of estrogen for the regulation of the skeleton, not only in women, but also in men (82).

Today, we know that in men, serum levels of E2 are strongly associated with bone mineral density (BMD) (20), that low levels of E2 are associated with increased risk of fractures (135), and that there is a causal effect of serum E2 on BMD as shown using Mendelian randomization (136). In addition, several experimental animal studies have shown that E2 treatment increases bone mass in males (19, 20). Thus, estrogen is an important regulator of bone metabolism in both genders.

1.13 HORMONE REPLACEMENT THERAPY

At menopause, the E2 and progesterone production from ovaries decline drastically, which leads to menopausal symptoms (e.g., hot flashes and mood swings), atrophy of uterine endometrium and vaginal epithelium, increased risk of hypertension and atherosclerosis, loss of fertility, and accelerated bone loss. Hormone replacement therapy (HRT), usually consisting of estrogen in combination with progesterone, reduces menopausal symptoms and prevents bone loss.

The large Women’s Health Initiative (WHI) study aimed to assess the effect of continuous HRT (consisting of estrogen and progesterone) on coronary

heart disease (CHD) in postmenopausal women and to evaluate breast cancer risk. However, in 2002, the WHI study was stopped due to severe side effects, such as increased risk of breast cancer and venous thromboembolism, and lack of protective effects on CHD (137, 138).

The Million Women Study was also set up to investigate the effects of specific types of HRT (estrogen, estrogen in combination with progesterone, tibolone, and other types of HRT) on incident of fatal breast cancer. The result of the Million Women Study also showed an increased risk of breast cancer after HRT (139). In contrast, by treatment with only estrogen in the WHI study, CHD risk was not affected and breast cancer risk tended to be lower (140). However, both WHI and the Million Women study have received criticism regarding the inclusion of subjects, with inclusion of rather old women (up to 79 years of age) in the WHI study and inclusion of biased subjects in the Million Women study (141, 142). Today, HRT is not recommended for treatment of osteoporosis, but short term HRT can be used to treat menopausal symptoms.

1.14 SELECTIVE ESTROGEN RECEPTOR MODULATORS (SERMs)

SERMs are synthetic estrogen-like molecules that can bind to an ER and they are used for several therapeutic purposes including osteoporosis. SERMs can act as agonists or antagonists to ER in a tissue-specific manner. The tissue-specificity depends on many factors including (i) relative binding affinity for ERα and ERβ, (ii) relative expression levels of ERα and ERβ, and (iii) co-regulator availability (143). Unlike the E2 molecule structure, SERMs have a long bulky side chain that affects the conformation of ER upon binding. This bulky chain affects the AF-2 interaction with coactivators or corepressors (71). The results of paper II and previous publications suggest that both the AF-1 and AF-2 regions of ERα are important in mediating the anti-osteoporotic effects of SERMs in mice (144-146).

There are several SERMs used in clinical practice, including tamoxifen, raloxifene, lasofoxifene, and bazedoxifene. Tamoxifen was the first commercially used SERM for treatment of ER-positive breast cancer (147). Tamoxifen was shown to be an agonist in bone but also an agonist in uterus, leading to increased risk of endometrial cancer (148, 149). Raloxifene was the first approved SERM for treatment of postmenopausal osteoporosis which also proved to be effective in prevention of breast cancer (150, 151). Lasofoxifene was the first SERM preventing non-vertebral fractures, and it

also decreases the risk of ER-positive breast cancer (152, 153). Bazedoxifene, which is not an ER agonist in uterus or breast tissue, is currently used in EU and Japan, and it prevents vertebral and non-vertebral fractures in high risk patients (154, 155).

1.15 ESTROGEN DENDRIMER CONJUGATE (EDC)

The estrogen dendrimer conjugate (EDC) consists of estrogens attached to a large, positively charged nondegradable poly(amido)amine (PAMAM) dendrimer via hydrolytically stable linkages (156). This molecule enables the separation of nuclear and extra-nuclear signaling pathways, since it lacks the ability to enter the nucleus. Experiments in breast cancer cells have shown that EDC is highly effective in stimulating membrane-initiated signaling but inefficient in affecting nuclear ER target gene expression (156). Other studies have shown that EDC promotes cardiovascular protection but not uterine or breast cancer proliferation in mice (157). In vitro studies have demonstrated that EDC, like E2, can decrease osteoblast apoptosis and promote osteoclast apoptosis (85, 158), and in a recent study, Bartell et al. showed that EDC prevents cortical bone resorption caused by estrogen deficiency in female mice (159). In paper III of this thesis, we have used EDC to evaluate the importance of membrane-initiated estrogen signaling on bone mass in male mice.

2 AIM

The general aim of this thesis was to characterize different ERα signaling pathways in bone and other organs in order to increase the knowledge regarding the mechanisms behind the protective effects of estrogen on bone mass versus adverse effects in other organs. The specific aims for each paper included in this thesis are listed below.

Paper I

To evaluate the role of ERα expression in two distinct hypothalamic nuclei – the arcuate nucleus (ARC) and the ventromedial nucleus (VMN) – in the regulation of bone mass in female mice.

Paper II

To evaluate the role of different domains of ERα for the effects of E2 and SERMs on bone mass in male mice.

Paper III

To determine the importance of extra-nuclear estrogen effects on bone mass in male mice and to determine the role of ERαAF-1 for mediating these effects.

Paper IV

To investigate the role of membrane-initiated ERα (mERα) signaling for skeletal growth and maintenance and for estrogen treatment response in male mice.

3 METHODOLOGICAL CONSIDERATIONS

Studies of intracellular ERα signaling in bone versus other organs require *in vivo* experiments. The experimental methods employed in each study are described in detail in their respective papers. Here follows an overview of the most relevant aspects of the methods used in this thesis. Care of animals and procedures were approved by the University of Texas Southwestern Medical Center (paper I) and the local ethics committee at the University of Gothenburg (paper I-IV).

3.1 ANIMAL MODELS

Mice are the most commonly used animal model for studying human biology and human diseases. The similarities between the mouse and human genome, anatomy, and physiology, as well as the short life cycle of mice, their small size, and cost benefits are advantages that make mice the most widely used *in vivo* animal model. In addition, the most important benefit of using the mouse as animal model is that the mouse can easily be genetically manipulated. By using transgenic techniques, we can study the expression of genes of interest either by deletion (knock out) or enhancement (overexpression) of the genes. Despite many advantages, it can always be questioned whether animal models can reliably be compared to the situation in humans or not. In addition, regarding bone physiology, there are differences between mice and humans. For example, in mice, the growth plates are never fully closed, while in humans, the growth plates close after puberty due to elevated E2 levels. However, high dose E2 treatment can fuse the growth plates in adult mice. In addition, mice do not experience menopause as women do. However, gonadectomy in mice leads to sex steroid deficiency and a bone loss similar to the decreased bone mass seen after menopause or castration in humans.

