Regulation and modulation of growth : insights from human and animal studies

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Chapter 1

General introduction

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The blueprint for human postnatal growth is defined by the genetic background of an individual, but is fine-tuned by hormonal, environmental, psychosocial and nutritional factors. As the result of a process called endochondral ossification taking place in the epiphyseal growth plates, long bones elongate and the size of the skeleton increases. Successive phases of growth can be distinguished, each characterized by specific hormonal regulation [1). In the intrauterine phase and neonatal period, up to the age of approximately 3 year, a high but rapidly declining growth velocity is observed. It is assumed that prenatal growth is mainly regulated by insulin-like growth factors (IGFs), insulin and nutrition. From the second semester onward growth hormone secretion plays an important role, and in the first 2-3 years the correlation between the child's length and parental height gradually increases, suggesting a growing influence of genetic factors.

From the age of 3 years to puberty, growth continues at a lower and gradually diminishing rate, predominantly under the influence of thyroxin and growth hormone (GH). During pubertal development, sex steroid secretion and concomitant upregulation of GH-IGF-I signaling causes an increased growth rate, also known as the pubertal growth spurt. In both genders, estrogen-induced maturation and closure of the epiphyseal growth plates at the end of pubertal development is associated with a rapidly declining growth rate and the termination of growth, inevitably determining adult height (2). In contrast, it has also been suggested that growth deceleration precedes epiphyseal fusion (3).

Although many scientific research projects have been dedicated to unraveling the regulatory pathways underlying growth, there are still many remaining questions. Also, the pathophysiological mechanisms underlying the majority of growth disorders are still to be elucidated and more effective modes of treatment need to be developed.

As this thesis touches upon several aspects of growth regulation and modulation, this first introductory chapter is subdivided in three parts. Part A reviews the background of idiopathic short stature (ISS) and growth hormone (GH) treatment. Part B recapitulates evidence from animal studies regarding alternatives for GH treatment. Part C addresses the need for development of an appropriate model system for studying growth regulation in humans.

A. Idiopathic short stature (ISS)

Definition and subcategories

Short stature is one of the most common reasons for referral of a child and his parents to a pediatric endocrinologist for diagnostic evaluation and advice. A large variety of congenital or acquired conditions that can be classified as primary, secondary or idiopathic growth disorders can cause shortness (4). A primary growth disorder, such as achondroplasia, is due to an intrinsic defect in the growth plates of long bones. Secondary growth disorders result from conditions that negatively affect growth plate physiology (e.g. endocrine disorders, chronic illness, malnutrition). In industrialized countries, celiac disease, Turner syndrome and growth hormone deficiency (GHD) are the main causes for short stature. In the majority of cases of short stature, however, an elaborate assessment of the child's medical history, physical examination, laboratory tests, and radiological examinations does not reveal any clue for an underlying pathological mechanism, leaving the patient with the diagnosis ISS, short stature of unknown origin (5).

ISS is a condition defined by an individual's height more than 2 standard deviation (SD) below the corresponding mean height for a given age, sex, and population group (thus a height standard deviation score (SDS) of <-2) without evidence of systemic, endocrine, nutritional, or chromosomal abnormalities (4;6-9). Children with ISS have a normal birth size and body proportions, are not growth hormone deficient and have no psychiatric disorder associated with poor growth. By definition, ISS is a diagnosis of exclusion, as it does not rely on the identification of certain features characteristic for ISS, but rather on excluding all currently known other causes of short stature. Since approximately 80% of the short children referred to a pediatric endocrinologist meets the criteria for ISS (10), the clinical relevance of this entity is evident.

The label ISS presumably unifies a variety of different causes of short stature that are unknown at present. Subdivision of this group in broad categories may therefore seem arbitrary, but is considered useful from a diagnostic and prognostic point of view (11). The most important distinction is between familial and non-familial short stature (FSS and NFSS, respectively), based on how the child's growth pattern relates to his target height. The conditional target height (cTH) for short children, that takes into account the effect of assortative mating and parent-offspring correlations, can be calculated according to the formula of Hermanussen and Cole (12): 0.72 × average of parental height SDS. The lower limit of the target height range is defined as cTH minus 1.6 SD.

