DEPARTMENT OF WOMEN'S AND CHILDREN'S HEALTH
Karolinska Institutet, Stockholm, Sweden

ESTROGEN ACTIONS IN THE GROWTH PLATE CARTILAGE

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Stockholm 2020

Cover photo: Estrogen receptor alpha expression in the control human growth plate tissue and the hormones and exogenous factors affecting bone growth.

Published by Karolinska Institutet.
Printed by Eprint AB 2020

ISBN 978-91-7831-801-8

Estrogen actions in the growth plate cartilage

THESIS FOR DOCTORAL DEGREE (Ph.D.)

Wednesday 15th of April, 2020, 9.30 a.m.

Karolinska Universitetssjukhuset Solna, BioClinicum J3:12,
Conference room Nanna Svartz, Solnavägen 30

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To my beloved family

I am neither at the beginning of the story nor at the end. Hopefully, I am at the end of the good beginning!

ABSTRACT

Estrogens may influence bone growth locally or systemically via the estrogen receptors alpha (ERα), beta (ERβ) and G protein-coupled estrogen receptor 1 (GPER-1). **In Paper I**, our study showed that the treatment of ovariectomized C57BL/6 mice with a selective ERα agonist 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT) reduced growth plate height and hypertrophic zone height suggesting that the effect was induced via ERα. Furthermore, chondrocyte proliferation in the growth plate was also inhibited by 17β-estradiol (E2) or PPT as evaluated by proliferating cell nuclear antigen (PCNA) staining. Furthermore, tibiae and femur bones were shorter in E2- or PPT-treated mice when compared to vehicle-treated controls. In contrast, bone lengths in mice treated with a selective ERβ agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) were similar to bone lengths in controls. These results showed that estrogenic effects on bone growth and growth plate maturation are mainly mediated via ERα. In contrast, the selective GPER-1 agonist G1 had no effects on either metatarsal bone growth ex vivo or tibia and femur growth in treated metatarsals or mice when compared to control groups. Thus, the results from **Paper I and II** demonstrated that, ligand stimulation of GPER-1 and ERβ does not influence bone growth in mice.

In **Paper III**, target genes and signaling pathways affected by E2 were identified. The enriched pathways inhibited by E2 included estrogen response early and late, apoptosis, epithelial mesenchymal transition and angiogenesis. Also, the mammalian target of rapamycin (mTOR) signaling pathway, which regulates chondrocyte proliferation and differentiation, was significantly inhibited by E2. Among the most strongly affected genes, the expression of peptide YY, a negative regulator of bone formation and mineral density, was inhibited by E2 treatment. Furthermore, epidermal growth factor and oxidative phosphorylation signalling pathways and subgroups of genes regulated by Myc and genes important for mitotic spindle assembly were among the enriched pathways upregulated by E2.

Our data showed that E2 actions on bone growth and growth plate maturation are mainly mediated via ERα. In contrast, ligand stimulation of either ERβ or GPER-1 did not influence bone growth in mice. Also, our study has identified target genes and pathways influenced by E2 in the growth plate. Further studies are required to determine the specific mechanisms involving E2-regulated genes. Our findings may have direct implications for the development of new and more selective treatment modalities of extreme tall stature using selective ER modulators that may have fewer side effects than high-dose E2 treatment.

LIST OF SCIENTIFIC PAPERS

I. **Regulation of bone growth via ligand-specific activation of estrogen receptor alpha**
MARYAM IRAVANI, Marie Lagerquist, Claes Ohlsson and Lars Sävendahl
J Endocrinol. 2017 Mar;232(3):403-410. doi: 10.1530/JOE-16-0263. Epub 2016 Dec 20

II. **Effects of the selective GPER-1 agonist G1on bone growth**
MARYAM IRAVANI, Marie K Lagerquist, Elham Karimian, Andrei S Chagin, Claes Ohlsson and Lars Sävendahl
Endocr Connect. 2019 Aug 1. pii: EC-19-0274.R2. doi: 10.1530/EC-19-0274

III. **Estradiol-mediated gene expression profiles in human growth plate cartilage**
MARYAM IRAVANI, Phillip T. Newton, Artem Artemov, Andrei S. Chagin and Lars Sävendahl
Manuscript

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LIST OF ABBREVIATIONS

AF-1	Activation function 1
ALPL	Alkaline phosphatase
AP1	Activator protein 1
AR	Androgen receptor
AXIN2	Axis inhibition protein 2
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
Col2α1	Collagen Type II Alpha 1
CYP19A1	Cytochrome P450 Family 19 Subfamily A Member 1
DMEM/F12	Dulbecco's modified Eagle medium and Ham's F-12
E2	17β-estradiol-3
ECM	Extracellular matrix
ephA2	Ephrin type-A receptor 2
ERα	Estrogen receptor α
ERβ	Estrogen receptorβ
ERβKO	Estrogen receptor β knockout
ERK	Extracellular signal-regulated kinases
FGF	Fibroblast growth factors
FGFR3	Fibroblast growth factor receptor-3
DAPI	4′,6-diamidino-2-phenylindole dihydrochloride
DBD	DNA-binding domain
DPN	2,3-bis(4- hydroxyphenyl)-propionitrile
GPER-1	G protein-coupled estrogen receptor 1
GPER-1-KO	G protein-coupled estrogen receptor 1 knockout
GH	Growth hormone
HOXB7	Homeobox 7
IGF-1	Insulin-like growth factor I
IGFBP4	IGF binding protein 4
Ihh	Indian hedgehog protein
JAK-STAT1	Janus kinase-signal transducer and activator of transcription 1
LBD	ligand binding domain
MMP	Matrix metalloproteinase

MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
Nr5a2	Nuclear Receptor Subfamily 5 Group A Member 2
NTD	NH2-terminal domain
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OVX	Ovariectomized
Pax7	Paired box protein 7
PCNA	Proliferating cell nuclear antigen
PI3K/Akt	Phosphatidylinositol 3-kinase/Akt
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related peptide
PPT	1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole
PYY	Peptide YY
RNA-seq	Ribonucleic acid sequencing
Runx2	Runt-related transcription factor 2
SARM	Selective androgen receptor modulators
SERM	Selective estrogen receptor modulators
Sox	Sex-determining region Y-related high mobility group box
Sp1	Specificity protein 1
SP6	Transcription factor SP6
TGF-β	Transforming growth factor β
TUNEL	Terminal deoxynucleotidyltransferase (TdT)-mediated deoxy-UTP nick end labeling
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 PHYSIOLOGY AND STRUCTURE OF THE GROWTH PLATE

1.1.1 Functional morphology of the growth plate

Longitudinal bone growth originates in the epiphyseal growth plate and is regulated by various mechanisms, which control the size and the shape of the body [1]. The growth plate is not vascularized and is formed by chondrocytes surrounded by extracellular matrix (ECM). The growth plate closure in most species follows skeletal maturation. The primary phase of longitudinal bone growth includes deposition of cartilage. This process is followed by cartilage degradation caused by calcification and infiltration by bone-building cells, which construct osseous tissue. These mechanisms of degeneration and regeneration take place within the growth plate located between the epiphysis and metaphysis at the ends of the long bones [2].
Histologically, the growth plate is mostly formed by chondrocytes located in columns along the axis of the bone. There are three different zones in the growth plate containing cells with different stages of differentiation [3]. These zones include: a) the resting zone, formed by stem cells; b) the proliferative zone, containing flat cells; c) the hypertrophic zone, containing hypertrophied cells. During endochondral ossification, newly generated cartilage develops into the bone tissue. The direction and degree of the growth depend on the processes of proliferation and the activity of extracellular matrix. The cells of the resting zone serve as a constant supply for bone growth. The chondrocytes of the resting zone eventually migrate to the proliferative zone, where they elevate their division rate. Moreover, in the proliferation zone, the chondrocytes form dense cellular columns. The spatial orientation of these columns dictates the future direction of the bone growth. In the hypertrophic zone, chondroblasts differentiate into hypertrophic chondrocytes by undergoing significant phenotypic changes [4]. These transitional changes include cytoplasm expansion and upregulation of cartilage matrix genes at mRNA level.

1.1.2 Regulation of chondrocyte proliferation and hypertrophy

After undergoing hypertrophy, chondroblasts cease the expression of matrix genes and differentiate into prechondrocytic cells under control of Sex-determining region Y-related high mobility group box 5 (Sox5) and Sox6 [5]. Chondroblasts also proliferate under the control of Runx2, which regulates the positioning of columns by mediating cell proliferation.

Moreover, Runx2 was found to be active in hypertrophic chondrocytes, but was suppressed in chondroblasts [6]. Thus, Runx2 is considered as an activator of maturation markers expressed by chondrocytes. After the increased division in the proliferative zone, the chondrocytes abandon cell cycle and commence to augment in the hypertrophic zone. Ultimately, the chondrocytes become apoptotic and enhanced tissue vascularization and calcification adjourn the growth plate activity.