In this thesis, we have used mice that are genetically manipulated by different techniques, and they are all on C57BL/6 background (Table 2). Different substrains of C57BL/6 have been used and these substrains have minor genetic differences due to accumulated spontaneous mutations over time. Since we use the same substrain in each study, we can ignore the differences.

Table 2. Mouse models in different studies.

Papers	I		II & III	IV
Mouse	POMC-ERα$^{-/-}$	VMN study	ERα$^{-/-}$, ERαAF-1^{0} & ERαAF-2^{0}	NOER
Mouse background	C57BL/6J	C57BL/6N	C57BL/6N	C57BL/6NTac
Gene modification method	Conditional KO by Cre-loxP	Inducible by AAV-shRNA	Global KO	Point mutation
Previous publications	(160)	(161)	(83, 162)	(121)

KO; knockout, AAV; adeno-associated virus, shRNA; short hairpin RNA

In paper I, we have used two different techniques to delete expression of ERα in two distinct hypothalamic nuclei, ARC and VMN. By using the Cre-loxP system, we generated female mice lacking ERα expression in proopiomelanocortin (POMC) neurons of the ARC nucleus. The Cre-loxP system is a site-specific recombinase technology that is widely utilized to modify genes. This technique is based on the use of bacteriophage P1 cyclic recombinase (Cre). The Cre enzyme recognizes DNA sequences called locus of crossing over (loxP) and cleaves DNA sequences that are flanked by two loxP sites. By the Cre-loxP system, we can express or delete the gene of interest in a tissue- and time-specific manner.

To induce ERα deletion in VMN, we used an adeno-associated viral vector containing short hairpin RNA (AAV-shRNA). A basic AAV vector containing ERα-shRNA under control of the U6 promotor, has been used to specifically induce gene silencing in the VMN of adult WT mice. AAV-vectors can be used to efficiently silence ERα in hypothalamic VMN (161). The AAV-ERα-shRNA and AAV-scramble-shRNA (control) were injected into the VMN of female mice, by stereotaxic operation.

In papers II and III, we used male mice lacking total ERα, ERαAF-1, or ERαAF-2, by global deletion of the whole ERα gene or a part of it. In Paper IV, male mice with a point mutation in palmitoylation site C451 of ERα (nuclear only ER [NOER]) was used. All these mouse models were generated by use of homologous recombination, in which the target deletion was inserted into the esr1 locus in embryonic stem (ES) cells. The ES cells were then injected into blastocysts of mice to generate the transgenic knockout (KO) mouse model. Corresponding control littermate mice were used in each experiment.

3.2 GONADECTOMY AND E2 TREATMENT

Sex steroid deficiency, in both men and women, causes imbalance between bone formation and resorption, leading to decreased bone mass and strength and increased risk of osteoporotic fractures. Gonadectomy (ovx in female and orx in male mice) enables studies of sex-steroid deficiency. Mice do not experience menopause as women; however, all major characteristics of bone loss induced by sex-steroid deficiency in humans can be mimicked in mice by gonadectomy (163, 164). Gonadectomy leads to a substantial decline in serum sex steroid levels. In humans, the production of estrogen is not totally eliminated, since adrenal androgens, after aromatization, can be transformed to estrogens. However, in mice, the production of androgens in adrenals is considered insignificant.

Gene manipulation in some of our transgenic mouse models disturbed the negative feedback regulation of sex steroids, leading to elevated serum levels of sex steroids and confounding effects on bone parameters. To avoid this, we ovx female mice (paper I) and orx male mice (paper II-IV) and treated with slow-release pellet (paper I, II, and IV) of E2 or placebo, or used osmotic minipumps (paper III) to deliver E2 or vehicle, to examine their responses to estrogen treatment. The E2 doses used in the papers of this thesis (Table 3) were based on previous experiments. The E2 doses in paper I, II, and IV are slightly supraphysiological, while the E2 doses in paper III is higher than the required dose for E2 replacement in orx mice and is considered pharmacological.

Table 3. E2 doses per mouse and per day in different studies.

Papers	**I**	**II**	**III**	**IV**
E2 doses	0.5 µg	167 ng	6 µg	167 ng
Previous studies	(160)	(83)	(159)	(165)

3.3 MEASURMENTS OF BONE PARAMETERS

3.3.1 DUAL ENERGY X-RAY ABSORPTIOMETRY (DEXA)

DEXA is a widely used technique for measuring BMD and body composition both in the clinical setting and animal research. In the clinical setting, DEXA measurements of lumbar spine and femoral neck BMD are the current criteria for the diagnosis of osteoporosis, assessment of fracture risk, or monitoring of response to treatment.

The underlying principle of DEXA measurements is that different tissues absorb energy to different degrees. From an X-ray source, a dual-energy spectrum is created, which passes through the body. The amount of this energy is then detected by sensors. Because of the two emitted X-ray beams with different energy levels, the DEXA can distinguish between bone and soft tissues. The advantage of using DEXA is that it is a non-invasive and painless method that can be used for longitudinal studies. The limitation of the DEXA technique is that it renders two-dimensional (2D) – length and width – images, and no consideration is given to the third dimension – depth or volume – of the bone. This is particularly a problem when measuring growing animals with major skeletal changes in size. The measured BMD by DEXA is therefore areal BMD (aBMD, g/cm^2) and should not be mistaken for the true volumetric BMD (vBMD, g/cm^3). The DEXA analyses in papers II and IV were performed using the Lunar PIXImus mouse densitometer (Wipro GE Healthcare, Madison, WI, USA), which was calibrated before use.

3.3.2 PERIPHERAL QUANTITATIVE COMPUTED TOMOGRAPHY (pQCT)

pQCT is a useful tool for measuring bone compartments in both humans and animals. A rotating X-ray device provides a three dimensional measurement of the bone. The classical pQCT measurement of cortical bone is in the mid-diaphyseal region of the long bones. The trabecular bone is determined as the inner 45% of the total cross-sectional area, in the metaphyseal region of long bones, with the growth plate as a reference point. The advantages of using pQCT are: (i) it can measure the true vBMD and (ii) it separates the trabecular bone from the cortical bone. The limitations of using pQCT in contrast to DEXA are: (i) it has a slightly higher radiation dose, and (ii) in animals, *in vivo* longitudinal measurements are somewhat difficult to perform. In this thesis, the pQCT analyses in papers II and III were

performed *ex vivo*, using the pQCT XCT RESEARCH M (version 4.5B, Norland, Fort Atkinson, WI, USA), operating at a resolution of 70 µm.