A child with FSS grows within the target height range of the family, but is short in comparison with the appropriate reference population. In contrast, NFSS is characterized by short stature for the normal population as well as for the genetically defined familial growth potential. The diagnostic work-up in children with FSS may be limited, since the chance of finding a pathological mechanism underlying short stature is small in those cases, given that no pathological causes for parental short stature are present. However, the probability of a dominant genetic disorder (e.g. hypochondroplasia) is higher in case of one short parent (height SDS <-2.0 SDS) and a more elaborate diagnostic evaluation is then warranted.
A second subdivision can be made according to the tempo of maturation of the child. Girls reaching breast development stage 2 later than the age of 13 years and boys with a testicular volume smaller than 4 ml at the age of 14 years are considered to have delayed puberty in the Netherlands and in many other industrialized countries (11). Information on pubertal onset is usually not available at the moment of diagnostic evaluation, as most short children referred to a pediatric endocrinologist are prepubertal. Therefore, it is impossible to ascertain pubertal delay before the age of 13 years in girls and 14 years in boys. The determination of skeletal age may render additional information, as pubertal delay is more likely in the presence of delayed bone maturation (bone age minus chronological age < 0 years). However, in some children with delayed skeletal age puberty is not delayed, and vice versa.
Whereas the demarcation line between FSS and NFSS is sharply, though arbitrarily defined before puberty, the presence of normal and delayed puberty can only be definitively established in retrospect after the onset of puberty. Still, it is valuable to consider the possibility of delayed maturation from a prognostic perspective. In a child with NFSS and a positive family history of late puberty, the likelihood of delayed pubertal maturation is high and the pediatrician may choose to limit the number of diagnostic tests and follow an expectative line of treatment and advice. In case of normal pubertal timing and development in the parents, a pathological process underlying the clinical presentation of the child is suspected and additional investigations should aim at finding this mechanism.

Impact on psychosocial functioning

Children with short stature may experience psychosocial stress attributed to shortness such as social immaturity, infantilization, low self-esteem, being bullied or not accepted by their peers, and an overprotective parenting style that may hamper development of autonomy (13;14). In most clinic-based studies parents indeed report increased psychosocial stress in their short children (15-19), whereas peers and teachers do not mention a relevant decrease in social competence (15;18). Unfortunately, the experience of the children themselves is usually not

investigated (14). The impression from population-based studies is that children and adolescents with ISS may experience varying degrees of stature-related stress, but generally are functioning within the normal range (20).
Studies on psychosocial well-being of short individuals in adulthood are inconclusive, some reporting a lower chance of getting married, a higher percentage of unemployment and more self-reported problems in social functioning (21-24), whereas others find no difference between short and normal-sized adults (25;26). In the absence of conclusive evidence that ISS has a persistent negative impact on psychosocial functioning, it is important that the potential psychological burden for each patient referred for ISS is individually assessed (13).

Indications for growth-promoting treatment

Once the presence of ISS has been established, various treatment options may be considered (13;27). The decision whether or not treatment is indicated in an individual patient is based on a careful evaluation of auxological, psychosocial, ethical and financial arguments (13;27). Biochemical parameters have not been recognized to justify initiation of pharmacological interventions. Ideally, treatment results in alleviation of psychosocial stress, attainment of a normal adult height and preferably also a normal height during childhood. It should be stressed that a taller stature per se will not necessarily result in an improved quality of life or psychosocial functioning (14). For a short child that is not concerned about or hampered by his height in any way, treatment is usually not recommended. On the other hand, when a child evidently suffers from stress attributed to shortness, treatment can be considered. The patient and parents should be provided with a realistic perspective of the results that can be expected regarding height gain, the variability of clinical outcome, the risks, benefits, costs and possible treatment alternatives.

Growth hormone treatment

Many clinical trials with GH treatment for ISS have been performed since recombinant GH became available in 1985. GH on average increases AH of the majority of ISS children with 3-7 cm after 4-7 years of treatment, compared with historical, placebo-treated or non-treated controls or with predicted adult height before the onset of treatment (27). The effectiveness of GH treatment has been attributed to the notion that most children with ISS have a certain degree of GH insensitivity that cannot be counteracted by endogenous GH secretion, but can be overcome by GH replacement.

