Regulators of growth plate maturation

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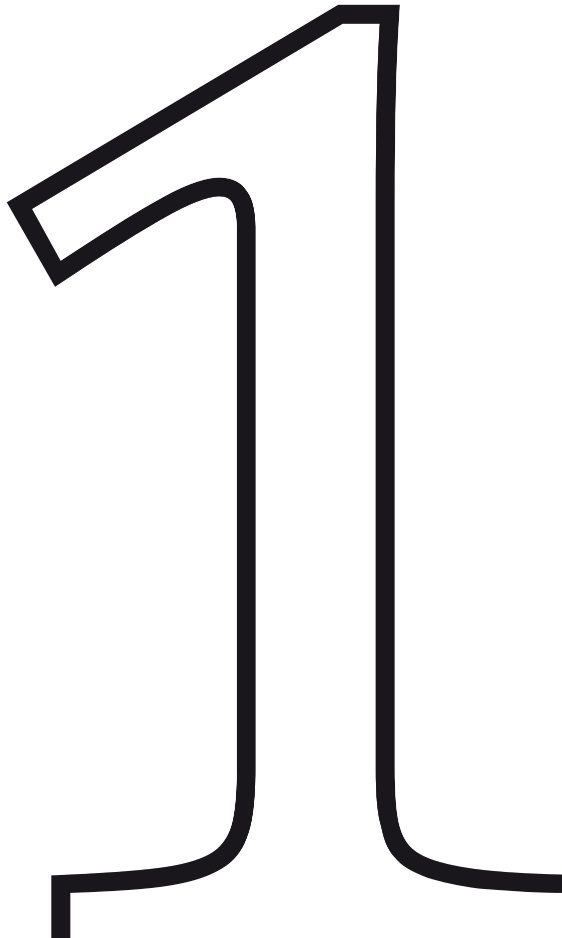

General introduction

Longitudinal bone growth and the growth plate

Longitudinal growth occurs at the epiphyseal plate or growth plate, a thin layer of cartilage entrapped between epiphyseal and metaphyseal bone, at the distal ends of the long bones. This growth plate is highly organized and consists of chondrocytes in three principal layers: the resting zone, proliferative zone and hypertrophic zone (fig 1). The resting zone is located at the epiphyseal end of the growth plate and contains resting chondrocytes, originating from mesenchymal stem cells, forming a proliferative reservoir for the entire growth plate. Resting chondrocytes enter the proliferating zone in organized columns and undergo cell-divisions in longitudinal direction regulated by a multitude of hormones and growth factors. In this zone chondrocytes start producing significant amounts of extracellular matrix proteins (ECM) essential for the structure of the growth plate. More towards the hypertrophic zone chondrocytes lose their proliferating capacity and begin to increase in size while they differentiate into hypertrophic chondrocytes. In the hypertrophic zone chondrocytes increase their size 6-10 fold and secrete a large amount of matrix proteins. Longitudinal bone growth is the result of proliferation, enlargement of hypertrophic chondrocytes and production of ECM proteins in the proliferative and hypertrophic zone.
From the adjacent metaphyseal bone osteogenic progenitors and osteoclasts are recruited to remodel the newly formed cartilage and deposit trabecular bone at the bottom of the growth plate. During childhood the growth plate matures, its total width decreases and eventually it disappears at the end of puberty with complete replacement by bone along with cessation of longitudinal growth (1).