3.3.3 MICRO COMPUTED TOMOGRAPHY (µCT)

The µCT is a technique that can obtain 3D images of bone, including the microarchitecture of the bone, without the need for destructive sectioning. Like pQCT, the µCT separates the trabecular and cortical bone. In addition, it provides information about the trabecular network by calculating the trabecular number and thickness.

A rotating stage is located between an x-ray source and a charge-coupled detector (CCD) array. The bone is placed on the rotating stage. Three factors determine the spatial resolution of the image: (i) the focal size of the x-ray source, (ii) the detector's array resolution, and (iii) the bone position with respect to the source and the detector. The imaging system provides a series of x-ray projections from a range of angles around the bone. Each projection represents the value of the x-ray source to the x-ray element. Imaging the bone at the equiangular-spaced views over 180 degrees provides a complete set of projection data. Image reconstruction creates a 2D image from the measured projection data and a 3D image is calculated by reconstructing and stacking individual 2D slices. Different algorithms are then used to calculate several bone parameters. The resolution of µCT used in this thesis was at a 4.48 µm. The advantages of using µCT technique are that it gives 3D images with higher resolution than pQCT and information about the microarchitecture of the bone. The limitation is that the µCT analyses are more time consuming compared to the pQCT analyses.

In papers I, III, and IV, the µCT analyses were performed by using an 1172 model µCT (Bruker MicroCT, Aartselaar, Belgium) and in paper II a model 1072 scanner (Skyscan N.V., Aartselaar, Belgium) was used.

3.3.4 HISTOMORPHOMETRY

Bone histomorphometry is the classical method to examine bone parameters in undecalcified bone and to obtain quantitative information on bone structure and remodeling. The bone of interest is fixed and embedded in plastic (e.g., White Resin; Agar Scientific), sectioned, and analyzed under a light microscope. To separate different bone compartments or cells, different kinds of staining (e.g., Masson-Goldner's Trichrome) are used.

Bone histomorphometry parameters are divided into two categories; static and dynamic. The static parameters include the quantity of trabecular or

cortical bone volume and the number of osteoclasts and osteoblasts per bone perimeter. Dynamic histomorphometry includes parameters that show changes in bone over time. The dynamic parameters can be studied after injections of labeling fluorochromes. In paper I and IV, the mice were injected with calcein at day 1 and 8 before termination, leading to double labeling. The calcein is incorporated on the bone surface and the amount of new bone formed in one week can be measured.

One of the advantages of the histomorphometry method is that it provides information regarding bone cells. In contrast to CT methods, it also provides information about dynamic bone parameters (e.g., bone formation rate). However, the bones are sectioned and longitudinal measurements are therefore not possible using histomorphometry.

3.3.5 MECHANICAL TESTS

Three-point bending is a method to evaluate the quality of a bone by measuring its mechanical properties. The bone of interest is placed in the machine, and load is applied to the bone until it breaks. The three-point bending tests in paper I, II, and IV were done by the Instron 3366, Instron Corp. (Norwood, MA, USA) testing machine. The load deformation curves from the three-point bending are registered and from these the biomechanical parameters are calculated by using raw-files produced by the Bluehill 2 software version 2.6 (Instron Corp., Nowood). Since the bone breaks in this method, it cannot be used for further analysis, which is a limitation of using three-point bending. In addition, the test is performed on the mid diaphysis of a long bone and the test therefore mainly corresponds to the mechanical strength of the cortical bone. However, it is possible to examine the mechanical strength of trabecular bone in vertebrae, using a compression test.

3.3.6 FOURIER TRANSFORM INFRARED (FTIR) MICROSPECTROSCOPY

FTIR is a powerful tool that provides information about the quality of the bone. This analytical technique measures the absorption of infrared radiation by the sample material versus wavelength. The infrared absorption bands identify molecular components and structures. The bone material parameters measured by FTIR in this thesis were: mineral to matrix ratio, mineral maturity, collagen maturity, and crystallinity (paper I). To analyze these parameters by FTIR, the bone of interest was fixed, dehydrated, embedded in plastic (Technovit 9100, Heraeus-Kulzer), and sectioned by a microtome. The bone sections were placed on IR transparent barium fluoride windows and the spatial molecular bone composition was assessed by means of FTIR

microspectroscopy. The sectioning of bone is a limitation of using FTIR and other analyses should be done prior to FTIR.

In paper I, we have used FTIR microspectroscopy at beamline D7, (MAX IV Laboratory, Lund University, Sweden) using a Hyperion 3000 microscope and a Bruker IFS66/v FTIR spectrometer.

3.4 REAL-TIME PCR

Real-time PCR is an extremely sensitive method to measure mRNA levels and thus to evaluate the relative expression levels of a specific gene of interest. In this method, the mRNA is prepared from the tissue or cells and then reverse transcribed into cDNA. Thereafter, a specific cDNA sequence (corresponding to the mRNA from the gene of interest) is amplified, and the amount of that specific cDNA is then measured. To amplify the cDNA sequence of interest, a set of primers (complimentary DNA strands) are used to guide replication to the cDNA sequence of interest. Detection of the amplified cDNA sequence can be performed in several ways. In our studies, we have used the TaqMan method, in which a probe (complimentary DNA strand) attaches to the amplified cDNA sequence. A fluorescent dye and a quencher of fluorescence are both attached to the probe. Due to the quencher, no fluorescence is emitted when the probe is intact. During replication of the amplified cDNA, the probe is cleaved and the quencher is detached and separated from the fluorescent dye, and fluorescence is emitted. The emitted light intensity is proportional to the amount of amplified cDNA. The amplification of cDNA is exponential because the amount of amplified cDNA is doubled in each cycle and this can be followed over time. In this thesis, we have analyzed two different mRNA simultaneously; the gene of interest and an internal standard. In papers I and IV, RT-PCR analyses were performed using the ABI Prism 7000 sequence Detection System (PE Applied Biosystem) and 18S as internal standard.

3.5 SERUM MEASUREMENTS

In this thesis (paper I and IV), we have used gas chromatography-tandem mass spectrometry (GC-MS/MS), the most sensitive technique for steroid measurements (47), to measure serum levels of E2, T, and dihydrotestostrone (DHT). After isotope-labeled standards are added to the samples, the steroids are extracted to chlorobutane and purified on a silica column and then derivatized. In this thesis, steroids were analyzed in multiple reactions monitoring mode with ammonia as reagent gas using an Agilent 7000 triple

quadrupole mass spectrometer equipped with a chemical ionization source. A benefit of using GC-MS/MS compared to other techniques is its multianalyte capability, which allows multiple sex steroids to be quantified from a single sample with high selectivity, sensitivity, precision, and accuracy.