The applied dosage is an important determinant for the response to treatment. It has been demonstrated that higher GH dosages result in a more pronounced acceleration of growth velocity and a taller adult height than lower dosages (28;29). The currently known most effective treatment regimen is 1.4 mg/m^2/day (equivalent to 50 µg/kg/day) subcutaneously. This is approximately twice as high as the substitution dose, as assessed by studies on spontaneous secretion (30), but only slightly higher than the regular GH 'replacement' dosage prescribed in GHD in the United States. The long-term outcome of such a regimen has been reported by several authors (reviewed in (27)). The data on the effects of even higher dosages are contradictory. In one study, a dosage of 2 mg/m^2/day (equivalent to 75 µg/kg/day) appeared to accelerate bone maturation and advance pubertal onset (31;32), whereas no such effects were observed in other studies that applied up to 2.8 mg/m^2/day (equivalent to 100 µg/kg/day) (33;34), nor in a recent Swedish study that used a dosage of 1.9 mg/m^2/day (equivalent to 67 µg/kg/day) (35). In the latter three studies the average age at onset of therapy was close to 11 years, while average age in the first study was 8.7 years. Premature advancement of maturation may limit the effectiveness of GH treatment, as the timeframe reserved for growing is limited once puberty has commenced.

Even if corrected for the dosage effect, there is still a large interindividual variation in growth response. It is therefore estimated that there are many other predictive factors, the majority of which still unknown. Factors with a positive influence on growth response are a younger age, larger bone age delay, initial height and midparental height, a higher body weight and a relatively large difference between initial height and cTH at start of treatment. Also, a better response to treatment in the first year is associated with adult height outcome (27). The explained variance of these variables is still rather low (40-60%) (36).

Several parameters can be used for the evaluation of the success of treatment. For assessing the first year's growth response, the change in height SDS, height velocity, height velocity SDS and the change in height velocity may all be useful, provided that these parameters are corrected for the age, pubertal stage, and degree of growth retardation of the individual patient (27). Whether or not treatment was successful in the long term can be objectified with the auxological parameters adult height SDS, adult height SDS minus height SDS at start of GH treatment, adult height SDS minus predicted adult height SDS, and adult height minus conditional target height (27).

The absence of a circumscript defect in the GH-IGF-I axis in most children with ISS, the large inter-individual variation in response to GH-treatment and the high cost of GH have prompted the search for alternatives for GH treatment. An additional reason to do so is that GH treatment is not registered for ISS in the Netherlands and other European countries.

Gonadotropin releasing hormone agonists (GnRHa)

At pubertal onset, a steep increase in the level of circulating estrogen results in acceleration of bone maturation, which preludes epiphyseal fusion and determines a person's adult height. The development of pharmaceutical compounds that can delay or inhibit pubertal development has received increasing interest. Postponing pubertal development would hypothetically result in prolongation of the timeframe reserved for growth, leading to an increased adult height gain. In children with central precocious puberty (CPP), suppression of gonadal sex steroid synthesis effectively halts pubertal development and augments adult height (37;38).
The beneficial effects of GnRHa treatment on height gain in CPP has prompted attempts to apply this treatment strategy in children with short stature without precocious puberty as well. In general, GnRHa monotherapy in children with ISS results in a marginal increase in adult height of 0-4 cm (39-41). The efficacy of GnRHa depends on the duration of treatment. Short term GnRHa (2 years) does not improve adult height, due to a balance between decreased growth velocity and arrested bone maturation, whereas in long term treatment (3-4 years) the decreased but continuous growth in the absence of bone age progression augments adult height with approximately 1 cm per treatment year (42).
The possibility of adding GH to GnRHa can be considered, as the decreased growth velocity observed during GnRHa treatment may limit height gain (43;44). In such a scenario, GnRHa ideally extends the period available for growth while GH simultaneously preserves growth velocity, ultimately leading to a taller adult stature. Combined GH+GnRHa treatment was shown to augment adult height by approximately 4-5 cm in two controlled clinical trials (45;46), but analysis of large databases of GH-treated children that were also treated with GnRHa has revealed no effect (47-49). Combined GH+GnRHa treatment may be considered in children with ISS and a pronounced growth retardation at pubertal onset (27). In the absence of information on the possible adverse effect of sex steroid deprivation on bone mineralization and of postponing puberty on psychosocial well-being, GnRHa treatment, whether or not combined with GH, should not be considered routine treatment for children with ISS (27).