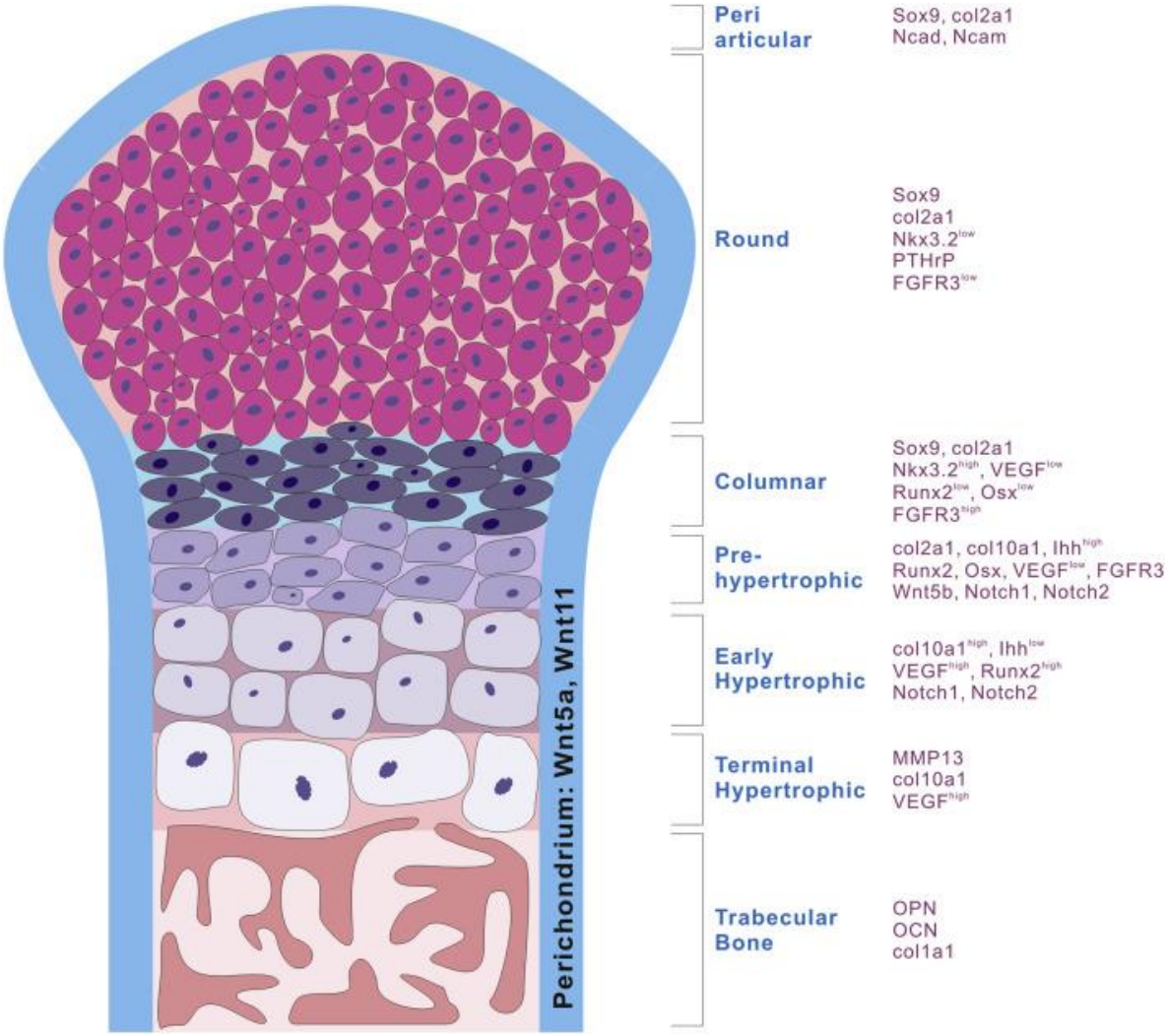

Figure 1. Regulatory gene expression in the growth plate zones and the perichondrium [7]. There is a variety of physiological genes and signaling pathways regulating chondrogenesis. These growth regulators include Hedgehog, bone morphogenic proteins (BMPs), fibroblast growth factors (FGFs) and vasculogenic signaling pathways.

1.2 LOCAL AND SYSTEMIC FACTORS REGULATING BONE GROWTH

1.2.1 Nutrition, exercise and bone health

Nutrition and exercise are the two factors, which are vital for bone development and bone health. The importance of the key nutrients, such as calcium, magnesium, phosphorus, potassium and other microelements and vitamins D, A, K, C and B has been emphasized by multiple studies [8]. Vitamin D regulates calcium and phosphate absorption and calcium release from the bone [9]. In line with this, vitamin D deficiency induces rickets and osteomalacia, which are represented by impaired mineralization during skeletal development and adulthood, respectively [10]. Moreover, lack of vitamin D blocks chondrocyte maturation followed by chondrocyte hypertrophy and broadening of the growth plates.

Exercises have been shown to protect bone health against skeletal disorders, such as osteoporosis [11]. Physical activity might positively influence bone health by affecting multiple factors, such as release of hormones, cytokine secretion and activation of regulatory signaling pathways. In particular, exercises were shown to activate the production of several hormones regulating bone growth such as estradiol (E2) and parathyroid hormone (PTH). Moreover, there was a clear correlation between exercises characterized by elevated forces applied on the body and increased bone mass density and strength [12]. Also, physical exercises have been demonstrated to enhance bone formation [13]. Furthermore, mechanical loadings caused by exercises activate osteoblast differentiation and regulate bone metabolism [14].

1.2.2 Systemic factors regulating bone growth

Bone growth and metabolism are regulated by a spectrum of various hormones. Thyroid, parathyroid and sex hormones show the most notable effects. Among them, thyroid hormones play a crucial role in the development, growth and metabolism of bone tissue. The expression of thyroid receptors was found in the chondrocytes located in the proliferative zone of the growth plate and osteoblasts [15]. Being the main regulator of calcium and phosphate levels, PTH influences bone homeostasis acting via specific receptors expressed by osteoblasts. Several in vivo studies have demonstrated that PTH enhances bone growth via stimulation of proliferation and inhibition of apoptosis in osteoblasts and their precursors [16].

Among other hormonal factors, growth hormone (GH)/insulin-like growth factor 1 (IGF-1) axis is vital for the longitudinal bone growth. During prepubescence, GH is similarly produced in boys and girls. However, both GH and IGF-1 production raises earlier in girls than in boys during puberty [17]. Under the regulation by GH produced in the hypophysis, the hepatocytes

secrete IGFs, which regulate the bone and cartilage physiology. Noticeably, both GH and IGF-1are locally produced by osteoblasts and chondrocytes [18]. Both GH and IGF-1 deficient animal models demonstrate diminished periosteal and longitudinal bone growth. Furthermore, GH receptor or IGF-1 receptor deficient models showed that GH regulates synthesis of IGF-1 in the liver and IGF-1 exert its effects both locally and systemically.

Also, androgens were shown to enhance longitudinal bone growth. The effects of androgens in the bone are mediated via androgen receptors (ARs) expressed in both osteoblasts and osteocytes [19]. Moreover, ARs, which were detected in osteocytes, are involved in the regulation of skeletal integrity [20]. An impaired bone development due to affected osteoblasts differentiation was found AR-deficient mice [21]. Furthermore, selective androgen receptor modulators (SARMs), which stimulate ARs, were demonstrated to elevate BMD and bone strength in animal models [22]. In contrast, a study by our group has shown that AR modulation by neither androgens nor the selective AR inhibitor affect growth of rat metatarsal bones in vitro [23].

1.2.3 Signaling pathways regulating bone growth

Differentiation of chondrocytes is a crucial step in bone development. A plethora of local and systemic regulatory signaling factors, which control the differentiation of chondrocytes from mesenchymal progenitors to mature hypertrophic chondrocytes, includes Indian hedgehog protein (Ihh), fibroblast growth factors (FGF), SRY-related high-mobility group-box gene 9 (Sox9), bone morphogenic proteins (BMP), parathyroid hormone-related peptide (PTHrP), and others (Fig. 1).

Among these factors, Indian Hedgehog (Ihh) is one of the key regulators of osteoblast differentiation. The expression of Ihh was found in the prehypertrophic chondrocytes in the growth plate. The interplay between Ihh and parathyroid hormone-related peptide (PTHrP) regulates the differentiation processes in the growth plate and longitudinal bone growth [24]. In particular, Ihh activates PTHrP expression in chondrocytes. As a consequence, PTHrP is transported to the growth plate to stimulate chondrocyte proliferation. Further, chondrocytes halt the cell cycle and proceed to hypertrophy when PTHrP expression declines [25]. Also, previous studies have shown that Ihh-deficient mice have increased numbers of hypertrophic chondrocytes caused by early migration of chondrocytes from the group of proliferating growth plate cells. This event is induced by the inability of the cartilage in Ihh-deficient mice to produce PTHrP. Moreover, since Ihh is necessary for osteoblast development, these mice show decreased osteoblast fraction [26]. In addition, the bone development depends on the interactions between Ihh and Wnt/β-catenin signaling. Moreover, overreactivity of Ihh and

Wnt/β-catenin signaling leads to either deficient bone growth due to osteoporosis or ossification loci in different tissues.

In addition to Ihh pathway, fibroblast growth factor (FGF) signaling is an important pathway controlling chondrocyte expansion and differentiation. During chondrocytes development, FGF receptor-3 (FGFR3) is expressed in proliferating cells, while FGF receptor-1 is detected in hypertrophic cells. Also, FGF receptor-3-deficient mice demonstrate an enhanced chondrocyte proliferation [27]. Moreover, mutations in human FGFR3 suppress chondrocyte proliferation and induce disorganization of chondrocyte columns [28]. Several studies have shown that FGFR3-mediated suppression of proliferation by FGF is regulated via stimulation of JAK-STAT1 signaling pathway [29]. Overall, FGF makes chondrocyte columns shorter by suppressing proliferation and Ihh expression.

1.2.4 Functions of BMPs, VEGFs and mTOR in the regulation of bone growth

Also, bone morphogenic proteins (BMPs), which belong to the TGFβ family of transcription factors, are vital for cartilage and bone development. Signaling by TGF-β/BMPs includes Smad-dependent and Smad-independent pathways. The development of skeleton involves the activation of both Runx2 and TGF-β/BMPs-stimulated Smads [30]. The expression of BMP family members, BMP2, -6 and -7 was found in both proliferating and hypertrophic chondrocytes. Treatment with BMPs was demonstrated to elevate chondrocyte proliferation and suppress differentiation of hypertrophic chondrocytes [31, 32]. Noticeably, BMP and FGF signaling pathways demonstrate antagonistic effects on cellular proliferation in the growth plate [33]. As a growth activator, exposure to BMP2 stimulated chondrocyte proliferation primarily reduced in a mouse model of achondroplasia [31]. BMP proteins were shown to activate condensation of mesenchymal stem cells and enhance chondrocyte proliferation.

Although chondrocytes and osteoblast belong two distinct cellular lineages, the precursors of osteogenic and chondrogenic cell lineages were found to differentiate from mesenchymal stem cells under the regulation of multiple transcriptional factors [34, 35]. Thus, mesenchymal stem cells demonstrate high chondrogenic potential (Fig. 2). In contrast, bone resorbing osteoclasts might originate from the bone marrow hematopoietic stem cells [36].

In addition, the mechanisms of bone remodeling are controlled by growth factors, such as vascular endothelial growth factor (VEGF), which are crucial for regulation of vasculogenesis

in the bone. With regard to bone formation, VEGF is known to stimulate cartilage development and its replacement by bone [37]. Furthermore, mTOR signaling pathway has recently been found to be important for bone development, since mTOR deficiency led to bone growth impairment [38]. Moreover, activation of chondrocyte proliferation and suppression of terminal differentiation by mTORC1 has recently been shown [39]. In addition, the mice deficient in TSC1, a suppressor of mTOR, showed increased chondrocyte proliferation but inhibited chondrocyte differentiation.

The extracellular matrix of the growth plate consists of collagen types II, VI, X, and XI and proteoglycans. Collagen type X is produced by hypertrophic chondrocytes in the part of the growth plate which undergoes matrix calcification [40, 41].