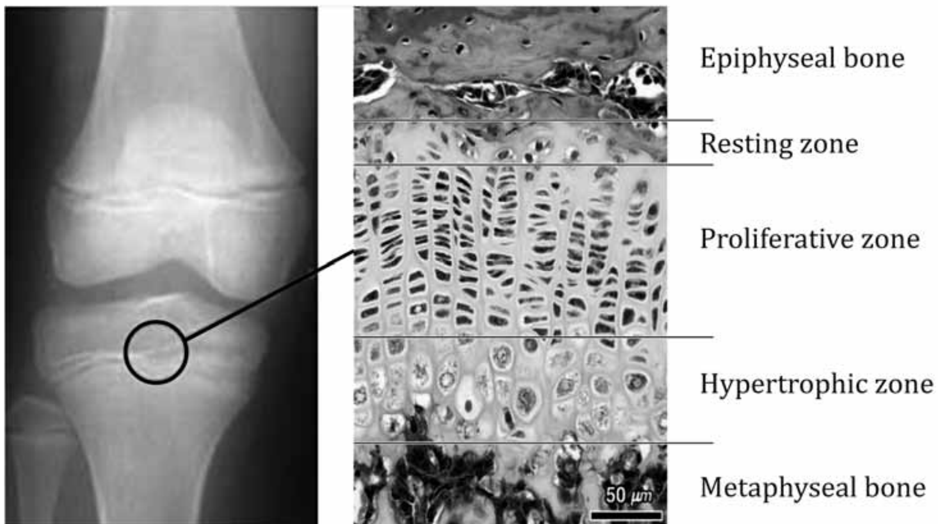

Figure 1: The structure of the growth plate

Extracellular matrix (ECM) proteins

Chondrocytes are embedded in an extracellular matrix consisting of various large and multifunctional molecules. An important group consists of the collagens, of which collagen type II is most abundantly present throughout the proliferative and hypertrophic zone. Another important collagen in the growth plate, often used as a marker for hypertrophic chondrocytes, is collagen type X that is solely expressed in the hypertrophic zone of the growth plate. Dysplasias caused by mutations in these collagens are associated with growth disorders and short stature (2;3). Another group of ECM molecules are the glycosaminoglycans (GAGs), heteropolysaccharide molecules. The majority of GAGs in the body are linked to core proteins, forming large mucopolysaccharide proteoglycans cross-linking the matrix. Members of this proteoglycan group are aggrecan, biglycan, glypican and chondroitin.

Within the ECM there are endopeptidases called Matrix Metalloproteinases (MMPs), remodelling these molecules, and tissue inhibitors of MMPs, preserving the integrity of the ECM (4). In addition the MMPs initiate angiogenesis for vascular invasion of growth plate cartilage together with various growth factors like vascular endothelial growth factor (VEGF) produced by chondrocytes in the growth plate (4-6). Besides controlling angiogenesis, VEGF has also been shown to modulate chondrocyte differentiation and survival, osteoblast differentiation and osteoclasts recruitment (7-9).

The ECM is furthermore a reservoir for various growth factors, hormones and proteins like Transforming growth factor beta (TGF-β), Fibroblast growth factors (FGFs), Indian Hedgehog (Ihh), Parathyroid hormone-related peptide (PTHrP), Wnt signalling factors and Bone Morphogenetic Proteins (BMPs), interacting together to tightly control chondrocyte proliferation and differentiation in the growth plate (1).

Hormones and their influence on the growth plate

Longitudinal bone growth is influenced by a variety of hormones and growth factors acting directly or indirectly on the growth plate. A fine and tight control of several hormone systems is obligatory to control longitudinal bone growth. For example triiodothyronine (T3) and thyroxine (T4) in the hypothalamic-pituitary-thyroid axis are crucial for normal skeletal growth and bone maturation illustrated by cases of congenital or acquired hypothyroidism that show a severe retardation of growth and skeletal maturation, while hyperthyroidism is associated with an increased growth velocity and bone maturation (10-12). Glucocorticoids are strong inhibitors of growth and long-term treatment often leads to growth failure, which might be the result of an inhibiting effect on chondrocyte proliferation and a stimulating effect on apoptosis of growth plate chondrocytes (13;14). In children suffering from chronic inflammatory diseases growth failure is frequently observed. These disorders are associated with increased circulating cytokine levels acting locally in the growth plate or through systemic effects on other growth regulatory mechanisms (15).

Growth hormone and IGF-I are the most well-known hormones involved in longitudinal growth, whereas estrogen is thought to be the most important hormone during pubertal growth and epiphyseal fusion. Both hormonal systems are described in more detail in the following sections and interactions between these systems and the ones described above frequently occur.