Aromatase inhibitors

Bone age advancement and epiphyseal fusion are caused by the surge in estrogen secretion during puberty (50) as illustrated by unfused epiphyseal plates, ongoing growth into adulthood and extremely tall stature observed in patients with disruptive mutations in the aromatase gene (51-55) or estrogen receptor gene (56). Therefore, it has been postulated that blocking endogenous estrogen synthesis may result in delayed bone maturation, prolongation of the

timeframe reserved for growth and increased adult height (57). Aromatase inhibitors reversibly or irreversibly prevent the conversion of androstenedione and testosterone into estrone and estradiol, respectively.

Several studies have been conducted in order to establish whether aromatase inhibitors can be used as an alternative for GH treatment in children with short stature by various causes. Some studies resulted in no beneficial effects of aromatase inhibitor treatment (58-60), but most reported an increased predicted adult height (61-66). Due to differences in trial design, such as the presence or absence of a control group, inclusion of various types of short stature (GH deficiency, McCune-Albright syndrome, precocious puberty), employment of different classes of aromatase inhibitors or co-treatment with other compounds (GH or testosterone), it is difficult to draw conclusions on the efficacy of aromatase inhibitors. Moreover, only two trials were randomized controlled clinical trials (61;66). Adult height data of most of these studies have not yet been reported. In boys with short stature due to constitutional delay of growth and puberty, letrozole treatment was shown to increase near-adult height, without apparent detrimental effects on bone mineralization (67). However, it was recently reported that aromatase inhibition may predispose to vertebral disc deformities (68).

Estrogen receptors and aromatase activity are ubiquitously present throughout the body, illustrating that estrogen signaling is crucial and inhibition of estrogen biosynthesis may result in adverse effects in various tissues or organs. The clinical phenotype of estrogen-deficient men gives several clues to potential unwanted effects of pharmaceutically induced estrogen deficiency. Obesity, insulin resistance, steatohepatosis, and severely decreased bone mineral density have been described in these patients (52;54;55;69). In ISS boys anastrozole or letrozole treatment did not impair skeletal mineralization (59;61;70), but the bone turnover rate was diminished due to treatment (61). Furthermore, a high number of vertebral body deformities was described after letrozole treatment raising the concern that aromatase inhibition may impair vertebral body strength (68;71). A reduced HDL-cholesterol in the absence of other effects on the lipid profile (61;72), and decreased insulin sensitivity have also been described (72). Theoretically, estrogen deprivation might result in disturbances of cognitive function and fertility, but this has not been documented in human studies so far. A careful exploration of the potential side effects of aromatase inhibition is required before such treatment can be applied in clinical practice (73).

Aromatase inhibition has been tested for the treatment of precocious puberty in girls with McCune-Albright syndrome (58;60), but not for growth enhancement in girls with ISS. Potential adverse effects such as virilization due to hyperandrogenism and the development of ovarian cysts warrant a cautious approach.

B. Manipulation of growth in animal models

Rodent models

Animal studies have provided valuable insights into the process of postnatal growth regulation and modulation. Although growth can be studied in a variety of animals (e.g. goat, rabbit, pig), most information has come forward from studies involving rodent models and this segment will therefore focus on results from studies in the mouse and rat.

GH-IGF-I signaling and growth

The crucial role of GH and IGF-I signaling in the regulation of postnatal growth has been established by studies in various rodent models such as spontaneously mutated mouse models characterized by dwarfism, knockout mice for several constituents of the GH-IGF-I axis, mice transgenically modified to overexpress components of the GH-IGF-I family, and hypophysectomized rats with combined pituitary hormone deficiency.

Dwarf mouse models

Spontaneous mutations in the pituitary-specific transcription factors Pit 1 or Prop-1 result in underdevelopment of subsets of pituitary cells and render the mice deficient for GH, thyroid-stimulating hormone and prolactin (74). This combined pituitary hormone deficiency translates into a growth phenotype characterized by normal body size and weight at birth, but severe growth retardation afterwards (Snell dwarf, Ames dwarf). Similarly, the GH deficient Little mouse (mutation in the GH releasing hormone receptor) shows a progressively impaired growth pattern from the age of two weeks onward. The phenotype of the Little mouse was shown to be rescued by injection with a recombinant adenovirus containing rat GH and a human promoter sequence. This form of gene therapy resulted in elevated levels of GH and IGF-I, and a normalization of body weight and body length as compared with wild-type mice (75), highlighting the important role of GH signaling in postnatal growth.