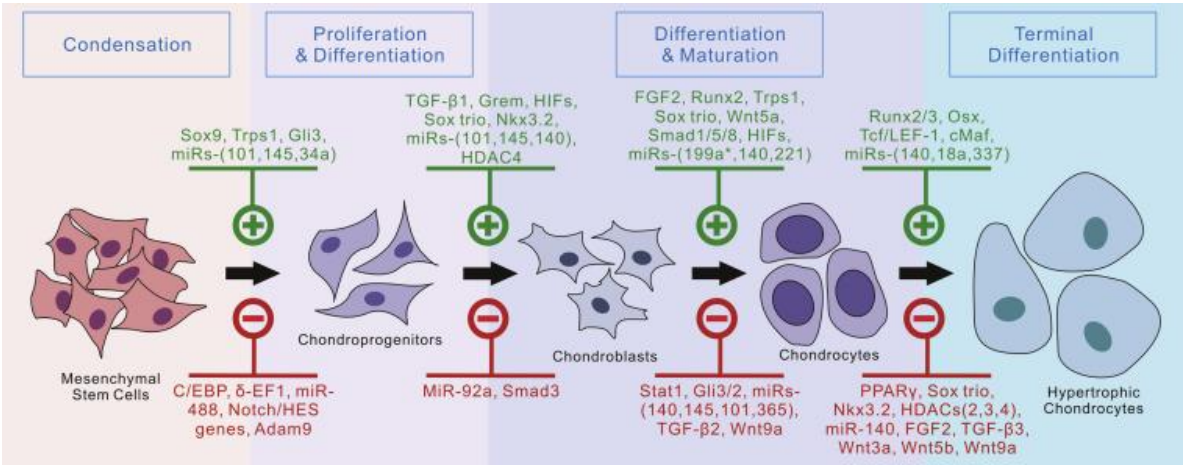

Figure 2. Currently known regulators of chondrogenic proliferation and differentiation from mesenchymal stem cells. The regulatory factors include transcription factors, signaling molecules and microRNAs. Positive regulatory factors are shown in green, while the inhibitory regulators are shown in red [7].

1.2.5 ESTROGENS AND THEIR RECEPTORS IN THE BONE

Sex hormones are known to influence bone growth and metabolism. It is well established that trabecular bone mass notably diminishes during menopause; however, this mechanism can be suppressed by estrogen treatment. Recent studies indicate that estrogens play an essential role in maintaining bone homeostasis also in men. Furthermore, E2 was suggested to regulate BMD in men, since raloxifene, a selective ER modulator, elevated BMD in hip and spine in male

patients [42]. In line with these findings, aged male patients, who were treated with an aromatase inhibitor anastrozole, had enhanced bone resorption and suppressed markers of bone development [43].

1.2.6 Effects of E2 on bone growth

Predisposition of men to bone loss and fractures might be related to variability in Cytochrome P450 Family 19 Subfamily A Member 1 (CYP19A1), which modifies serum E2 levels. Also, an association of a single-nucleotide polymorphism in CYP19A1 with increased E2 levels was observed in men [44].

Moreover, E2 decreases the degree of bone remodeling and assist to keep the equilibrium between bone resorption and development. These functions are mediated via the regulation of osteoblast and osteoclast proliferation. Besides, estrogens influence the growth of both osteoblasts and osteoclasts by inducing apoptosis in osteoclasts and inhibiting this process in osteoblasts. Decreased levels of E2 induce loss of bone tissue accompanied by the enhanced bone remodeling, elevated osteoblasts and osteoclasts counts and bone resorption [45]. Many physiological processes, including growth, development and differentiation, are regulated by estrogens. The main estrogen, E2, is primarily synthesized in the ovaries in premenopausal women, while in men and postmenopausal women E2 is converted from testosterone and androstenedione in extragonadal tissues [46]. Besides the effects on normal cells, E2 is also involved in various pathological processes, including malignancies [47].

1.2.7 Regulation of GH/IGF-1 signaling in bone by estrogens

Studies *in vitro* and *in vivo* have shown that estrogen can be produced by chondrocytes [48-51]. This is in line with the observations of P450 aromatase expression in both human [48] and rat [50, 51] growth plates. Besides, the locally produced estrogen is suggested to regulate proliferation of chondrocytes and to prevent chondrocytes from undergoing apoptosis [51]. In particular, low levels of E2 stimulate bone growth during early puberty, while high levels during late maturation suppress bone growth by inducing growth plate fusion [52]. Since E2 is known to influence the GH/IGF-1 pathway, the regulation of bone growth by E2 might be partially mediated via the effects on the GH/IGF-1 axis [53].

Notably, suppression of IGF-1 was observed in male ERα-deficient mice and aromatase inhibitor-exposed mice in spite of enhanced androgens, which suggested that androgens might regulate IGF-1 through stimulation of ERα and aromatization. In addition, E2 was shown to be

a crucial regulator of IGF-1 production in the liver of male GH-receptor-deficient mice, since E2 completely revived periosteal bone growth in these animals [54]. Thus, several studies have proved that the interactions between estrogens and the GH-IGH-1 pathway are vital in the determination of sexual dimorphism during puberty. Post-pubertal effects of estrogens via GH-IGF-1 remain to be verified.

1.2.8 Structure and functions of nuclear and membranous estrogen receptors

The nuclear estrogen receptors alpha (ERα) and beta (ERβ) are the main receptors mediating the effects of E2 [55]. Originally, ERα expression was found in the rat uterus in 1964 and extensively characterized afterwards [56]. In contrast, ERβ, another nuclear E2-binding receptor, was discovered in the rat prostate two decades later [57]. Both receptors were reported to act as transcription factors that bind estrogen response elements (ERE) in the E2-regulated genes.

Structurally, both ERα and ERβ consist of three domains: the NH2-terminal domain (NTD), the DNA-binding domain (DBD), and the ligand binding domain (LBD) [58] (Figure 1). The NTD contains an activation function (AF-1) region regulating ligand/independent transcriptional activation of target genes. The DBD regulates the binding of the ERs to the target DNA sequences called ERE. Upon binding to EREs, the ERs affect transcription of target genes. Alternatively, ERs regulate gene expression by interacting with the transcription factors, including the specificity protein 1 (Sp1), the activator protein 1 (AP1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and p53 [59]. In addition to direct effects on gene transcription, the ERs are involved in rapid cytoplasmic activation of phospholipase C and protein kinase pathways [60].

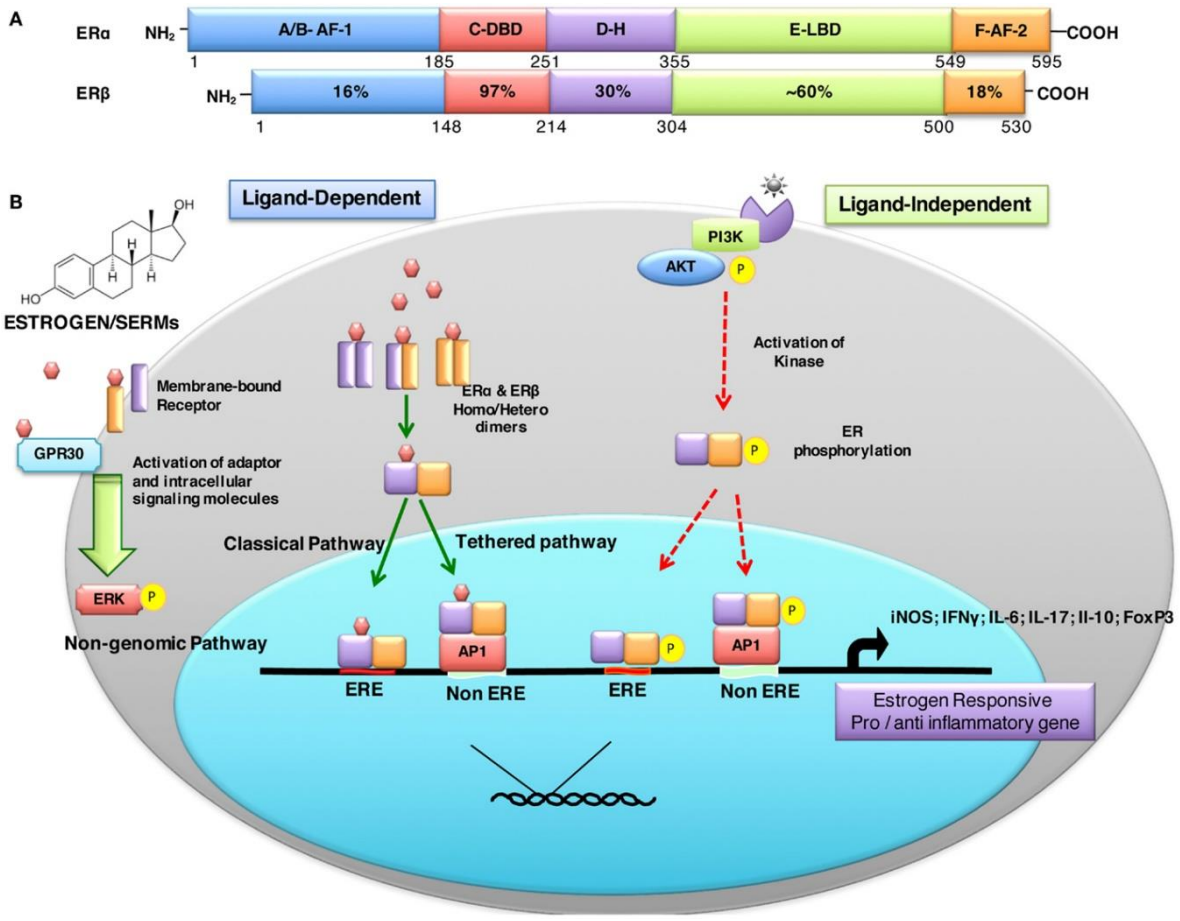

Figure 3. Structure of human ERα and ERβ and overview of ER ligand-dependent and ligand-independent signaling pathways. (A) Structural and functional ER domains: DNA-binding domain, hinge domain, ligand-binding domain and transcriptional activation domains AF-1 and AF-2. (B) ER ligand-dependent (green arrows) and ligand-independent (red arrows) signaling.

Although ERα and ERβ bind E2 with similar affinity, structural differences in the LBD domain allowed to develop subtype-specific selective ligands for ERα and ERβ [55]. Selective ER agonists are effectively used to identify the specific functions of the ERs. In particular, propyl pyrazole triol (PPT) has a 410-fold selectivity for ERα versus ERβ [61]. With regard to ERβ, 2,3-bis(4- hydroxyphenyl)-propionitrile (DPN) is a selective ERβ agonist which has a 70-300-fold selectivity and is widely used to study the functions of ERβ [62, 63]. Following the ligand-dependent binding, the nuclear ERs compose homodimers or heterodimers. Animal and *in vitro* studies have shown different transcriptional effects of ERα and ERβ [64] and different cellular reactions for each specific ligand [65].

In addition to the differences in transcriptional responses, the distribution of the ERs varies in different tissues. While ERα expression is mainly observed in the uterus, ovaries, breast, kidney, adipose tissue and liver, ERβ is primarily found in central nervous system,

cardiovascular system, lung, male reproductive organs, prostate, colon, kidney and immune system [55, 66]. With regard to bone, both ERα and ERβ are highly expressed in the growth plate [67].

In addition to nuclear ERs, membrane-expressed GPER-1 was demonstrated to bind E2 with high affinity *in vitro* [68] and was originally identified in lung, heart, brain and lymphoid tissues [69, 70] and breast cancer and Burkitt's lymphoma cell lines [70, 71]. Previous studies have shown that activation of GPER-1 by E2 or selective GPER agonists activates cAMP production and proto-oncogene tyrosine-protein kinase Src, leading to the stimulation of MMPs. A series of the following events leads to the activation of MAPK and PI3K/Akt signaling pathways resulting in changes in gene transcription [72].