In all papers, serum concentrations of bone markers were assessed by using commercially available radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) kits.

3.6 IMMUNOHISTOCHEMISTRY

Immunohistochemistry is a method to determine the presence of an antigen in cells or tissue sections using specific antibodies that bind to the specific antigen. This antibody-antigen binding is visualized by detection molecules and visible under a microscope.

In paper I, immunohistochemistry was used to evaluate the expression of green fluorescent protein (GFP) and ERα in the injected area of hypothalamus. The protocol was optimized from previously established protocols (161, 166-168). Based on previous studies, we have used free-floating brain sections (168). The antibodies that we have used were also examined in earlier studies (161). Images were analyzed using a Carl Zeiss LSM 780 confocal microscope.

3.7 FLOW CYTOMETRY

Flow cytometry is a technique to sort and characterize cells from a heterogeneous mix of cells. The cell suspension is labeled with fluorophore-conjugated antibodies that are directed to a specific cell antigen. The cell suspension is injected into a flow cytometer, pass an excitation source, and then fluorescence is emitted from the cells. The emitted light is collected by detectors and presented on a plot. A limitation with this technique is the autofluorescence which is the endogenous fluorophores that can interfere with the specific fluorescence emission, leading to false positive signals. In paper II, B lymphocytes in bone marrow cells were detected by staining with phycoerythrin-conjugated antibodies to CD19. We have used the FACSCalibur (BD Pharmingen) and analyzed the data with the FlowJo software.

3.8 STATISTICS

Statistical calculation can be based on either parametric or non-parametric tests. In parametric tests [e.g., Student's t-test and analysis of variance (ANOVA) (95)], the statistical method assumes normal distribution of data. In non-parametric tests, any particular distribution of data is assumed and the statistical method is based on a ranking of individual observations.

In all four papers included in this thesis, we have used Student's t test to compare two groups. In paper II, we have used Student's t test with Bonferroni correction for three comparisons between treatment groups. In paper I and IV, we have used the interaction term from a two-way ANOVA analysis to determine the effect of treatment between two groups. In all tests, $P < 0.05$ was considered statistically significant. All statistical calculations in this thesis were performed in GraphPad Prism 7.02 Windows version.

4 RESULTS

The main results of each paper are briefly described below. For more details, see the full papers in the end of the thesis.

4.1 PAPER I

The aim of this paper was to test the hypothesis whether ERα expression in POMC neurons of ARC or ERα expression in VMN is involved in the regulation of bone mass in female mice.

The gonadal intact POMC-ERα$^{-/-}$ female mice had increased cortical bone mass and mechanical strength. They also had slightly elevated serum levels of E2, indicating that their feedback regulation of serum sex steroids was modestly disturbed. Thus, to avoid the possibility of confounding effects of elevated serum E2 levels in the gonadal intact POMC-ERα$^{-/-}$ mice, we compared the estrogenic responses in ovx POMC-ERα$^{-/-}$ and ovx control mice. E2 treatment resulted in increased cortical and trabecular bone mass in both ovx POMC-ERα$^{-/-}$ and controls. Importantly, the estrogenic responses on cortical bone mass and mechanical strength were substantially augmented in ovx POMC-ERα$^{-/-}$ compared to the estrogenic responses on the same parameters in ovx control mice. In contrast to cortical bone, the estrogenic responses on trabecular bone mass were unchanged in the axial skeleton and only modestly increased in the appendicular skeleton in ovx POMC-ERα$^{-/-}$ compared to the estrogenic response in controls.

To achieve site specific silencing of ERα in the VMN, we exploited RNA interference mediated by AAV-shRNA. As demonstrated previously (161), suppression of ERα in the VMN resulted in increased body weight. Interestingly, silencing of ERα in VMN did not affect trabecular or cortical bone mass.

In summary, ERα expression in POMC neurons located in the ARC, but not ERα expression in the VMN, is involved in bone mass regulation. Mice lacking ERα in POMC neurons in the ARC display substantially enhanced estrogenic response on cortical bone mass and only modestly increased estrogenic response on trabecular bone mass, while mice with silenced ERα in hypothalamic VMN have no changes in the bone phenotype.

4.2 PAPER II

To evaluate the role of different domains of ERα for the effects of E2 and SERMs on bone mass in males, two separate experiments were performed:

1. Three mouse models lacking (i) ERα-AF1 (ERαAF-1^0), (ii) ERα-AF2 (ERαAF-2^0), or (iii) the total ERα (ERα$^{-/-}$) and their corresponding controls were orx and treated with E2 or placebo.
2. ERαAF-1^0 and control mice were orx and treated with the SERMs Raloxifene (Ral), Lasofoxifene (Las), Bazedoxifene (Bza), or vehicle.

Serum T levels were elevated in gonadal intact ERα$^{-/-}$ male mice compared to controls. In order to avoid confounding effects from elevated serum T, all three mouse models in the first experiment were orx and treated with either E2 or placebo. As expected, E2 treatment increased total body aBMD and cortical and trabecular bone mass in orx control mice. E2 treatment in orx ERαAF-1^0 mice resulted in increased total body aBMD and cortical bone mass, while trabecular bone mass was not affected. In contrast, orx ERα$^{-/-}$ and ERαAF-2^0 mice did not respond to E2 treatment. We also investigated the E2 response in the immune system of these three mouse models. As expected, in orx control mice, E2 treatment decreased thymus weight, bone marrow cellularity, and the frequency of B lymphocytes in the bone marrow. E2 treatment in orx ERαAF-1^0 resulted in decreased bone marrow cellularity, while no E2 response was seen on thymus weight. Similarly, as seen for bone parameters, no E2 responses were seen for the evaluated parameters in orx ERα$^{-/-}$ and ERαAF-2^0 mice.

In the second experiment, all three SERMs increased total body aBMD and trabecular vBMD, to a similar extent, compared to vehicle treatment in orx control mice. When evaluating cortical bone parameters, only Las increased cortical thickness and only Bza increased bone strength significantly in orx control mice. ERαAF-1^0 mice did not respond to any of the SERMs.

In summary, orx ERαAF-2^0 mice, similarly to orx ERα$^{-/-}$ mice, do not respond to E2 treatment. The E2 responses in male ERαAF-1^0 mice are tissue dependent. All SERMs increase total body aBMD and trabecular vBMD, while only Laz and Bza increase cortical bone mass. In addition, all SERM-effects are absent in the orx ERαAF-1^0 mice.