Growth hormone and insulin-like growth factor-I (IGF-I)

Growth hormone and IGF-I are important stimulators of longitudinal bone growth. Both GH and IGF-I receptors are expressed in the growth plate (16). Defects in GH and IGF-I pathways result in marked growth disorders (17-20). GH can stimulate skeletal growth directly by local action in the growth plate and indirectly by liver-derived IGF-I (21;22).
The effect of GH on longitudinal growth has been a captivating subject of research through the years. The original hypothesis was the *somatomedin hypothesis* in which it was thought that GH stimulates hepatic production of somatomedin (IGF-I), which in turn stimulates chondrocytes locally in the growth plate (23). In 2001 this hypothesis was questioned by Le Roith et al who found little effect on the growth rate in mice with a selective *igf1* deletion in their liver while their circulating IGF-I was 20-25% of normal (24). The terminal hypertrophic zone in the growth plates of these mice was reduced and therefore it was thought that IGF-1 is responsible for chondrocyte differentiation (24).
The majority of circulating IGF-I is bound to a complex with IGF binding protein 3 (IGFBP-3) and the acid labile subunit (ALS). Knocking out the *als* gene or the *igf1* gene results in just a small effect on longitudinal growth in mice (24;25). On the contrary, a deletion of the *igfbp-3* gene results in an increase in linear growth in mice. A double knock out mouse of *als* and *igf1* as well as a triple knockout of each gene results in significant growth retardation, suggesting that there might be a threshold level of circulating IGF-I to control skeletal growth and that both ALS and IGF-I are crucial participators (26).
Nowadays it is well known that GH not only generates liver produced IGF-1, but also acts locally on chondrocytes in the growth plate. These findings were described in the *dual effector hypothesis,* in which GH acts locally on chondrocytes in the resting zone to enhance the recruitment of resting chondrocytes in the proliferative state and to stimulate local IGF-I production, resulting in an increase in proliferation by both GH and IGF-I stimulation (21;27-29). Several clinical reports and laboratory results have demonstrated the importance of both IGF-I and GH in the regulation of longitudinal bone growth, however the exact contributions and roles of circulating and local effects of these hormones still need to be clarified in more detail.

Estrogen and its effect on longitudinal bone growth

Estrogens are known to play a key role in longitudinal bone growth through growth plate maturation, epiphyseal fusion and augmentation of accrual of bone. The main active form of estrogen is 17β-estradiol (E_2) that can be synthesized from estrone and testosterone (figure 2). Other estrogen metabolites are less active and have less affinity for the estrogen receptors. The effects of estrogen have been suggested to be mediated mainly through the nuclear receptors ERα and ERβ, both expressed in growth plate chondrocytes of several species, including the human (30-33). Expression patterns co-localize throughout the whole growth plate with a more abundant expression in the resting and proliferative zone (32). The two receptors show high conservation in their DNA-binding domain (97%) and share considerable homology (55%) in their ligand-binding domain (34). Recently a new membrane G protein-coupled estrogen receptor was discovered, the GPR30 receptor, which was shown to be expressed in human and mice and was shown to be

functional in the growth plate of mice (35;36). Estrogen receptor gene polymorphism is reported to have an effect on adult height. There are two studies demonstrating that a mutation in a certain region of the estrogen receptor allele can result in an average of 3 cm increase in adult height (37;38).

The important role of estrogen is supported by several clinical observations. Premature estrogen exposure in for example precocious puberty accelerates skeletal maturation, whereas on the contrary hypogonadism results in delay in skeletal maturation. Smith et al in 1994 described a male with an inactivating mutation in the estrogen receptor a that showed no pubertal growth spurt and continued to grow into adulthood due to absence of growth plate fusion, resulting in tall stature (210 cm) and osteoporosis (39). A similar phenotype was described in a second report of two boys with a mutation in the aromatase gene (40). Aromatase catalyzes the conversion of androgens into estrogens, thus also in these boys there was a lack of estrogen action. Thirdly, from clinical observations it is known that high levels of estrogen inhibit longitudinal bone growth (41).