With regard to the bone, GPER-1 was found to be expressed in the epiphyseal growth plate with the highest level of expression in hypertrophic chondrocytes [73]. Moreover, the expression of GPER-1 was found in human osteoblasts, osteoclasts, and osteocytes [74]. This membrane ER is involved in rapid non-genomic E2 signaling and is highly expressed in the hypertrophic zone of the bone growth plate.

The expression of GPER-1 is downregulated toward puberty, indicating that GPER-1 may be involved in the regulation of longitudinal bone growth [73]. Moreover, GPER-1 may mediate E2 effects in the growth plate, since E2 does not affect bone growth height and bone length in the GPER-$1^{-/-}$ mice [75].

1.2.9 ER target genes and pathways in bone

Although the target genes regulated by estrogens in the bone have not been clearly identified, E2 has been demonstrated to suppress the bone resorption through ephA2/ephrinA2 signaling pathway [76]. Furthermore, the protective effect of E2 on cartilage via ERK-mammalian target of rapamycin (mTOR) signaling has also been shown [77].

In addition to the effects on bone remodeling, the development of osteoporosis has been known for decades to be highly dependent on E2 levels. A potential mechanism of osteoporosis might be related to the lower sensitivity of bone tissue to mechanical loading. The decrease in sensitivity in this case is caused by E2 deficiency. In line with this statement, ERα is known to stimulate the sensitivity of bone to mechanical loading by stimulation of the Wnt signaling [78], although the influence of ERα on mechanical loading did not necessitate ligand binding.

1.2.10 ERα target genes in osteoblasts

With regard to ERα target genes in osteoblasts, ChiP-on-chip analysis has revealed that ERα regulates the expression of a large number of crucial osteoblast genes, such alkaline phosphatase Alpl, receptors GPER-1 and Nr5a2 and several transcription factors (Pax7 and Sox5) [79]. Also, the IGF binding protein 4 (*IGFBP4*) was proved to be an ERα target, which demonstrates the interactions between ER and IGF signaling [80]. In addition, ERα-mediated effects on matrix metalloproteinase 3 influence FasL-mediated apoptosis in osteoclasts [81].
High doses of E2 were shown to decrease height in young females with constitutional tall stature [82, 83]. However, therapy with high doses of E2 may have potential side effects, including decreased fertility [84], elevated risk of breast and ovarian cancers [85, 86], malignant melanoma [86] and vein thrombosis [87]. On the contrary, inhibition of E2 synthesis with an aromatase inhibitor increased predicted adult height in young patients with idiopathic short stature [88]. Although, treatment with an aromatase inhibitor was shown to increase the prevalence of vertebral deformities [89].

1.2.11 Effects of SERMs on bone growth

Several generations of selective estrogen receptor modulators (SERMs) are currently available for treatment of bone growth disorders. Tamoxifen, a first-generation SERM, is used in the treatment of breast cancer [90]. In addition to anti-cancer applications, tamoxifen was used to treat young males with gynecomastia [91, 92] and McCune-Albright syndrome [93]. With regard to the effects on bone growth, tamoxifen had either no effect or decreased longitudinal growth [91-93]. In addition, tamoxifen was shown to suppress the growth of longitudinal bones in young male rats by increasing chondrocyte apoptosis, decreasing growth plate height and the levels of IGF-I [94]. Moreover, tamoxifen decreased both endosteal and periosteal bone growth in young male rats [94]. These results suggest that treatment with tamoxifen may negatively affect bone growth in young patients.

With regard to other SERMS, raloxifene acts as an ER agonist in bone and is used to prevent postmenopausal osteoporosis. Also, it was shown to suppress longitudinal bone growth in rats [95]. However, it accelerates epiphyseal fusion in ovariectomized (OVX) rabbits [96].

In addition, aromatase-deficient male patients were found to have decreased bone mass. Since aromatase mediates conversion of androgens into estrogens, estrogen was suggested to regulate the development of male bones [97]. In male skeleton, the local production of estrogens from

testosterone is catalyzed by aromatase and supports protective functions of estrogens. Notably, aromatase-deficient individuals demonstrate symptoms of osteoporosis and unclosed epiphyses similarly to patients lacking ERα [98].

1.3 FUNCTIONS OF THE ERS IN THE REGULATION OF BONE GROWTH

1.3.1 ERα is the predominant ER in bone

Several studies of patients with inactive ERα gene or aromatase deficiency suggested that E2 regulates growth plate closure [99-102]. In addition, a clinical report of an 18-year-old female patient with mutated ERα gene indicated that ERα mutations might induce E2 resistance. Moreover, the clinical symptoms of the patient with the ERα mutation were similar to the abnormalities found in the phenotypical changes in the mouse ERα knockout [102].

In addition to clinical data, studies using ERα, ERβ and GPER-1 deficient mouse models showed that the effects of E2 in both trabecular and cortical bone are mainly mediated through ERα. OVX mice were shown to lose bone mass, which can be restored by E2 treatment. However, in ERα$^{-/-}$ mice treatment with E2 did not restore the bone mass, suggesting the importance of ERα for bone growth [103].

In contrast, both ERβ and GPER-1 were not shown to be important for mediating estrogenic effects on bone growth [103]. The studies using female ERβ$^{-/-}$ mice demonstrated that ERβ has only moderate effects in bone by modulating ERα actions in female mice [103]. Moreover, studies in ERα$^{-/-}$, ERβ$^{-/-}$ and ERαβ$^{-/-}$ mice found that ERβ inhibits ERα-mediated gene transcription in bone when ERα is expressed [104].

Estrogens are proposed to affect bone growth and growth plate closure either via GH and IGF-I or via ERα which is highly expressed in the chondrocytes of the growth plate (Fig. 4) [103]. Thus, low levels of E2 in early sexual maturation stimulate bone growth through the GH/IGF-I axis, while the high levels of E2 during late sexual maturation or exposure to high doses of E2 suppress bone growth directly by activating ERα locally expressed in the growth plate.

A mouse model with selective knock-out of ERα in cartilage (Col2α1-ERα$^{-/-}$) was generated to study these bone growth regulatory pathways [105]. Comparison of male ERα$^{-/-}$ and male Col2α1-ERα$^{-/-}$ mice showed a reduction of bone growth in ERα$^{-/-}$ mice, while the bone length

was not affected in Col2α1-ERα$^{-/-}$ mice [105]. The suppressed bone growth in male ERα$^{-/-}$ mice correlated with decreased serum concentration of IGF-I and reduced GH secretion, suggesting that in male mice the GH/IGF-I pathway mediates the regulation of bone growth via ERα and does not require ERα expression in the growth plate [18].

To study the potential functions of local ERα in the process of growth plate closure, gonadectomized Col2α1-ERα$^{-/-}$ mice were exposed to a high dose of E2. No changes were detected in Col2α1-ERα$^{-/-}$ mice, while the vehicle-treated group showed shortened growth plate height in response to E2 treatment. These results suggested that the growth plate height in both male and female mice is reduced by the E2 action via locally expressed ERα. Also, knock-in mice with an ERα impaired of DNA binding (NERKI mice) showed several bone abnormalities, such as defective bone development [106] and suppressed osteoblast activation markers [107]. In addition, ERα, locally expressed in the growth plate, was shown to mediate age-related decrease of longitudinal bone growth, since both female Col2α1-ERα$^{-/-}$ and female ERα$^{-/-}$ showed continued longitudinal bone growth [105, 108].

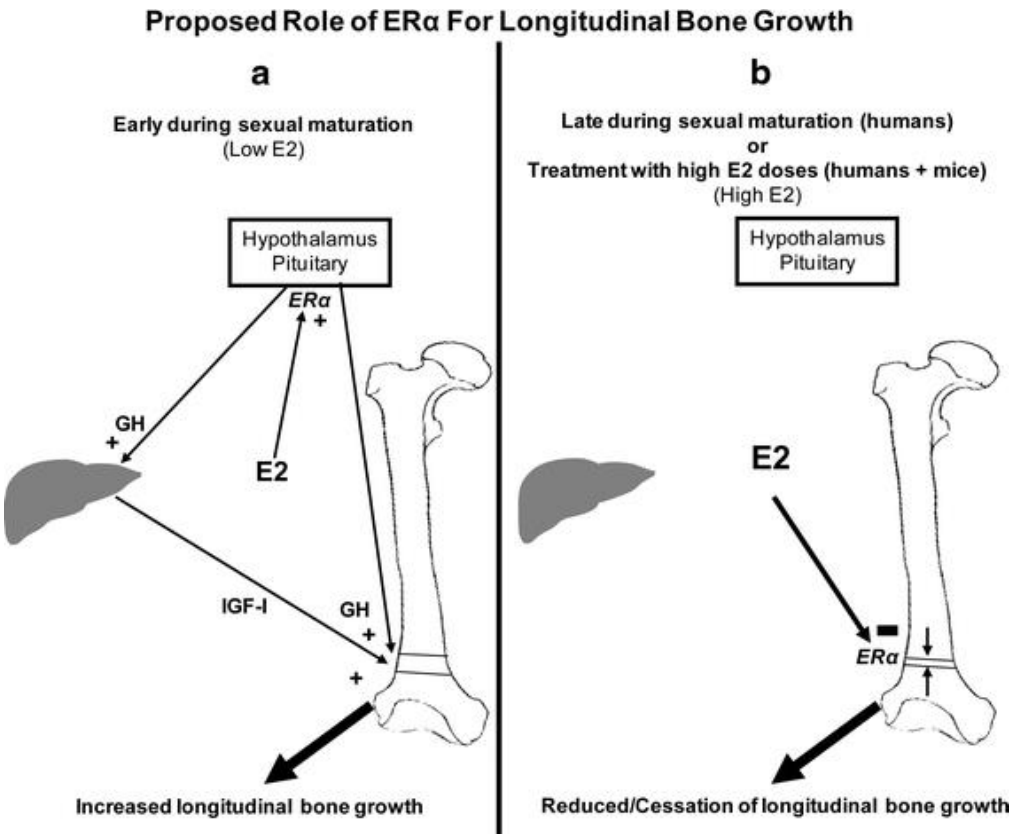

Figure 4. The role of ERα in longitudinal bone growth. The regulation of bone growth by ERα includes two potential mechanisms: **a.** Indirect GH/IGF-I-mediated effects, which regulate bone growth

during early puberty associated with low serum E2 levels and do not involve ERα in the growth plate, and **b.** Direct actions of locally expressed ERα, which is activated by high doses of E2 and suppresses growth plate height in mature mice and longitudinal bone growth in elderly mice. + Stimulatory effects, - suppressive effects [108].