4.3 PAPER III

The aim of this paper was to determine whether extra-nuclear estrogen signaling affects the skeleton in males and whether ERαAF-1 is involved in mediating these effects.

E2 treatment in orx control mice resulted in increased cortical and trabecular bone mass. As expected, and consistent with the results of paper II, E2 treatment in orx ERαAF-1^0 mice resulted in increased cortical, but not trabecular bone mass. EDC treatment in orx control mice resulted in a moderate increase in cortical thickness, while trabecular bone parameters were unchanged. EDC treatment did not affect cortical or trabecular bone parameters in orx ERαAF-1^0 mice.

In summary, EDC treatment affects cortical bone, but not trabecular bone, in orx control mice, and this effect is abolished in orx ERαAF-1^0 mice.

4.4 PAPER IV

To investigate the role of mERα signaling in the physiological regulation of bone mass in males, we studied gonadal intact male NOER mice. Furthermore, to evaluate the role of mERα signaling for estrogen treatment response, we studied orx NOER mice treated with either E2 or placebo pellet.

Serum T and DHT levels were comparable between gonadal intact male NOER mice and controls. This finding indicates that the feedback regulation of sex hormones was normal and thus excludes the possibility that a disturbed feedback regulation of sex hormones could interfere with the interpretation of our findings.

The gonadal intact male NOER mice had reduced total aBMD in repeated measurements at three, six, and nine months of age. Detailed analysis of the excised long bone showed that both cortical and trabecular bone parameters were decreased in male NOER mice compared to controls. The static and dynamic histomorphometry analysis on trabecular bone showed decreased number of osteoblasts and osteoclasts per bone perimeter, decreased bone formation rate, and decreased mineralized bone surface. In addition, the mRNA expression levels of the osteoblast-related gene coding for collagen type Iα1 were reduced in NOER male mice compared to controls.

E2 treatment increased total aBMD as well as trabecular and cortical bone mass in orx male NOER mice and controls. However, the estrogenic

responses in these skeletal parameters were significantly decreased in orx NOER mice compared to estrogenic responses in orx control mice.

In summary, mERα signaling is involved in developing and maintaining a normal total aBMD as wells as a normal cortical and trabecular bone phenotype in male mice. mERα signaling is also crucial for a normal estrogenic response in both cortical and trabecular bone in males.

5 DISCUSSION

Osteoporosis in both genders is one of the most highly recognized public health problems because of the high fracture risk, which in turn often leads to high morbidity and mortality (169-171). Sex steroid deficiency, old age, and lack of physical activity are critical factors for the development of osteoporosis (172). Estrogens are crucial in the regulation of bone mass in both genders. After menopause, estrogen deficiency increases bone turnover rate which can result in low BMD. In men, declining sex steroid levels, especially decreased bioavailable estrogen levels, contribute to age-related bone loss (172). Estrogen treatment prevents bone loss in both females and males but leads to an increased risk of adverse effects in both sexes. Thus, it would be beneficial to develop bone-specific estrogen treatments. Considerable evidence based on human and animal studies indicates that ERα mediates the main protective effects of estrogen in bone in both genders. Thus, a better understanding of how estrogen signaling via ERα regulates bone metabolism would help to develop bone-specific estrogen treatments.

The brain – the master regulator of homeostasis in peripheral tissues – has long been known to affect the skeleton. The importance of neuronal regulation of bone remodeling has now been outlined by a number of *in vivo* animal studies (100, 173). CNS is a target for estrogen and ERα is widely distributed in the CNS (17). ERα is highly expressed in hypothalamus (160); a region exposed to hormones and nutrients with a leaky blood-brain-barrier (174).

Local injections of estrogen restore bone loss caused by estrogen deficiency (175). Furthermore, inactivation of ERα in bone cells leads to decreased bone mass (79). Thus, peripheral (local) estrogen signaling has a positive effect on bone mass. Our group has previously shown that specific inactivation of ERα in nervous tissue (using the Nestin-ERα$^{-/-}$ mouse model), leads to a high bone mass phenotype (101). This finding demonstrates that central (neuronal) ERα signaling exerts an inhibitory effect on bone mass, in contrast to peripheral estrogen signaling that exerts a stimulatory effect on bone mass. Ohlsson et al. suggested that the main mechanism for the increased bone mass in Nestin-ERα$^{-/-}$ mice is mediated via decreased central leptin sensitivity in the hypothalamus, leading to increased leptin secretion from adipose tissue and thereby increased leptin-induced bone formation. However, the primary target cells in the brain for these central inhibitory effects of estrogen on bone mass were not determined.

In this thesis, we have confirmed the previously published results about the inhibitory effects of central estrogen signaling on bone mass. In addition, we now show that these inhibitory effects of estrogen signaling are mediated via ERα in POMC neurons, mainly located in the hypothalamic ARC. The female POMC-ERα$^{-/-}$ mice in our study were healthy in general with normal body weight, fat mass, and bone growth in both the axial and appendicular skeleton. In addition, they had normal ERα expression levels in bone, making this model suitable for evaluating the role of ERα in POMC neurons for bone metabolism. The ERα expression was previously reported to also be reduced in POMC expressing pituitary cells (ACTH-cells) in POMC-ERα$^{-/-}$ (160). However, several measured parameters including ACTH expression and corticosterone levels were similar between POMC-ERα$^{-/-}$ mice and controls. This indicates that bone phenotypes of POMC-ERα$^{-/-}$ mice are due to lack of ERα in POMC neurons in the ARC and not in pituitary cells. Nevertheless, we cannot fully rule out the possibility of confounding effects of ERα deletion in POMC neurons in pituitary cells.

The gonadal intact female POMC-ERα$^{-/-}$ mice displayed increased cortical bone mass and mechanical strength. Analysis of sex steroid levels in serum using GC-MS/MS demonstrated slightly elevated E2 levels, and this may be a confounding factor when interpreting the high bone mass phenotype in female POMC-ERα$^{-/-}$ mice. To avoid this, we compared the estrogen treatment responses in ovx POMC-ERα$^{-/-}$ mice with the estrogen treatment responses in ovx control mice.