Transgenic and knockout mice

Additional insights into growth regulation by the GH-IGF-I axis have been gathered from studies in transgenic and knockout mice (76). GH insensitivity due to knockout of the GH receptor/binding protein gene (GHR; Laron mouse) or caused by transgenic overexpression of

an antagonizing GH analog results in decreased IGF-I levels and dwarfism (77-79). In contrast, gigantism is observed in transgenic mice that overexpress GH or hypothalamic GH releasing hormone (GHRH) (80;81).
IGF-I knockout mice have demonstrated that postnatal growth is mainly determined by GH-dependent IGF-I, whereas both hormones also act independently. This was shown by observations of compromised intrauterine and postnatal growth in IGF-I knockouts, but an even more pronounced postnatal growth retardation in IGF-I and GHR double knockouts (82). Partial reduction in IGF-I signaling in heterozygous IGF-I receptor knockout mice leads to an apparently normal phenotype, although there are also clues that a reduced growth potential may be present (82;83). Formation of a ternary complex with IGF binding protein 3 (IGFBP-3) and acid-labile subunit (ALS) normally increases the half-life of IGF-I in the systemic circulation. Overexpression of IGFBP-3 results in pre- and postnatal growth retardation, whereas ALS overexpression results in a moderate delay of postnatal growth. When both ALS and IGFBP-3 are overexpressed, growth inhibition is more pronounced (84). On the other side of the spectrum, transgenic mice overexpressing IGF-I show somatic overgrowth (85).
The findings in GH and IGF-I knockout mice are confirmed by observations in hypophysectomized rats that serve as a model for GHD. A markedly retarded somatic growth is seen in these animals compared with wild-type littermates. Both GH and IGF infusion in these rats results in stimulation of longitudinal growth. However, the effect of GH is more pronounced than that of IGF-I (86).

Representativeness of models for the influence of GH-IGF-I on growth

In the human, disorders or gene mutations leading to GH or IGF-I deficiency or resistance all result in various degrees of shortness, generally similar to the phenotypes observed in rodent counterparts (87). Patients with primary IGF-I deficiency or GH deficiency show a strikingly similar degree of growth retardation (87). Suppletion of GH or IGF-I in these patients results in stimulation of growth, but with GH treatment having a superior effect (88). In summary, both an effect of GH-dependent IGF-I and a separate, direct effect of GH have been demonstrated to be essential for coordinated postnatal growth in humans, which is in agreement with collective conclusions drawn from rodent animal models. Therefore, it can be concluded that rodent animal models are suitable for studying the effects of deregulation of the GH-IGF-I axis and treatment strategies designed for counteracting its effects on postnatal growth.

Sex steroid signaling and growth

In the following paragraphs the results of experiments on steroid signaling and growth will be reviewed. After the growth hormone (GH)-IGF-I axis, sex steroid signaling is the second major determinant of postnatal growth, especially during pubertal development. Many rodent models have been developed and applied to assess the influence of sex steroids on the regulation of growth, amongst which knockout mice for the estrogen receptor α and β, androgen receptor, and aromatase genes, and the ovariectomized or orchidectomized mouse and rat.

Estrogen receptor knockout mice

The actions of estrogen in the human and rodent are mediated by two receptors, the estrogen receptors alpha and beta (ERα and ERβ). Three knockout mouse models have been generated, the partially estrogen-resistant αERKO and βERKO mice (89), and the completely estrogen-resistant double knockout (α/βERKO) mouse (90). The effects of ER inactivation depend on sex and age of the mice (91).
In female mice, axial growth is unaffected in αERKO, βERKO and α/βERKO at all stages of life (92), although during the postpubertal phase a tendency towards slightly increased growth is observed in βERKO (92). During the period of sexual maturation and in adulthood, appendicular growth is decreased in αERKO, but increased in βERKO mice, with a more pronounced effect in adulthood. The phenotype of α/βERKO mice is intermediate between those of αERKO and βERKO in all stadia (92). A direct correlation between appendicular length and IGF-I levels has been noted, with high levels stimulating growth (93). Decreased growth of the axial bones is accompanied by a smaller width of the growth plate (94). Knockout of ERβ does not affect body weight gain, whereas obliteration of ERα or both receptors results in increased body weight (92). In adult αERKO and βERKO mice, a higher BMD is reported than in wild-type littermates, with a more prominent effect in αERKO mice. BMD in double knockouts is comparable to wild-type BMD (92;93). Apart from the growth effects, αERKO and α/βERKO mice also show a distinct ovarian phenotype characterized by enlarged, hemorrhagic and cystic follicles and are anovulatory (89).
Male αERKO and double knockout mice display decreased growth of the axial and appendicular skeleton, narrow epiphyseal growth plates, low IGF-I levels, less body weight, and a decreased bone mineral density (BMD) compared to wild-type littermates (92-94). Ablation of ERβ has no effect on growth parameters in male mice. Male αERKO mice show testicular atrophy, reduced sperm count and viability, altered sexual behavior and decreased fertility (89).