Growth plate closure in rodents may be induced by high doses of E2 [109]. To study the involvement of local ERα in the mechanisms regulating growth plate closure, male and female Col2α1-ERα$^{-/-}$ and WT mice were castrated and treated with high doses of E2 or vehicle. Both Col2α1-ERα$^{-/-}$ and WT mice showed similar effects of E2 on bone mass and uterus [105]. While WT mice had decreased growth plate height in response to E2 treatment, Col2α1-ERα$^{-/-}$ mice did not show any changes in growth plate size after they were treated with E2, indicating that reduction of growth plate height by E2 involves ERα, locally expressed in chondrocytes [105]. In addition, the histological evaluation of growth plates in WT mice revealed that the reduction of the growth plate height was related to a decrease of the proliferative zone with the size of the hypertrophic zone being unchanged. In contrast, no reduction was observed in either the proliferative or hypertrophic zones in the growth plates in Col2α1-ERα$^{-/-}$ mice [105]. Interestingly, the mice with an inactivation of AF-1 (ERαAF-1^{0}) also showed a decreased size of the proliferative zone before closure of the growth plate, indicating that the senescence of the growth plate is regulated via local ERα independently of ERα AF-1. [108]. These findings in mouse models, including Col2α1-ERα$^{-/-}$ mice, indicate that ERα, expressed in the growth plate, may be crucial for growth plate senescence in humans and that the reduction of bone growth induced by high levels of E2 is mediated directly through locally expressed ERα [105]. Thus, ERα, expressed in growth plate chondrocytes, is suggested to be the main ER regulating growth plate senescence and cessation of longitudinal bone growth.

The actions of ERα in the bone might also depend on the involvement of ERα AF-1 and AF-2. In particular, gonadectomized AF-2-deficient mice do not respond to E2 treatment, suggesting the important functions of ligand-activated AF-2 in bone physiology [110].

1.3.2 Functions of ERβ in bone

No effects of ERβ on bone growth were observed using knockout models, since no bone impairments were found in male ERβKO mice [111]. Moreover, the trabecular response to E2 in OVX female ERβKO mice was found to be normal [112]. The expression of was found in

cancellous bone, while no was detected in cortical bone tissue [113]. A few studies have shown increased of longitudinal bone growth and decreased bone resorption, which was associated with elevated ERα RNA expression, in female ERβKO mice [114, 115]. In addition, decreased trabecular bone loss and enlarged cortical bone area have been detected in OVX ERβKO mice over long-term observation [116]. Moreover, ERβ-deficient mice showed faster regeneration after fracture [117]. Also, the mice with ERβ-deficient bone cells had enlarged trabecular bone volume [107]. A recent study has shown that in femoral bone tissue mRNA expression of several genes, which were affected by ovariectomy, was restored by the treatment with the selective ERβ agonist DPN. The study indicated that ligand-activated ERβ might regulate bone biology [118].

Among the membranous and nuclear ERs, ERα appears to be the predominant regulator of E2 effects on bone growth. Furthermore, ERβ demonstrates moderate inhibitory effects on ERα effects. However, the functions of ERβ in growth plate remain to be identified.

1.3.3 Functions of GPER-1 in bone

Membranous ER, GPER-1, might also play an important role in the regulation of bone growth. The regulation of rapid E2-mediated activation of signal-regulated kinases via this receptor, at that time known as GPR30, was initially demonstrated in 2000 [119]. Accumulated data indicated that GPR30 was an E2-binding receptor, which led to its entitlement as GPER-1 in 2007. Although, the functions of GPER-1 have been determined for various tissues, its effects in the bone remain to be uncovered. Notably, lower body weight, shorter body length and lowered IGF-1 in serum were observed in female GPER-1-KO mice [120]. In addition, ovariectomy prevented E2-regulated growth plate closure in GPER-1-deficient mice [75]. Also, another study found suppressed growth plate closure in GPER-1-KO mice [121]. The development of puberty was shown to negatively correlate with GPER-1 expression in the human growth plate [73]. Thus, the functions of ERβ and GPER-1 on human bone growth remain to be identified.

2 AIMS OF THE THESIS

Sex hormones are known to affect longitudinal bone growth. Recent studies demonstrate that estrogens play an important role in maintaining bone homeostasis. The effects of estrogens are mediated via estrogen receptors. All three main estrogen receptors, ERα, ERβ and GPER-1 are expressed in the growth plate. However, their functions in bone physiology have not yet been determined. Also, the target genes of E2 in the growth plate chondrocytes are not well known. The overall aim of this thesis was to investigate the ER-mediated effects of E2 in the growth plate cartilage.

The specific aims of this work were:

- To investigate the functions of ERα and ERβ on bone growth and in the growth plate cartilage (**Paper I**).
- To study the effects of activated GPER-1 on bone growth and in the growth plate cartilage (**Paper II**).
- To identify target genes and signaling pathways affected by E2 in human growth plate cartilage (**Paper III**).

3 MATERIALS AND METHODS

3.1 MATERIALS FOR IN VIVO AND EX VIVO STUDIES

3.1.1 MICE

For in vivo experiments, twelve-week-old female C57BL/6 mice (n = 9–10/group) and four-day-old mouse pups C57BL/6 were purchased from Charles River Laboratories. The adult animals were housed in the animal facility at the University of Gothenburg and kept in 12-h day and night condition with standard chow and tap water *ad libitum*.

3.1.2 METATARSAL CULTURE

For *ex vivo* organ cultures, the three middle metatarsal bones obtained from four-day-old mouse pups C57BL/6 were dissected out from each hind paw and cultured as described below.

3.1.3 PATIENTS

Human growth plate tissue samples were obtained from one 12-year and 3-month-old girl and one 13-year and 5-month-old boy, who underwent epiphyseal surgery due to constitutional tall stature. Growth plate biopsies from the proximal tibia and distal femur were taken using bone marrow biopsy needles (7 gauge; Gallini Medical Products and Services, Modena, Italy). The study was approved by the local ethics committee at Karolinska University Hospital, Stockholm, Sweden (ethical permit number: 97-214). The informed consent was obtained from both the patients and their parents.

3.1.4 REAGENTS

17β-estradiol-3-benzoate (E2) was purchased from Sigma-Aldrich and the ERβ-selective agonist DPN (2,3-bis (4-hydroxyphenyl) propionitrile) and the ERα-selective agonist PPT (1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole) were obtained from Tocris Bioscience (Bristol, UK). G protein-coupled estrogen receptor 1 (GPER-1) selective agonist G1 was obtained from Merck Chemicals.

Bovine serum albumin (BSA), β-glycerophosphate disodium salt hydrate and 17β-estradiol-3-benzoate (E2) were purchased from Sigma-Aldrich. Phosphate buffered saline (PBS), DMEM/F12 phenol red-free medium and gentamycin were bought from Invitrogen. l-Ascorbic acid A4403 and β-glycerophosphate disodium salt hydrate G 9422 were purchased from Sigma. Primary rabbit anti-PCNA antibody (RbPAb + PCNAab 18197) was provided by Abcam. Normal donkey serum and secondary CY3-conjugated AffiniPureF (ab) Fragment Donkey Anti-Rabbit IgG antibody were purchased from Jackson ImmunoResearch Laboratories. Fluorescence mounting medium was obtained from Dako. 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Sigma-Aldrich.

3.2 METHODS

3.2.1 Ovariectomy and treatment

Mice were randomized into 5 different groups. Four groups were OVX and one group was sham-operated. Both ovariectomy and sham operations were performed at twelve weeks of age under intraperitoneal anesthesia with ketamine (Ketalar; Pfizer) and medetomidine (Domitor; Orion Pharma, Espoo, Finland). Carprofen (Orion Pharma) was used postoperatively for pain relief. A midline incision was followed by flank incisions of the peritoneum, and the ovaries were removed with sterile scissors. The skin incision was closed with metallic clips. The sham-operated mice were treated in the same way, except that the ovaries were not removed. Sham-operated mice were subcutaneously (s.c.) injected with 100 µL of vehicle (10% ethanol and 90% Miglyol 812, Omya Peralta, Hamburg, Germany) (n = 10). The OVX mice were treated s.c. with 100 µL of E2 (1 µg/mouse; 0.04 mg/kg/day) (n = 9), PPT (175 µg/mouse; 7 mg/kg/day) (n = 10), DPN (105 µg/mouse; 4.2 mg/kg/day) (n = 10) or vehicle (n = 10). All the groups received injections 5 days per week for 4 weeks. Alternatively, OVX mice were treated s.c. with 100µL of E2 (1µg/mouse; 0.04mg/kg/day) (n=9), G1 (5µg/mouse; 0.2mg/kg/day) (n=9) or vehicle (n=10). The activity of G1 at the chosen dose has earlier been shown in vivo [122, 123]. All groups received treatments 5 days per week for 4 weeks.

3.2.2 Metatarsal culture *ex vivo*

After dissection, metatarsal bones were transferred to 24-well plates and cultured in 1 mL of phenol red-free and estrogen-free DMEM/F12 medium supplemented with 0.1% BSA, 1 mM β-glycerophosphate, 0.05 mg/mL ascorbic acid and 20 μg/mL gentamicin added at 37°C under a humidified atmosphere containing 5% CO_2 following the protocol established by our group [124]. The bones were co-cultured with either vehicle (DMSO) or G1 (1, 10, 100 and 300 nM) dissolved in DMSO.

3.2.3 Body weight and uterus, femur and tibia measurements

Body weight was measured at the beginning and the end of the experiment. Also, at the end of the experiment, mice were killed and uteruses were weighted. The length of the femur and tibia was measured *ex vivo* using a pocket vernier caliper (Helios-Preisser, Gammertingen, Germany).

3.2.4 Immunofluorescence for the detection of the ERs and PCNA expression and TUNEL assay

The femur and tibia were dissected out and fixed in 4% phosphate-buffered formalin for 24 h. Decalcification was done in 10% EDTA for 2 weeks and bones were stored in 70% ethanol. For histological analysis, tibias were embedded in paraffin. Five micrometer-thick sections were prepared from the paraffin blocks. Uteruses were collected and weighed prior to analysis.

For immunohistochemistry, the tissue sections were deparaffinized for 40 min at 60°C, rehydrated in xylene, 100% ethanol, 95% ethanol and 75% ethanol, for 5 min in each solution and finally washed with water. Antigen retrieval was performed in sodium citrate buffer (10 mM, pH 6.0) for 15 min at 95°C. After retrieval, the slides were incubated with 1.5% donkey serum in PBS for 1 h at room temperature (RT), with primary rabbit anti-PCNA antibody (1:250 dilution) overnight at 4°C and with secondary donkey anti-rabbit antibody (1:250 dilution) for 1 h at RT. Nuclear staining with DAPI was performed for 15 min at RT. After the staining, the slides were mounted with Dako Fluorescence Mounting Medium. The TUNEL assay was performed on growth plate sections according to the manufacturer's instructions. Briefly, apoptotic chondrocytes were identified by terminal deoxynucleotidyltransferase

(TdT)-mediated deoxy-UTP nick end labeling (TUNEL) immunohistochemistry applying the TdT-FragEL DNA fragmentation kit (Oncogene Research, Boston, MA, USA) as described previously by our group [125]. Alexa-546 (Invitrogen) (red fluorescence) positive cells represented apoptotic chondrocytes.