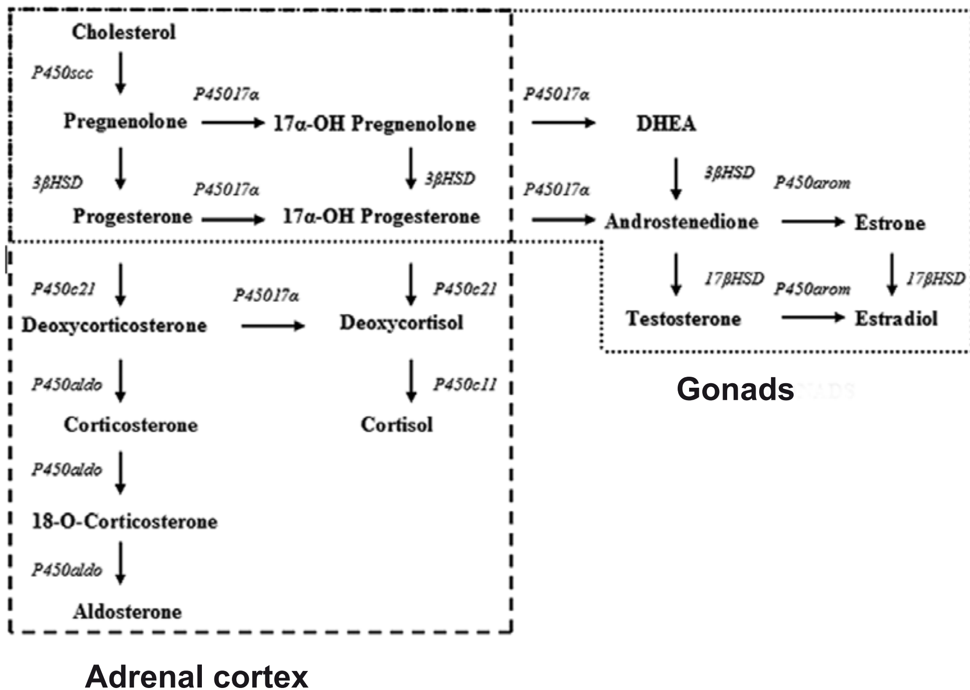

Figure 2: Steroid hormone synthesis pathways.

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Estrogen action

Estrogens can exert their effect through several mechanisms. In the classical way estrogen diffuses through the plasma membrane into the cytosol where it binds cytoplasmic receptors to form hormone-receptor complexes. These complexes dimerize and are translocated into the nucleus where they bind estrogen response elements in or close to a promoter region of a specific gene to activate gene transcription (42).
In a non-classical way estrogen can also act without binding to the DNA. Estrogen receptors can have an effect through interaction with DNA-bound proteins that function as transcription factors or co-regulators like cyclic AMP-response element binding protein (CREB), specificity protein-1 (SP-1) and activator protein-1 (AP-1) (43-45). Alternatively, estrogens act through interaction with membrane-bound receptors, like GPR30 that results in a relatively rapid non-genomic estrogen response through intracellular signaling pathways (46).
Not only do estrogenic ligands regulate ER-dependent gene expression, but the ER can also be phosphorylated and activated by a variety of signaling pathways such as pathways involving IGF-1 and epidermal growth factor (EGF) (47;48). Vice versa estrogens can influence the GH-IGF-I axis, demonstrated by increasing GH but decreasing IGF-1 levels in patients with oral estrogen treatment (49;50). High dose of estrogen treatment decreases IGF-1 levels by 35% (51). However, transdermally administered estrogens increase IGF-1 levels (52). In addition, in puberty an increase in the amplitude of GH-secretion is seen and blocking estrogen signaling results in a drop of GH level (53). It has been suggested that estrogen can act on an AP-1 motif in the promoter site of the IGF-1 gene (54). Another indirect effect of estrogen on longitudinal growth might be through interference with components of the Indian Hedgehog/PTHrP growth restraining feedback loop suggested by experimental data in rats where estrogen increased mRNA expression of PTHrP and its receptor (55).