The estrogenic response was substantially increased in cortical bone in POMC-ERα$^{-/-}$ compared to controls, while only modestly augmented in trabecular bone in the appendicular skeleton, and unchanged in the trabecular bone in the axial skeleton. This suggests that central ERα activity in POMC neurons mainly targets cortical, and not trabecular, bone. Several clinical and animal studies have suggested that cortical and trabecular bone compartments are separate functional entities that are regulated by different mechanisms. For example, detailed clinical investigations have revealed that trabecular bone loss begins in sex hormone replete young adults of both sexes, while the start of cortical bone loss is closely tied to estrogen deficiency (19, 176). In addition, both ERαAF-1^{0} and steroid receptor coactivator 1 (SRC-1) KO mice (74, 83, 144), and many cell-specific ERα KO mouse models display different phenotypes, or different estrogenic responses, in cortical versus trabecular bone (177). Our study further strengthens the idea of trabecular and cortical bone being separate entities and suggests that central ERα activity is involved in this bone compartment specific regulation.

Several other studies have confirmed that hypothalamic POMC neurons in the ARC have a role in the regulation of bone mass. It has been shown that modulation of the activity of the transcription factor AP1 in POMC-neurons affects bone formation (178). Furthermore, both melanocortin 4 receptor (MC4R) KO mice and humans with a MC4R mutation, in which the POMC-neuron-derived ligand α-melanocyte-stimulating hormone (αMSH) cannot bind to its receptor (MC4R), have a high bone mass phenotype (179, 180). Thus, our results, together with other studies, demonstrate that hypothalamic POMC neurons are important regulators of bone mass.

We have tried to find possible mechanisms for the POMC ERα-mediated effects on cortical bone. In contrast to the elevated serum leptin levels and decreased central leptin sensitivity found in Nestin-ERα$^{-/-}$ mice, these factors were unchanged in female POMC-ERα$^{-/-}$ mice compared to controls. Thus, leptin signaling is not involved in the high bone mass phenotype of POMC-ERα$^{-/-}$. Interestingly, in bone, Nestin-ERα$^{-/-}$ mice displayed a decrease in mRNA expression levels of CIITA – a factor involved in T-cell activation – and in our study we found that the decline in CIITA expression in cortical bone after E2 treatment was significantly increased in ovx POMC-ERα$^{-/-}$ mice compared to controls. This finding suggests that the central ERα effects could be mediated via altered T-cell activation. However, this hypothesis/suggestion needs further investigation.

In addition to the hypothalamic ARC, ERα is also highly expressed in hypothalamic VMN. In this thesis, we evaluated the possible role of ERα in VMN for bone mass. By using an AAV vector, the ERα expression was silenced specifically in VMN in adult females. Consistent with other studies, we confirmed that ERα expression in hypothalamic VMN is involved in body weight regulation (161). In contrast, we observed no changes in cortical or trabecular bone mass, indicating that ERα in hypothalamic VMN neurons is not involved in the regulation of bone mass. Other studies, confirming our results, demonstrate that: (i) the central E2 effects on fat mass, but not on bone, are partly mediated through the VMN (in references 178-180, the authors refer to VMN as the ventromedial nucleus of hypothalamus [VMH]) (181) and (ii) genetic depletion of Nkx2-1 neurons in the VMN does not affect bone mass, although ERα neurons in VMN were greatly reduced (182). However, it has been demonstrated that selective ablation of neurons in VMN blocked the central leptin-induced bone loss, indicating a role of VMN neurons for bone mass (183).

We propose that normal ERα-mediated effects in bone require a balance between the inhibitory central and stimulatory peripheral estrogen signaling.

Thus, our finding that central E2 signaling targets bone via ERα in POMC neurons of the ARC might be useful for development of SERMs. This kind of ERα-specific SERM should have reduced penetrance to the ARC, resulting in only stimulatory peripheral ERα-mediated effects.

Osteoporosis in men is less common than in women, but still constitutes a major burden for public health. Several elegant rodent and human studies have established that estrogens regulate skeletal development and bone maintenance in males (10, 19, 20, 135, 184, 185). However, E2 treatment causes feminization and other adverse effects in men and it is therefore not desirable for clinical use in men (13). In addition to E2 treatment, many SERMs also have beneficial effects on the male skeleton; however, the use of SERMs should be under careful consideration due to reported side effects (186). Thus, it is essential to find a bone-specific treatment with minimal adverse effects for an ageing male population. In order to achieve this, it is important to characterize the estrogen and SERM signaling pathways in the male skeleton.

ERα mediates the beneficial effects of estrogen in the skeleton of both sexes, while ERβ is only involved in the regulation of the female but not the male skeleton (5, 8, 11, 81, 84, 187). Consistent with previous studies, deletion of ERα in male mice in paper II resulted in elevated T levels due to disturbed negative feedback regulation of sex steroids (81). The high serum T levels activates the androgen receptor (AR), leading to compensatory effects on the skeleton (7, 81). We found that male mice lacking ERα had decreased cortical thickness but unchanged trabecular bone volume per tissue volume (BV/TV). Thus, high serum T levels through binding to the androgen receptor (AR) have compensatory effects on trabecular bone, while ERα is required to maintain cortical bone (144). To avoid these compensatory effects, all parameters in paper II were evaluated in orx and E2/placebo treated mice. We have only measured the serum sex steroids in $ER\alpha^{-/-}$ and not in $ER\alpha AF\text{-}1^0$ or $ER\alpha AF\text{-}2^0$. Other studies have reported that deletion of AF-1 or deletion or mutation of AF-2 causes both male and female infertility, suggesting that AFs are also involved in negative feedback regulation of serum sex steroids (83, 188-190). Since we wanted to compare the estrogenic responses in these two KO models ($ER\alpha AF\text{-}1^0$ and $ER\alpha AF\text{-}2^0$) with the estrogenic responses in $ER\alpha^{-/-}$ mice, we decided to orx all mice.

Studies of the role of AFs of ERα are important in order to understand the mechanisms behind E2 and SERM effects on bone versus other tissues. In paper II, we have first evaluated the role of ERαAF-1 and ERαAF-2 for the E2 response in male mice. In addition, we have also evaluated the role of

ERαAF-1 for SERM effects in male mice. These questions have also been investigated in female mice (83, 145). In our ERαAF-1^0 and ERαAF-2^0 mice; ERα is only partially deleted (i.e. the AF-1 and AF-2 domain); all other domains of ERα remain intact and ligand or DNA binding of the receptor is not affected by the specific deletion of AF-1 and AF-2 (73, 83, 191-193). Thus, the phenotypes of ERαAF-1^0 and ERαAF-2^0 are due to lack of AFs.