3.2.5 Quantitative histology of mouse and human growth plate tissues

Tibia growth plates were analyzed *ex vivo*. For quantitative histology of tibia growth plates, 5-µm-thick paraffin-embedded tibia sections were prepared and stained with Alcian blue/van Gieson. The quantitative analysis was performed by measuring two-third of the growth plate sections at 10× magnification. Images were captured with a Nikon Eclipse E800 light microscope (Nikon) connected to the digital camera (Hamamatsu C4742-95, Hamamatsu City, Japan) with a digital color camera system (Olympus DP70). Olympus MicroImage software (version 4.0; Olympus Optical) was applied for imaging. The height of the whole tibia growth plates was calculated as an average of 20 measurements per growth plate. The height of the proliferative and the hypertrophic zones was measured in 20 columns per tibia growth plates and was presented as an average. Hypertrophic chondrocytes were defined as cells with height bigger than 7 µm. All the measurements were obtained in a blind manner by Image Pro Plus, version 6.3 software.

The human growth plate tissue samples were fixed in 4% phosphate-buffered formalin for 24h, embedded in paraffin and 5 µm thick sections were prepared from the paraffin blocks. Images were captured with a Nikon Eclipse E800 light microscope (Nikon) connected to the digital camera. All measurements were made in a blind manner using the Image Pro Plus, version 6.3 software.

3.2.6 Quantitative histology of mouse metatarsal bones treated *ex vivo*

Digital pictures were captured on day 0, 2, 5, 7, 9, 12 and 14 using a Hamamatsu C4742-95 digital camera attached to a Nikon SMZ-U microscope. The length of each metatarsal bone was measured blindly using the ImageJ software (NIH). The increase in bone length was expressed as a delta of the length at the day of the dissection (day 0 = baseline).

3.2.7 *Ex vivo* treatment and RNA extraction

Human growth plate tissue samples were obtained from patients with extremely tall stature and treated with 10nM E2 or vehicle in phenol red-free DMEM/F12 medium supplemented with 0.1% BSA, 1 mM β-glycerophosphate, 0.05 mg/ml ascorbic acid and 20 μg/ml gentamicin added at 37°C under a humidified atmosphere containing 5% CO_2 for 24 hours. Following the treatment, the tissue samples were divided in half and used for either RNA extraction or fixation and paraffin embedding. The isolation of mRNA from the growth plate tissues was performed using TRIzol Reagent (Invitrogen) and followed by the RNA-seq analysis.

3.2.8 RNA-seq analysis

Base-calling and demultiplexing were performed using Illumina Casava1.7 software. The data were mapped to hg19 genome (bowtie2-indexed reference downloaded from ftp://ftp.ccb.jhu.edu/pub/data/bowtie2_indexes/hg19.zip) with STAR software 2.5.2b with default parameters [126]. Read counts per gene were counted with the featureCounts tool (version 1.5.3) from the Subread package [127]. Differential expression analysis was done using DESeq2 package [128]. DESeq2 was applied to compare two models predicting the expression levels of each gene: the null model accounted for the patient, the non-trivial model used both patient identity and the presence of estradiol treatment as predictors in order . to account for the natural variance between patients.

None of the genes passed the FDR<0.05 cutoff after Benjamini-Hochberg multiple testing correction, since the effect size was small. Nevertheless, we were able to conduct a GSEA-like gene set enrichment analysis with fGSEA package [129] using the results of the DESseq2 test. The further analysis targeted gene sets and pathways that were enriched by the genes activated or suppressed by E2 exposure. The fGSEA analysis was applied to investigate whether the genes in a particular set have either high or low ranks in gene list. As a reference database, we used MSigDB Hallmark gene set (human_H_v5p2, http://software.broadinstitute.org/gsea/msigdb/) [130].

3.2.9 Statistical analysis

In Paper I and II, the data were presented as means ± S.E.M., and the differences were calculated by one-way ANOVA using the SPSS software by multiple comparisons vs control group (Holm–Sidak method). ***$P < 0.001$, **$P < 0.01$ were considered as statistically significant.
In Paper II and III, Student *t* test was used for statistical analysis.
Mean values ± standard deviations were presented. *$P < 0.05$ was considered as statistically significant.

4 RESULTS AND DISCUSSION

4.1 THE EFFECTS OF LIGAND-ACTIVATED ERA AND ERB IN THE GROWTH PLATE

In the **Paper I**, we have studied the actions of ligand-activated ERα and ERβ in the growth plate. We observed shorter tibiae and femur bones in both E2- and PPT-treated mice when compared to control mice, whereas treatment with DPN did not affect bone lengths. Moreover, mice treated with E2 or PPT had reduced growth plate height and hypertrophic zone height when compared to those treated with DPN, suggesting that the effect was mediated through ERα (Fig. 5). Notably, the hypertrophic zone showed higher reduction than the proliferative zone by either E2 or PPT treatment. Moreover, PCNA staining revealed suppressed proliferation of chondrocytes in the tibia growth plate in PPT- or E2-treated mice compared to controls.

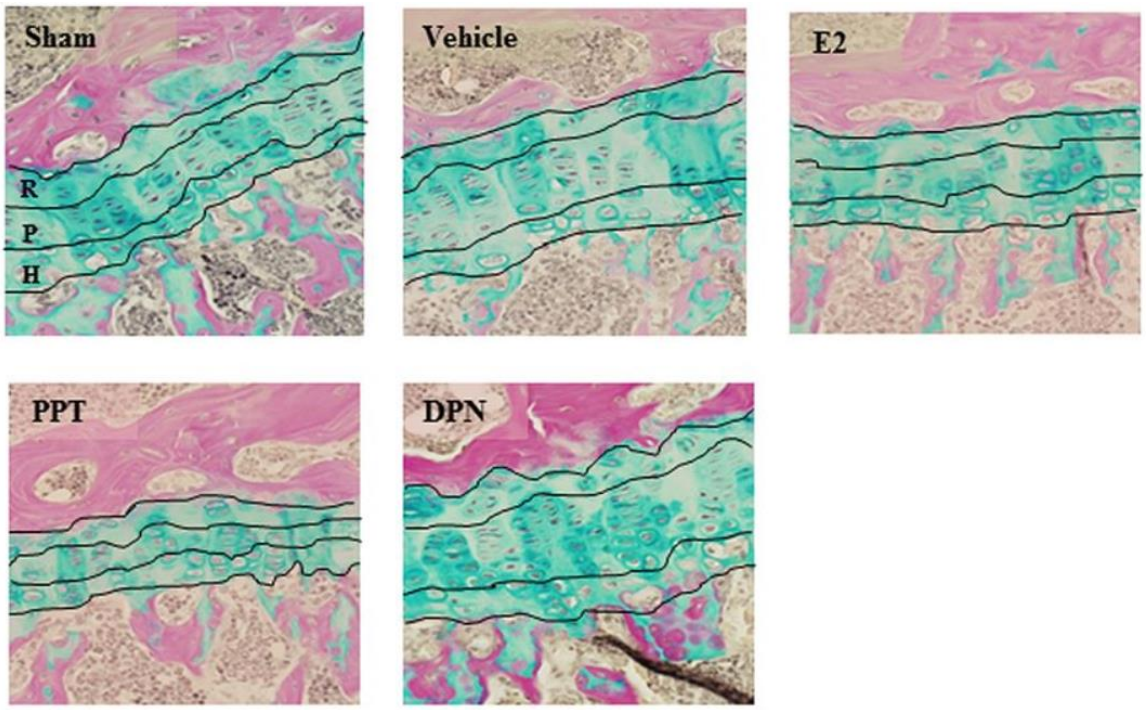

Figure 5. Effects of E2 and ERα and ERβ agonists on tibia growth plate length and proliferative and hypertrophic chondrocytes. Alcian blue/van Gieson staining of mouse proximal tibia growth plate. R = resting zone; P = proliferative zone and H = hypertrophic zone

Our report of ERα-mediated regulation of bone growth is the first to show that activation of ER by a selective ligand induces inhibition of bone growth. This finding is in line with the

previous study demonstrating that the number of chondrocytes is diminished in mice with the expression of active mutant ERα in chondrocytes [131].

In contrast to the influence on the proliferation, no effects of a selective ERα agonist PPT on apoptosis were observed, though it was difficult to make a clear conclusion regarding apoptosis without gene expression analysis. Notably, apoptotic genes were earlier reported to be affected by ERα in bone [132]. In contrast, no changes in apoptosis were found in ERα-deficient mice when compared to WT mice [108]. The identification of E2-targeted genes in the study described by Paper III provided partial understanding of the potential targets of ERα, since it appears to be the main functional ER in the growth plate.

Importantly, our results showed that despite the significant inhibition of bone growth, the selective ERα agonist PPT had only minor uterotropic effect. This finding suggested that in future studies one might choose to adjust the doses of PPT or another similar selective ERα agonist in order to selectively inhibit chondrocyte proliferation while being neutral towards uterus, ovaries and mammary glands.

4.2 THE EFFECTS OF LIGAND-ACTIVATED GPER-1 ON BONE GROWTH

In **Paper II**, we have studied if GPER-1 may regulate bone growth. Earlier studies have found the suppression of bone growth by E2 in WT mice but no effects in GPER-1-deficient mice [75]. Also, GPER-1 has been shown to influence transcription activation by E2 [133]. Moreover, inhibition of mesenchymal stem cell differentiation into chondrocytes has been demonstrated *in vitro* [134]. Therefore, we hypothesized that ligand-activated GPER-1 may affect chondrocyte proliferation in the growth plate. However, the results showed that none of the concentrations of G1 had a direct effect on metatarsal bone growth when compared to control. Similarly, G1 did not influence tibia and femur growth in treated mice (Fig. 6). Thus, our current study did not find any effects of the GPER-1 agonist G1 on the growth plate in *ex vivo*-treated metatarsal bones.

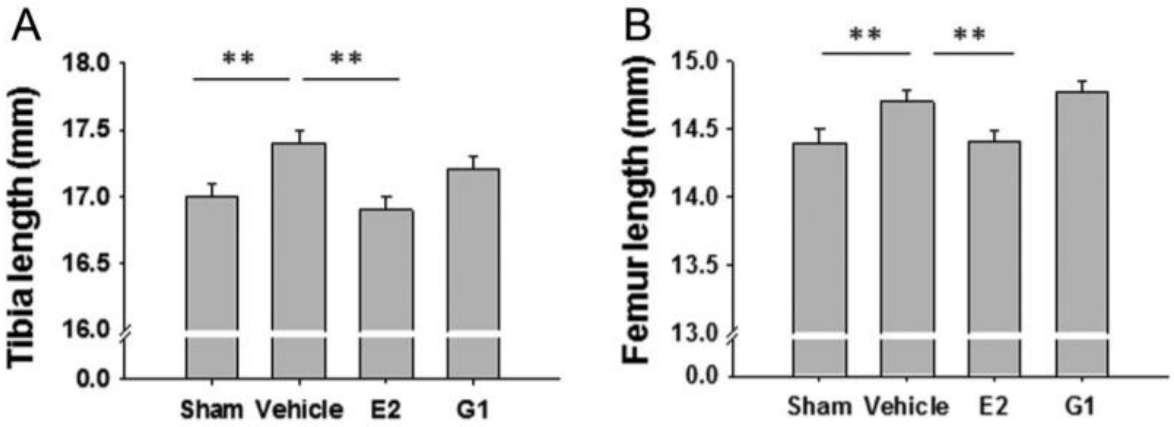

Figure 6. Effects of E2 and G1 on tibia and femur lengths. The study groups included ovariectomized mice treated with E2 (0.04 mg/kg/day), G1 (0.2 mg/kg/day) or vehicle alone and sham-operated mice. Vehicle vs E2, vehicle vs sham, $^{**}P < 0.01$. Tibia length (A) and femur length (B) ($n = 9–10$). All the data are presented as means ± S.E.M., and the differences were calculated by one-way ANOVA.