Senescence

The mechanism by which estrogens exert their effect on longitudinal growth and finally growth plate fusion is not fully understood. One of the postulated hypothesis is that estrogen accelerates the senescent decline (56). Senescence is a term for the structural and functional changes over time in the growth plate, such as a gradual decline in the overall growth plate height, proliferative zone height, hypertrophic zone height, size of hypertrophic chondrocytes and column density (56). Growth plate transplantation experiments showed that the growth rate of a transplanted growth plate depends on the age of the donor and not on the age of the recipient, suggesting that growth velocity in time is regulated by a local mechanism intrinsic to the growth plate besides hormonal regulation in time (57). It is believed that the growth plate fuses when senescence reaches a critical point in the growth plate. Recent evidence indicates that senescence might occur because stem-like cells in the resting zone have a finite proliferative capacity, which is gradually exhausted (58;59). Estrogen is thought to advance growth plate senescence, causing earlier proliferative exhaustion, and thus earlier fusion. This might explain that estrogen treatment does not induce fusion rapidly, but often must act for years before fusion occurs. In addition, the period of estrogen treatment required for growth plate fusion is longer in cases of precocious puberty and shorter in adults with a deficiency in aromatase than for normal individuals.

SERMS

Estrogen is known to be a major regulator of longitudinal growth and growth plate fusion. A low estrogen concentration increases growth velocity, while a high concentration inhibits growth (60;61). A high dose of estrogen is used in the treatment of extremely tall girls to reduce adult height. However, it not only affects longitudinal growth but has also potential side-effects like an increased risk for breast and uterus cancer and decreased fertility (62). These side effects can partially be circumvented by the use of selective estrogen receptor modulators (SERMs), that display both pro- and anti-estrogenic activity in a tissue-specific manner (63).
One of the best-studied SERMs is raloxifen, which prevents postmenopausal bone loss but has minimal effects on breast and uterus tissue (64). In rabbits it induces growth plate fusion without influencing uterus weight (65). Tamoxifen, a SERM widely used in the treatment of breast cancer appears to act as a estrogen antagonist at the growth plate in the treatment of progressive precocious puberty in McCune-Albright patients (66;67). A more detailed understanding of estrogen action and interactions on tissues is needed to enable the use of SERMs in the modulation of longitudinal growth.

Intracrinology

The major source of sex steroids acting on the growth plate is the gonads, which secrete sex steroids into the circulation in a classical endocrine fashion. In addition to this endocrine route, estrogen can also be produced locally. This phenomenon is seen with many other hormones as well and is called intracrinology. Sex steroids can be formed from various precursors by locally expressed enzymes in many tissues like adipose tissue, bone, breast tissue or brain (68;69). Oz et al showed aromatase expression in the human growth plate which was confirmed together with 17β-estradiol production in rat costochondral chondrocytes (70;71).
A few years later more of these converting enzymes, i.e. 17β-hydroxysteroid dehydrogenase, steroid sulfatase and type I 5α-reductase, were detected in the surroundings of the growth plate at time of sexual maturation, suggesting that bone cells possess the capacity to metabolize sex steroids necessary for skeletal growth (72). Van der Eerden et al showed that various enzymes involved in sex steroid metabolism are upregulated with puberty in the rat growth plate, suggesting a role for these enzymes and the steroids they produce during pubertal growth (73).

Catch-up growth

Following a period of growth inhibition, height velocity usually exceeds the normal range. This phenomenon is termed catch-up growth, and was first described by Prader et al. (74). Tanner proposed the hypothesis that catch-up growth is regulated by a neuroendocrine system that compares the individual's actual body size to the target size of the individual. This target size is described as the 'time tally' most likely localized in the brain. The growth rate is adjusted according to the degree of mismatch (75). More recent studies however suggest that catch-up growth is due to intrinsic factors of the growth plate like delayed growth plate senescence (58;76). Growth-inhibiting conditions slow growth plate chondrocyte proliferation, thus conserving the

proliferative capacity of the chondrocytes and consequently decreasing senescence of the growth plate. When growth inhibition conditions are abolished these chondrocytes are less senescent and retain a greater proliferative capacity resulting in a higher growth rate than expected for age, thus in catch-up growth (77). The relationship between catch-up growth and delayed growth plate senescence has only been studied in rabbits and rats so far (77;78). In catch-up growth after food restriction animal studies showed that HIF-1alpha protein and mRNA are upregulated in the growth plate together with IGF-1R and GHR protein levels (79;80). Recently Pando et al. reported an additional possible mechanism in nutritional catch-up growth involving microRNAs in the growth plate of rats.