The bone and the immune system parameters were evaluated in orx ERα$^{-/-}$, ERαAF-1^0, ERαAF-2^0, and control WT mice treated with either E2 or placebo. The expected normal E2 responses in bone and immune system parameters of orx WT mice were: an increase in total aBMD, cortical thickness, trabecular BV/TV, biomechanical properties, and a decrease in thymus weight, bone marrow cellularity, and frequency of B lymphocytes in bone marrow. Consistent with previous studies, orx ERα$^{-/-}$ did not respond to E2 treatment in any of these evaluated parameters, demonstrating that a normal E2 response in males requires ERα (7, 81). Similarly as in female mice (83), ERαAF-2^0 males did not respond to E2 treatment in any of the evaluated parameters, demonstrating that ERαAF-2 is crucial for the estrogenic effects in both genders. Similarly as in females, the role of ERαAF-1 in males was tissue specific, crucial for estrogenic effects on trabecular bone and thymus but not required for estrogenic effects on cortical bone, biomechanical properties, or the evaluated parameters in bone marrow (83). Thus, we suggest that (i) a normal estrogenic response is dependent on ERαAF-2 in all tissues and (ii) a normal estrogenic effect in some tissues (e.g., trabecular bone) are dependent on ERαAF-1, while they in other tissues (e.g., cortical bone) are completely independent of ERαAF-1. This indicates that the role of ERαAF-1 depends on the type of bone compartment, suggesting that there are different mechanisms of ERα signaling in cortical versus trabecular bone. The expression of cofactors could be different in different bone compartments, leading to different pathways activated by ERαAF-1. *In vivo* studies have demonstrated that SRC-1 KO female mice show similar skeletal estrogenic responses as in ERαAF-1^0 females (194). Since the E2 effects in ERαAF-1^0 females and males showed the same pattern, SCR-1 might be involved in AF-1 dependent E2 effects in trabecular bone in male mice. However, this hypothesis needs to be confirmed.

Several research studies during recent years have tried to answer the question regarding which cell type is responsible for ERα-dependent estrogenic effects on bone. By using the Cre-LoxP system, ERα has been deleted in different bone cell types in males and females. One of the advantages of using bone cell-specific ERα KO models is that the negative feedback regulation of serum sex steroid levels is normal. Deletion of ERα in osteocytes resulted in

decreased E2 response in trabecular bone but unchanged E2 response in cortical bone in female mice (92), a similar pattern as in female and male ERαAF-1^0 mice. In addition, ERα deletion in osteoblasts of male mice resulted in decreased trabecular bone and unchanged cortical bone (88). This finding suggests that ERαAF-1 activation in osteocytes and osteoblasts may be involved in mediating the estrogenic effects on trabecular bone in males.

Although SERMs are initially used for prevention and treatment of osteoporosis in postmenopausal women, animal and human studies have assessed the effects of SERMs on the male skeleton (186). In paper II, we evaluated, for the first time, the effects of the three SERMs Ral, Las, and Bza in orx male mice in the same study. We showed that all three SERMs increased total body aBMD and trabecular bone mass in orx WT mice to a similar extent. However, only Las and Bza-treated orx mice displayed increased cortical thickness and bone strength, respectively, while the effects of Ral treatment on the cortical parameters did not reach statistical significance. Interestingly, Las and Bza have significant preventive effects on non-vertebral fractures, while Ral has no effects (150-152, 155), which may reflect our finding on cortical bone.

In paper II, we also evaluated the role of ERαAF-1 for the SERM effects in orx male mice. We showed that ERαAF-1 is required for the effects of the evaluated SERMs on all assessed parameters, suggesting that ERαAF-1 is the main mediator of the effects of these SERMs in males. In contrast, we showed that E2 can affect cortical bone without interacting with ERαAF-1. Furthermore, SERM binding results in an inappropriate folding of helix 12, which in turn results in a limitation and/or loss of interaction between ERαAF-2 and its coregulators (71, 72, 195). Thus, to specifically target cortical bone, a new SERM could be designed that does not activate the ERαAF-1 or AF-2. In contrast to the SERMs used in our study, this new suggested SERM would (i) interact with other ERα domains than ERαAF-1 or AF-2, (ii) have positive effects on cortical bone and bone strength, and (iii) have fewer side effects in other tissues. Cortical bone is likely the major regulator of overall fracture risk and 80% of the skeleton consists of cortical bone (196). Thus, results of paper II could facilitate the design of new bone specific SERMs for male osteoporosis.

Although ERα is classified as a nuclear receptor and acts primarily as a transcription factor in the nucleus, extensive evidence has suggested that some effects of estrogens are mediated via extra-nuclear ERα signaling (197, 198). In this thesis, we have evaluated the role of extra-nuclear ERα signaling for the male skeleton using two different approaches. In paper III, we have

used the EDC compound, which selectively initiates extra-nuclear ERα signaling without involving nuclear ERα signaling. In paper IV, we have used a genetically modified mouse model (NOER) which has a disrupted mERα signaling but still a functional nuclear ERα signaling.

The results of paper III showed that EDC, similar as seen in female mice (159), could increase cortical thickness, but had no significant effect on the trabecular bone compartment. This suggests that part of the positive effects of estrogen on cortical bone is mediated via extra-nuclear actions of ERα, while extra-nuclear estrogen signaling alone does not affect trabecular bone. These results show that extra-nuclear estrogen signaling has different impact in cortical versus trabecular bone. This has now been shown to hold true in both genders and is further evidence that cortical and trabecular bone should be considered as separate functional entities and that estrogen signaling in these two bone compartments is mediated via different mechanisms.

We also evaluated the importance of ERαAF-1 for these extra-nuclear estrogen actions. E2 treatment of ERαAF-1^0 males resulted in increased cortical but unaffected trabecular bone, confirming our results in paper II that the estrogen response in cortical bone is independent of ERαAF-1, while the estrogen response in trabecular bone requires ERαAF-1 (144). Since E2 did not have any effects on trabecular bone when ERαAF-1 was deleted, we did not expect EDC treatment to have any effect in ERαAF-1^0 males on trabecular bone. As we expected, no effects were seen in trabecular bone parameters of EDC-treated ERαAF-1^0 males. Interestingly, EDC treatment, in contrast to E2 treatment, did not increase cortical thickness in ERαAF-1^0 males, demonstrating that extra-nuclear estrogen signaling effects on cortical bone is dependent on a functional ERαAF-1 in male mice.

In our study we found that EDC treatment, similar to E2 treatment, decreased serum CTx (degradation products of collagen c-telopeptides) levels, and a previous study has shown, *in vitro*, that EDC increases osteoclast apoptosis (85). These findings suggest that extra-nuclear effects of estrogen on cortical bone are mediated via effects on osteoclasts (159, 199). In line with this suggestion, we showed in paper III that extra-nuclear estrogen signaling via ERαAF-1 is essential for cortical bone (199). In addition, based on paper II and previous studies, we suggest that ERαAF-1 in osteoblasts and osteocytes mediate the estrogenic effects, both via nuclear and extra-nuclear signaling, on trabecular bone (79). These data together suggest that ERαAF-1 regulates cortical and trabecular bone via different signaling pathways and distinct bone cell types. A cross-talk between signaling pathways activated by

ERαAF-1 in different bone cells types may be needed for full estrogenic effects. However, further experiments are needed to confirm this hypothesis.