Studies of GPER-1 functions in the growth plate might have potential clinical applications, since GPER-1 was found to be expressed in both mouse and human chondrocytes [73, 135] and GPER-1-deficient mice had enhanced elevated bone mass and proliferation in the growth plate [121]. However, our *ex vivo* model revealed no direct actions of GPER-1 in the mouse growth plate in metatarsal bones. In line with this, ligand-mediated stimulation of GPER-1 did not show any effect on bone growth *in vivo*. Neither it affected growth plate chondrocyte proliferation or apoptosis. For our study, we choose the dose of G1, which was previously used in mouse models with clear systemic effects [123, 136].

Although GPER-1 does not seem to have any direct effects on chondrocyte proliferation in the growth plate, its potential interactions with other ERs expressed in the growth plate cannot be completely excluded until relevant studies would be performed. For instance, the potential cross-reactivity between GPER-1 with ERα [137]. Besides, ligand-independent actions of GPER-1 have been reported in ovarian tumor cells [138]. Thus, the findings of the Paper II did not reveal any effects of activated GPER-1 on bone growth. Our results indicate that E2 effects in bone and growth plate are mainly mediated via ERα.

4.3 IDENTIFICATION OF E2-TARGETED GENES AND SIGNALING PATHWAYS IN HUMAN GROWTH PLATE CHONDROCYTES

In **Paper III**, we have identified multiple enriched pathways inhibited by E2 treatment, such as mTOR, estrogen response early and late, apoptosis, epithelial mesenchymal transition and angiogenesis (Fig. 7).

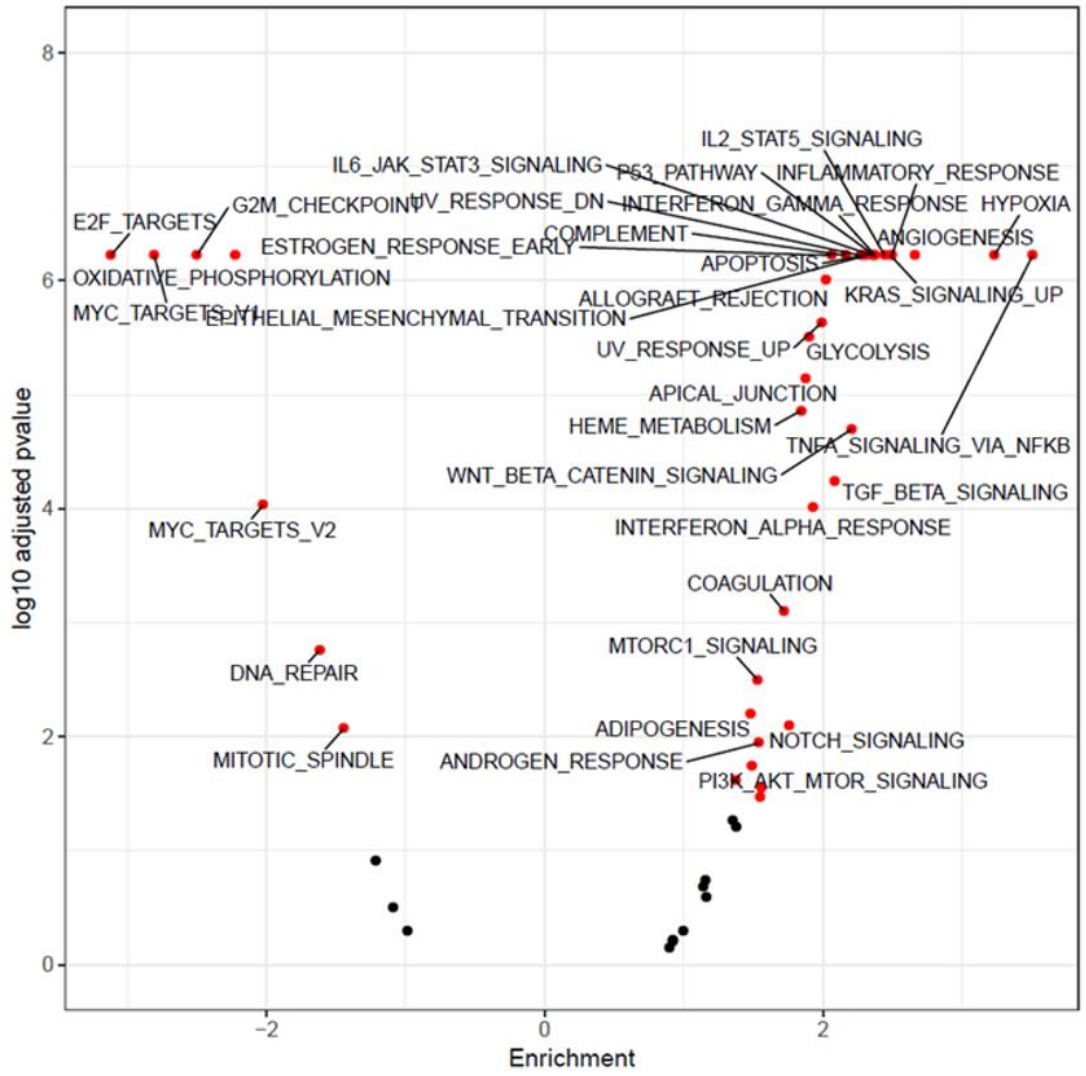

Figure 7. Pathway enrichment analysis. Volcano plot showing the significantly upregulated and downregulated enriched pathways in control vs. E2 treated human growth plate tissues.

Among the highly affected genes, peptide YY (PYY), which negatively regulates bone mineral density and bone mass, was significantly downregulated by E2 treatment. Since PYY-deficient mice showed increased osteoblast activity, PYY was suggested to inhibit osteoblast functions [139]. Moreover, overexpression of PYY suppressed osteoblasts via Y1 receptor-mediated MAPK activation. In addition, expression of Homeobox 7 (HOXB7), which stimulates

osteogenic differentiation of mesenchymal stem cells, was strongly inhibited by E2. HOXB7 was shown to activate osteogenesis via RUNX2 [140].

Also, the mTOR signaling pathway was significantly inhibited by E2. Stimulation of mTOR has been reported to regulate chondrocyte proliferation and differentiation [39]. Furthermore, mTORC1 was found to control preosteoblast differentiation [141]. Moreover, our group has earlier demonstrated that activation of mTORC1 caused disorganization of the resting zone of the growth plate [142]. Furthermore, TGFβ1 also was among the genes suppressed by E2. TGFβ/BMP signaling is known to regulate osteoblast differentiation [30].

Ihh signaling pathway, which has a pivotal role in the regulation of the differentiation in the growth plate, also was among the significantly enriched pathways downregulated by E2 in human growth plate (Paper III, Fig. 1). These results are in line with earlier findings demonstrating the inhibition of Ihh genes by treatment with both the selective ERα agonist PPT and the selective ERβ agonist DPN in the rat uterus [143]. Furthermore, the bone growth has been found to depend on the interactions between Ihh and Wnt/β-catenin signaling pathways. Our study has revealed that Wnt/β-catenin signaling was also suppressed by E2 in the growth plate (Paper III, Fig. 1). Furthermore, AXIN2 expression was strongly inhibited by E2 according to the RNAseq data. Axin2 has previously been shown to suppress maturation of osteoblasts via regulation of Wnt signaling [144]. Other pathways, which regulate bone growth and development and were downregulated by E2, include TGFβ, Notch and epithelial-mesenchymal transition signaling [30, 145, 146].

The vascular network supplies the bone with cells, growth factors and minerals essential for growth, mineralization and development of extracellular matrix. Vascular endothelial growth factors (VEGF) directly regulate differentiation of osteoblasts and osteoclasts [147]. Our findings have revealed that treatment with E2 might suppress angiogenesis in the growth plate, since RNA-seq data indicated the suppression of VEGF-C, one of the main vasculogenic factors and angiogenesis was among the enriched pathways suppressed by E2 treatment (Fig. 7). Furthermore, E2 was demonstrated to suppress VEGF expression and angiogenesis in tumor cells [148]. Therefore, VEGF-C might be another potential target of E2 for the regulation of bone growth.

In addition, E2 significantly suppressed apoptosis in the growth plate as observed by RNA-seq analysis (Fig. 7). In line with this, TUNEL assay showed inhibition of apoptosis in growth plate tissue exposed to E2 when compared to control.

Since nuclear and membranous ERs are the direct targets of E2 in the growth plate, we have investigated whether the expression of these receptors may be affected by E2 treatment. The results of the immunofluorescence revealed that the treatment with E2 significantly suppressed the expression of both ERα and GPER-1. These data might be explained by potential compensatory downregulation of ER expression as a consequence of E2 treatment. To summarize, the results of Paper III identified target genes and pathways affected by E2 in human growth plate. Notably, several signaling pathways, which are vital for bone development and growth, were suppressed by E2 treatment.

4.4 STUDY LIMITATIONS

There are several limitations of our studies due to the observed differences in E2 effects in mice and humans, potential interactions between different ERs within the growth plate, previously reported ligand-independent actions of the ERs and limited number of clinical samples analyzed by RNA-seq.

Extrapolation of these results obtained in experimental animals to humans should be done with caution, since high doses of E2 cause growth plate closure in humans but not in mice [149]. As to the results described in **Paper I**, considering ligand-mediated activation of ERα for therapeutic purposes, toxicological evaluation would be required, since ERα has shown potential involvement in tumorigenesis in breast cancer development [150].

With regard to the second study described in **Paper II**, the potential interactions of GPER-1 with other ERs should be taken into account, since GPER-1 has been shown to affect ERα. Furthermore, GPER-1 might function in a ligand-independent fashion in the bone. In particular, GPER-1 was reported to activate proliferation in ovarian cells [138].