Puberty and epiphyseal fusion

Puberty is a complex biologic process involving sexual development, adrenal maturation, gametogenesis and increase in growth velocity known as pubertal growth spurt which accounts for 15-20% of final height (81). The secretion of gonadotropin-releasing hormone by the hypothalamus represents the first step, initiating release of the gonadotropins (LH and FSH) that in turn stimulates the gonads to secrete sex steroids (estrogen and testosterone). Increase in sex steroids level induces the external signs of puberty (secondary sex characteristics) and the accompanying pubertal growth spurt. Estrogen mediates pubertal bone growth in both males and females. Along with an increase in estrogen also GH secretion increases 1.5 to 3-fold in puberty together with IGF-I levels (82). Peak levels of circulating GH coincide with Tanner stage B3-4 in girls and at Tanner stage G4 in boys (83). Estrogen levels and GH concentration show a positive correlation through puberty in females, demonstrating the relationship in their actions (84). In addition, estrogen has also a direct stimulatory effect on the growth plate chondrocytes, as demonstrated in Laron patients with GH resistance that still undergo a pubertal growth spurt (85). Furthermore, estrogen receptor beta and GPR30 expression is downregulated in the human growth plate during pubertal progression (32;35). Estrogen stimulates differentiation of the chondrocyte rather than proliferation (86) resulting in growth plate maturation and eventually growth plate fusion with cartilage being replaced by bone. Although the exact role of the estrogen receptor beta is still unclear, from mice experiments a crucial role in growth plate fusion was hypothesized (87). However, the male patient with an estrogen receptor alpha mutation had incomplete epiphyseal closure and a history of continued linear growth into adulthood, suggesting a role for estrogen receptor alpha in growth plate fusion. This patient was still able to produce a 46-kDa isoform of the estrogen receptor alpha which is shown to suppress estrogen receptor activation (88;89). Chagin et al speculated that this isoform might as well suppress the estrogen receptor beta and this might be the cause of delay of growth plate fusion in this patient (90).
The final step in epiphyseal fusion is not completely understood up to now. In endochondral ossification, a nowadays generally accepted hypothesis is that terminally hypertrophic chondrocytes die by the process of apoptosis leaving behind a scaffold of cartilage matrix for osteoblasts that invade and lay down bone resulting in growth plate fusion (91-93). More recent studies have questioned that apoptosis is the final mechanism through which chondrocytes die in the terminal hypertrophic zone, since terminal hypertrophic chondrocytes do not display a typical apoptotic appearance (94-96). Apoptotic cells undergo typical morphological changes like cell shrinkage with intact organelles and integrity of membranes, pyknotic nuclei by aggregation of

chromatin, fragmented DNA, partitioning of the cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies) and absence of an inflammatory response (94;97-99). Roach et al reported autophagic vacuoles in terminal hypertrophic cells suggesting a role for autophagy in the final step of endochondral ossification (94;97;98;100). A third and oldest hypothesis is that terminal hypertrophic chondrocytes can transdifferentiate into osteoblasts (101-103). The mechanism by which terminal hypertrophic chondrocytes disappear at the chondro-osseus junction is believed to be related to the underlying cause of growth plate fusion.

Cell cycle regulators

The process of longitudinal bone growth requires precise regulation of chondrocyte proliferation which is regulated by intracellular and extracellular mechanisms. Extracellular mechanisms involve for example hormonal systems as described in the sections above. An important intracellular mechanism is the cell cycle which is controlled by orderly activation and inactivation of cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs). Cyclin-dependent kinases control specific points of the cell cycle (104). Activation of the CDKs promotes progression of the cell cycle eventually resulting in cell division. CKIs can bind and inactivate these CDKs, thereby maintaining growth arrest. In mammals, two known families of CKIs have been identified, INK4 and the Cip/Kip family. The Cip/Kip family consists of p21Cip1 (p21), p27Kip1 (p27), and p57Kip2 (p57) which can inhibit all G1/S-phase cyclin-CDK complexes and which are all expressed in the growth plate (105-109). Cip/Kip inhibitors appear to mediate the growth inhibiting and differentiation effects of different stimuli. For example a recent study suggests that the effects of PTHrP on both the rate and extent of chondrocyte proliferation are mediated through suppression of p57 expression (110). In addition, p27 expression is up-regulated during thyroid hormone-induced terminal differentiation of rat resting zone chondrocytes (111). Little is known about the exact role of cell cycle regulators in the regulation of skeletal growth.