To further dissect the role of extra-nuclear estrogen signaling, we have used a genetically modified mouse model in paper IV. NOER mice lack membrane-associated ERα and therefore have disrupted extra-nuclear ERα signaling, but they still have nuclear actions of ERα. In contrast to female NOER mice (165), male NOER mice have normal, physiological levels of serum sex steroids, which demonstrate that their feedback regulation of sex steroids is normal. This indicates that mERα signaling is involved in feedback regulation of sex steroids in females but not in males. The normal feedback regulation in NOER mice was further confirmed by Nanjappa et al. showing that male NOER mice have normal levels of luteinizing hormone and follicle-stimulating-hormone (200). In contrast to our results, the same study reported elevated T levels in male NOER mice (200). This discrepancy could be explained by the choice of technique for serum sex steroids measurements. In our paper, we have used the sensitive and specific GC-MS/MS technique, while Nanjappa and colleges used ELISA to measure serum T levels.

Gonadal intact male NOER mice displayed decreased total body aBMD throughout their lifetime, which was reflected in decreased cortical and trabecular bone parameters in both young and adult mice. This demonstrates that mERα signaling is required for normal development and maintenance of both cortical and trabecular bone in male mice. In addition, both the number of osteoclasts and osteoblasts, as well as the trabecular bone formation rate, was decreased in male NOER mice.

To investigate the role of mERα for the estrogen treatment response in bone and other tissues, we studied orx NOER and WT mice treated with either E2 or corresponding placebo pellet. The estrogenic response in cortical and trabecular bone was decreased in orx NOER mice compared to the estrogenic response in orx WT mice. Thus, mERα signaling is crucial for normal estrogen response in both cortical and trabecular bone. This new finding could lead to a novel target for treatment of osteoporosis, where the mERα signaling pathways could be targeted.

Estrogen is known to induce thymic involution in both females and males, and treatment with estrogen is also known to decrease fat mass (5, 201, 202). Similar as seen in female NOER mice, the estrogenic response in thymus of male NOER mice was decreased compared to WT mice, suggesting a similar mechanism for mERα mediated estrogenic effects on thymus in males and females (165). In contrast, the estrogenic response on fat mass was different

in male versus female NOER mice. In females, the estrogenic response on fat mass is independent of mERα signaling, while in male NOER mice it was dependent on mERα signaling, suggesting a gender difference in the mechanisms behind the estrogenic regulation of fat mass.

To summarize our studies regarding extra-nuclear, membrane-initiated ERα signaling in male mice, we suggest that: (i) extra-nuclear signaling alone cannot affect trabecular bone, suggesting that trabecular bone effects require nuclear estrogen signaling; (ii) cortical bone effects can be mediated via both nuclear and extra-nuclear estrogen signaling; and (iii) both nuclear and extra-nuclear estrogen signaling and probably a cross-talk between these two signaling pathways is required for full estrogenic effects on both bone compartments.

6 CONCLUSIONS

The results generated from this thesis may create a better understanding of how estrogen signaling via ERα regulates bone mass and they may also help in the design of bone-specific SERMs for treatment of osteoporosis in both females and males.

The results of paper I highlight the role of central estrogen signaling for bone mass and demonstrate that it is mediated via ERα in POMC neurons of the ARC. We show that these central estrogen effects mainly target cortical bone, which plays a major role in determining overall fracture risk. This novel knowledge can facilitate the development of new SERMs that affect mainly cortical bone. The new SERMs should have minimal penetrance to the ARC and thereby only activate the stimulatory peripheral ERα signaling pathways, or have antagonistic effects in hypothalamic ARC.

Osteoporosis in men constitutes a major burden for public health, although it is less common than in women. Increased knowledge regarding estrogen signaling via ERα in males would facilitate the development of new SERMs for male osteoporosis. Based on results of paper II and in contrast to the SERMs presently available, a new SERM could be designed so that it does not activate ERαAF-1. This SERM would have a tissue specific role with beneficial effects on cortical bone and bone strength in men. In women, this kind of SERM would also have beneficial effects on cortical bone with minimal adverse effects on reproductive tissues.

The results of paper III and IV recognize the importance of mERα signaling for the male skeleton. From these two studies, we have learned that mERα signaling alone cannot affect trabecular bone, but it can affect cortical bone, and that for full ERα-mediated effects, both nuclear and mERα signaling is needed. In fact, depending on which bone compartment we want to affect, different signaling pathways of ERα can be targeted by a novel SERM or a compound such as EDC. If we want to only affect cortical bone, we could either design a SERM that does not affect ERαAF-1, or a compound that only binds to mERα.

With this thesis, the puzzle of estrogen signaling pathways has gained new pieces. Design and improvement of bone-specific SERMs with minimal adverse effects is a challenge for future osteoporosis research and the new pieces contributed by this thesis may facilitate the strategies for designing new SERMs for treatment of both female and male osteoporosis.

7 FUTURE PERSPECTIVES

Although the conclusions from the papers included in this thesis increase our knowledge regarding the role of ERα in the regulation of bone mass, there is still much remaining work to be done in this field.

The conclusions from this thesis are based on interpretation of results obtained from mice with global and embryonic inactivation of the whole or part of the ERα gene. It is possible that such genetic event creates developmental consequences that could affect the bone phenotype observed in adults. To avoid this, gene manipulation by inducible methods could be used. For example, instead of using the POMC-ERα$^{-/-}$ mouse model in paper I, we could silence ERα in the ARC with the same approach as we did in the VMN study, AAV-shRNA. Another alternative to avoid confounding effects that result from developmental compensations is to use a bone-cell specific KO. For example in paper IV, we do not know whether a specific cell type is responsible for mediating extra-nuclear ERα signaling. Thus, further investigations in e.g., cell specific NOER mouse models, are required to dissect the role of mERα signaling in each specific cell type.

One of the most important and recent issues about osteoporosis is to separate osteoporosis induced by hormone depletion from age-related osteoporosis. Recent genetic studies using mouse models have demonstrated that effects of estrogen deficiency and aging on the skeleton are independent and result from distinct mechanisms (203). Moreover, results from mouse models and supportive human data have demonstrated an increase in senescent cells in the bone microenvironment with aging. Treatment strategies that target those cells have been shown to prevent age-related bone loss in mice (204). These new insights should be considered in future experiments.

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