In conclusion, the influence of estrogen signaling on growth in the mouse shows a prominent sexual dimorphism. Signaling through ERα stimulates axial growth in male rats, whereas it has no effect in females. Appendicular growth is repressed via αERKO in both genders. ERβ does not exert an effect on longitudinal growth in both genders, nor on radial growth in males, but it does result in growth retardation in females. As it was demonstrated that estrogen levels are markedly raised in female αERKO and α/βERKO, but normal in males, it has been speculated that hyperestrogenism may inhibit appendicular growth via ERβ signaling.

Aromatase knockout mice

As an alternative model for assessing the role of estrogen signaling on longitudinal growth, estrogen-deficient mice can be considered. Targeted disruption of the aromatase gene has resulted in the development of such a model, the ArKO mouse (95). Aromatase synthesizes estrogen from androgenic precursors. Male and female ArKO mice appear phenotypically normal at birth. Appendicular growth is significantly retarded in adulthood in male, but not in female ArKO mice (96). Effects on axial growth have not been reported, and it might therefore be concluded that such effects are either absent or marginal at most. Both genders display osteoporosis (97). Female ArKO mice have underdeveloped uteri and ovaries, and are sterile due to anovulation (95;98). Male mice are fertile, but to a lesser extent than wild-type littermates (97). Increased abdominal fat deposition and insulin resistance is present in both males and females (99) and it has been postulated that a disturbed androgen to estrogen ratio may promote visceral fat accumulation (99).
The differences between the phenotypes of ERKO and ArKO mice may rely on the fact that partial estrogen signaling is still possible in ERKO mice, whereas the influence of estrogen is completely abolished in the ArKO model. Additionally, the androgen to estrogen ratio is presumably more disturbed in ArKO than in ERKO mice, which may account for phenotypic variations due to elevated androgenic signaling.

Androgen receptor knockout mice

Besides the established role of estrogen in the regulation of growth, there is also compelling evidence that androgens have a unique function. Part of the effect of androgen on the growth plate is probably due to aromatization into estrogens, which is suggested by the presence of aromatase in rat and human growth plate cartilage (100;101). However, the androgen receptor (AR) has been detected in rat (102) and human growth plate cartilage as well (103;104),

suggesting that direct actions of androgen in growth regulation also occur. The observations that androgens accelerate growth in mice, and that a nonspecific ER blocking compound does not attenuate this effect are in support of a direct androgenic effect (105).
The androgen-resistant knockout mouse was generated to study the influence of androgens on bone and metabolism. A normal growth phenotype was observed in these mice. Male ARKO mice had female secondary sexual characteristics, late onset obesity and a marked loss of BMD (106;107). The absence of an impact on growth in ARKO mice suggests that androgen signaling is not crucial for the regulation of longitudinal growth. The observed growth-stimulating effect mentioned before may require supraphysiological levels of androgens.

Gonadectomy and estrogen treatment

Sex steroid deprivation can be effectively induced by gonadectomy. The gonads are the exclusive source of synthesis of androgen and estrogen precursors in rodents, therefore gonadectomy results in complete ablation of sex steroids (108). Ovariectomy (OVX) in rats removes the growth-inhibiting influence of estrogens resulting in augmented longitudinal growth and decreased bone quality (109;110). Administration of estrogen to OVX rats counteracts the growth stimulation induced by estrogen deficiency (111). Treatment of OVX rats with non-aromatizable androgens stimulates longitudinal growth, which demonstrates a direct influence of androgen signaling on growth as well (112). In contrast, the growth pattern of OVX mice is marginally or not altered (113), illustrating different roles for sex steroid signaling in growth regulation in rats and mice.
Orchidectomy (ORCHX) in male rats results in decreased longitudinal growth, impaired body weight gain and osteoporosis (109). The contrasting effects of sex steroid depletion in male and female rats points at the existence of gender-specific patterns of growth regulation.