Though we identified a number of genes and related signaling pathways influenced by E2 in the human growth plate in the study described in **Paper III**, additional studies are required to verify the key regulatory mechanisms of these E2 effects. Besides, RNA-seq data from only two patients were included in **Paper III**. The analysis of larger groups of patients would be expected as a continuation of our studies. Furthermore, the growth plate samples were treated *ex vivo*, and there might be differences in gene expression changes between experimental data and gene expression alterations happening in patients. Moreover, the concentration of E2 for the treatment of growth plate samples was selected as the most common dose of E2 in the *ex vivo* and *in vitro* studies. Notably, treatment with high doses of E2 would not be appropriate to use *in vivo* as these doses might cause cancerogenic effects. In addition, we chose only one dose of E2 for the *ex vivo* experiments since tissue materials were limited, although testing a range of E2 concentrations would be important.

5 CONCLUSIONS

5.1 PAPER I

- Our *in vivo* data in mice showed that the growth suppressive effects were mainly mediated via ERα.
- Treatment with E2 or PPT decreased growth plate height while ligand stimulation of ERβ did not influence growth plate height.

5.2 PAPER II

- Our results showed that the selective GPER-1 agonist G1 had no local effect on metatarsal bone growth when treated *ex vivo*.
- *In vivo* studies in mice confirmed that G1 did not affect tibia or femur growth when administered systemically.
- We conclude that ligand stimulation of GPER-1 does not affect bone growth and growth plate cartilage in these models.

5.3 PAPER III

- Target genes and signaling pathways affected by E2 were identified in cultured rare specimens of human growth plate cartilage.
- Enriched pathways inhibited by E2 included mTOR, estrogen response early, apoptosis, epithelial mesenchymal transition and angiogenesis.
- The expression of peptide YY, a negative regulator of bone formation and mineral density, was inhibited by E2 treatment.

Our results open for the development of novel therapies of growth disorders. Estrogen signaling plays an important role in the regulation of longitudinal bone growth originating from the growth plate. High doses of E2 were demonstrated to inhibit bone growth. Although several ERs were shown to be expressed in the growth plate, ERα is suggested to be the main ER mediating the E2 effects on bone growth. Both clinical data and studies using ERα-deficient mouse models indicated that the effects of E2 in bone are mediated via ERα. Regarding the

functions of other ERs analyzed in **Papers I and II**, treatment with selective ERβ and GPER-1 agonists did not show any significant effects on the proliferation in the growth plate. Nevertheless, ligand-independent effects of these ERs might still influence chondrocyte activity.

Altogether, these data suggest that selectively targeting ERα can provide an effective treatment of bone growth disorders characterized by excessive tall stature.

6 FUTURE PERSPECTIVES

Several research directions can be developed based on our current results. Our studies in **Paper I** have demonstrated that ERα is the main ER, which regulates longitudinal bone growth. However, the target genes and signaling pathways involved in bone growth regulation via ERα remain to be identified. Gene expression studies, such as RNA sequencing, could be used to detect target genes regulated via ERα in the growth plate. Considering the negative side-effects known to be associated with E2 treatment, further preclinical studies of selective ERα agonists should be performed prior to any clinical studies exploring their effectiveness and safety in extremely tall adolescents.

The study described in **Paper III** has identified several target genes and enriched pathways affected by E2. Future studies could include investigation of the specific mechanisms involving identified genes and verification of those mechanisms using in vitro and *in vivo* experimental models. Furthermore, additional studies are required to confirm gene expression changes in a large cohort of patients. Also, a comparison of gene expression profiles in normal human growth plate and growth plate from individuals with tall stature would be important for the understanding of mechanisms regulating longitudinal bone growth.

7 ACKNOWLEDGEMENTS

I am grateful and happy to have managed to reach this point. I have had the pleasure to work quite a while at Karolinska Institutet. I would like to thank Karolinska Institutet for the great opportunities and the KID funding and other foundations for supporting me and my projects financially during these years.

I would like to thank all those who really helped and supported me during my PhD time.

Lars Sävendahl, my main supervisor who kindly welcomed me to his lab. There have been plenty of ups and downs and lessons that I have learned. Thank you very much for all the good times that we shared during these years and I will try to view all the challenges as a part of the PhD tolerance test! Thank you very much for letting the Ph.D. journey end well at this point.

Claes Ohlsson, my furthest distance co-supervisor and the one to whom I owe a lot. Thank you for all the scientific discussions via email and phone. Of course, I have had the chance to meet you two times at the scientific seminar CORS series in Gothenburg.

Elham Karimian, my Iranian co-supervisor, thank you for your support mostly at the beginning of my PhD. You left the research at KI and started the clinic basically at the same time I started my PhD. Thank you for being keen on following up with me sometimes in between. I wish you success in your career.

Andrei Chagin, my co-supervisor, thank you for your support.

Marie Largerquist, I would like to thank you very much as we have two papers together. I really appreciate your great personality and support despite the long distance between us.

Henrik Wehtje, thank you very much for all the surgeries and providing the human growth plate samples.

Olle Söder, the former head of the Women's and Children's Department. I would like to thank you for your support.

Angelica, I am pleased by knowing you, although I would be happy to get to know you by a different reason that I have had. Your name very well matches your character: ‘’calm, gentle and positive’’. Thank you very much for all your kindness and support. It is unforgettable.

Artem Artemov, thank you for your contribution and support with RNA-seq data analysis for my last paper. I really appreciate that. I wish you the best with your research.

Sandra, our head of the administration, I am glad to have you as a supportive person and I am very thankful for all your support.

Jobel, our nice IT support. Thank you very much for always having time for me and all the support during these years.

To my Mentor, Professor **Joseph Rafter**, I got to know you from those beautiful days when I have just come to Sweden to do my Master studies. Those days were clearly different from these days, since I did not have so many responsibilities as I do now! I was accepted to the Master program and I have started my trip from Iran with a lot of energy and enthusiasm toward the science. I was truly ambitious. Thank you for always inspiring and supporting me. During my PhD time, there was a lot of time when I needed extra support, but I decided not to involve you very much and keep our memories as beautiful as they were. I have tried myself to go through all the challenges and I can just tell you that I successfully managed everything well. ☺

Bettina, we have started our journeys together as PhD students in the same group almost simultaneously. We had more or less similar experiences in different conditions. We had given birth to our kids almost at the same time and we shared a lot of good and bad stories during these years together. I would like to thank you very much for your friendship, support and understanding during these years. Wish you all the best. ☺

Emma, a former PhD student in our group, the one who showed me most of the reagents and software that I needed when I had started in the lab. You were a very calm and gentle person. Thank you for always being kindly available for the help.

Mariana, I am glad to have you around. You are such a calm, positive and helpful person. You were always available for the support. I wish you all the best.

Ahmed Osman, I am happy to know you as a supportive person during my last project and I would like to thank you for the great introduction to Confocal Microscopy and all the software and antibodies support I needed. I will not forget your kind support. Good luck in the new career journey.

Phillip, thank you for your support with my third manuscript.

Magdalena, you are a positive and supportive person. I really appreciate your manners and support. Wish you good luck in your private life and your professional career.

Abishek, you are the newest PhD student who joined our lab. I found you a kind, wise and supportive person. We usually talked shortly, but efficiently about science facts, conflicts or politics! You are still a very young person with a lot of positive energy. I wish you good luck in your future journey.

Therese, Lilly, Valentina, Dorina, Femke, Hajar, Elena, Lil, Cecilia, Yunhan, Karuna, thank you for your support and nice chats we have had. I was happy to have you around. I wish you all the best. **Alice**, you are a nice person and we usually met each other on the way, whenever I was thinking about you! **Shanie**, thank you for your kindness. I will not forget it. **Yeneneh**, you are a nice person, thank you for your friendship. **Anders Ihrfors**, thank you for your support.
To all my Iranian friends in Iran, around the world and in Sweden. I have known some of you from elementary school up to university time. I am still very happy to have you as loyal, supportive and encouraging friends. **Golbarg, Azita, Sormeh, Nazanin, Mana, Farrokh (you are a kind, smart and super supportive person), Mozghan, Samaneh, Mozhdeh, Marjan. Nasim, Sara, Maryam, Behnaz, Kamran, Farzaneh, Amirhossein, Azadeh, Yasi, Manizheh, Yaser, Amir, Helen**. Thanks a lot and I wish you all great future.

To all great former and new colleagues and lab members that I have met during this journey at the Pediatric Endocrinology lab in the Astrid Lindgren building and in a new BioClinicum building. Thank you very much for a positive work environment and kind support. Those years and your kindness will always be remembered with high appreciation.

To my beloved family,

To the memory of my **Mother**,

The most generous, gentle and kind person that I have ever seen in my life. To the one who inspired me a lot. Unfortunately, I was not so lucky to have your support up to now. The destiny made us apart from each other, but I feel your presence everywhere and I still see you in my dreams. The most beautiful person who raised me up in the best condition that she could do. The one who fulfilled most of my wishes and led me to be the person I am. You were the only one who really wanted me to reach to PhD level. My precious Mom, now I am really close to that point. I hope you are happy about me and the level I reached. I am truly thankful for all the support, motivations and difficulties that you have encountered raising us. Please pray for us and rest in peace till we will meet again.

To my **Father**,

I really appreciate all your hard work, support, motivation and freedom that you have given to me. Thank you for always trusting in me and letting me choose my own ways. As a girl, I was given the most freedom among my friends (girls) in Iran. I have always understood your worries despite the freedom you gave me. As a result, I have become an independent person today. You are the most supportive and kind father that I could imagine one can have. Thank you so much for everything, baba!

To my **Sister**,

Melissa, you are the only and lovely small sister that I have in my entire life. I am so grateful that I always have all your support, especially since the time I moved to Sweden. I know I kept you alone with a lot of responsibilities in Iran and I really appreciate all the hard work you have done. Apart from the distance between us, we are always in a close contact with each other and you are the only one who always would hear from me the first news! ☺ Thank you so much for all the motivation and understanding during all these years. Thank you for always listening to all my complaints! I wish you health, luck and success in your entire life. I Love you so much. ☺

To my beloved God-gifted sons **Aryan** and **Nikolai**,

You came into my life during the PhD time. You are pure miracles with tons of energy. All the words would not be enough to express my feelings towards you. My love is uncountable to both of you. Despite all the challenges, YOU BOTH are the whole meaning of my life and my desire to continue. I am reviewing and exploring my life one more time with you. I know that the world is not as nice as we expected, but still there are a lot of good things to focus. Your mother wishes that you will be great men. Be sure that all the good you do will come back to you one day. I would encourage you to be always positive and energetic, always look forward, learn lessons from the past and follow your dreams. Focus on your real interests and try to be the most qualified persons as you can. I look forward to seeing the most brilliant future for you. I pray for health, luck and prosperities for you both. ♥♥

To my beloved husband, **Konstantin**,

I would like to thank you very much for all your unconditional support during these years. I really appreciate your patience during this period. I am always amazed by your encyclopedic knowledge. It was not easy to cope with two energetic kids combined with my PhD journey and lots of other issues that we had to handle. Without you it would not be easy to pass through this challenge. We spent a large part of our private life discussing science and my projects. Thank you that you have never complained about it! I ask God health, luck and prosperity for our lovely family. ♥

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