Growth plate models and species differences

Studies on growth plate regulation and epiphyseal fusion are hampered by the fact that frequently used *in vivo* and *in vitro* animal models poorly represent the human situation. Mouse and rat for example do not fuse their growth plates at the end of puberty like humans (32;33). This indicates that rodents are not perfectly representative for studies on growth plate fusion, excluding the use of transgenic approaches. This is furthermore illustrated by the discrepancy in growth phenotype between ERa knock out mice and a male patient lacking functional ERa receptors (39;112). The patient did not fuse his growth plates and continued growing into adulthood. Gene ablation of the estrogen receptors in mice had only a marginal effect on longitudinal growth, which only became apparent in part of the long bones late in life. Other animal models occasionally used for studies on growth plate regulation are rabbits and piglets, however these are relatively expensive and few molecular biological tools are available for interventions. *In vitro* human models like primary cultures of human chondrocytes or chondrosarcomatous cells are limited in its representation of the *in vivo* situation and have other impairments like a rapid dedifferentiation in primary cultures, a high variability between donors and loss of differentiation capacity. A promising relatively new

human *in vitro* model is a model with Mesenchymal stem cells (MSCs). MSCs are multipotent cells with the ability to differentiate into several mesenchymal lineages including chondrocytes. They can differentiate from stem cells to proliferating chondrocytes to subsequently hypertrophic chondrocytes, in a similar fashion as observed in the human growth plate (113;114). MSCs were originally isolated from bone marrow (115), but they have also been isolated from other tissue sources such as fetal organs, placenta, umbilical blood and adipose tissue (113;116;117). The lack of representative animal models to study processes occurring in the human growth plate, has led to the realization that human based models are required to elucidate the molecular mechanisms involved in growth plate regulation and fusion.

Rationale and outline of this thesis

Estrogen is known to play an important role in longitudinal bone growth and growth plate maturation, but the mechanism by which estrogens exert their effect is not fully understood. In this thesis this role is further explored. **Chapter 1** contains a general introduction to longitudinal bone growth and the regulation of the growth plate in respect to relevant topics further studied in this thesis. Estrogen can act through a genomic or a nongenomic pathway. Both pathways are explored in rats at the onset of maturation in **chapter 2.** Estrogen stimulates VEGF expression in uterus and bone, which is an important growth factor for chondrocyte differentiation and chondrocytes survival in the growth plate. In **chapter 3** the effect of estrogen on VEGF expression in the growth plate was studied in the rat and human growth plate. Another effect of estrogen is that it accelerates growth plate senescence. Senescence is one of the postulated intrinsic mechanisms by which the growth plate matures and finally fuses. In **chapter 4** we investigated senescence in relation to proliferation, by investigating a cell cycle inhibitor p27Kip1. In animal models, catch-up growth is suggested to be caused by delayed growth plate senescence. In **chapter 5** this hypothesis was further tested in humans**.**

With puberty estrogen levels increase, the growth plate matures and at the end growth ceases with epiphyseal fusion through mechanisms not yet completely understood. In order to further explore growth plate maturation we subjected two growth plate tissues of the same patient, but with one year and one pubertal Tanner stage in between, to microarray analyses. Gene expression patterns and transcription factor binding sides in relation to pubertal maturation were studied in a longitudinal study within this single patient in **chapter 6**. In addition, we collected extra prepubertal and pubertal growth plate tissues and studied these samples with microarray techniques as well in **chapter 7**. In **chapter 8** the process of epiphyseal fusion and apoptosis was studied in human growth plates.

Animal models are frequently used but not fully representative for the human growth plate. Therefore we investigated a promising human *in vitro* model with multipotent mesenchymal stem cells (MSCs) that can differentiate into chondrocytes. MSCs can be isolated from various tissues. In **chapter 9** we investigated the chondrogenic potential of MSCs from different origins and in **chapter 10** we compared this model with the epiphyseal growth plate by analyzing gene expression patterns and pathways with micro-array analyses. **Chapter 11** contains general conclusions and a discussion regarding the results.

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