Pharmacological suppression of sex steroid biosynthesis or action

Instead of gonadectomy, gonadal sex steroid synthesis can also be obliterated by chemical castration with GnRHa. Triptorelin treatment in female prepubertal rats results in enhancement of body weight and length gain, comparable to the observations in the OVX phenotype. In male rats, GnRHa treatment stimulates body weight gain and also slightly improves length gain, which is in contrast with the observed growth retardation after ORCHX (114). It was speculated that GnRHa in rats may interfere with the hormonal regulation of food intake and weight control instead of having an effect on growth regulation (114).

Selective suppression of estrogen signaling can be established by treatment with an aromatase inhibitor. Several studies have analyzed the effects of such treatment on bone health and growth in male rats, but not in females. Young male rats treated with the aromatase inhibitor vorozole display decreased body weight and BMD, but normal femoral length (115). The selective estrogen receptor modulator (SERM) tamoxifen reduces body weight gain, axial and radial growth, and BMD in young male rats (62). These observations are in agreement with the phenotype of castrated male rats, albeit less prominently. In contrast, treatment with the aromatase inhibitor vorozole in male mice was shown to result in augmented body weight and tail length gain, an increased width of the epiphyseal growth plates and elevated GH levels (116). These results in mice are in contrast with the decreased growth patterns that characterize male estrogen receptor or aromatase knockouts that show normal to decreased growth.

Representativeness of models for the influence of sex steroids on growth

Estrogen has been identified as the main regulator of growth during the pubertal phase. Low levels of estrogen initiate the growth spurt at the beginning of puberty, whereas high levels at the end of this period arrest growth and result in epiphyseal fusion both in males and females (2). The growth-inhibiting actions of estrogen have been illustrated by the clinical presentation of male and female patients with estrogen deficiency due to inactivating mutations of the aromatase gene (52;54;55) and in the estrogen-resistant man with a mutation in the ERα gene (56). These patients all share a common phenotype with absence of a pubertal growth spurt, ongoing growth into adulthood, unfused epiphyses and a tall stature.

An *in vivo* model for studies on the effect of sex steroid signaling on growth would ideally recapitulate the hallmarks of human growth regulation. Rodents do not display the typical estrogen-driven signs of maturation as observed in humans. However, even though epiphyseal fusion never occurs in rodents, growth velocity approximates zero at the end of sexual maturation. Female OVX rats show stimulated axial and appendicular growth, and estrogen administration to intact male and female rats inhibits longitudinal and radial growth (110), pointing at an important role of estrogen signaling in growth regulation in the rat similar as in humans.

ERα, ERαβ, and aromatase gene knock-out as well as gonadectomized mice show either retarded or unaffected growth patterns compared with wild-type littermates. The contrast with the observed stimulation of growth in estrogen-devoid human patients implies that growth regulation in mice is markedly different than in humans, and argues against the use of mice models for studying human growth regulation by sex steroids.

C. *In vitro* models for the analysis of growth regulation

The epiphyseal growth plate, localized at the distal ends of long bones, is a complex structure that is crucial for skeletal growth (1;117). At this level, endochondral ossification takes place, during which cartilage is first formed and subsequently replaced by osseous tissue, resulting in bone elongation. The growth plate is a polarized, multilayered structure, that consists of chondrocytes at various stages of differentiation: the resting zone, with relatively quiescent, stem-cell like chondrocytes, the proliferative zone and the hypertrophic zone. Upon unknown triggers, chondrocytes from the resting zone are recruited to undergo proliferation, hypertrophic differentiation and programmed cell death. As a net result of this differentiation program, a cartilaginous scaffold is synthesized, that is subsequently invaded by blood vessels and bone cell precursors and then replaced by bone.
The highly complex process of endochondral ossification is regulated by the interplay between systemically circulating hormones and growth factors produced locally at the level of the epiphyseal growth plate. The exact mechanisms underlying the coordination of growth plate physiology remain to be elucidated in detail. Apart from *in vivo* models as described in part B, various animal-derived and human *in vitro* models have been developed and applied for studying growth regulation and physiology at the level of the growth plate.

Whole bone tissue culture

Explanted, cultured rodent bones such as the tibia or metatarsals can be easily manipulated by the addition of hormones, growth factors, cytokines, or pharmacological compounds to the culture medium. In most studies, the results of metatarsal culture are directly compared with those obtained in similarly designed experiments in cultured (human) chondrocytes. Valuable insights have emerged from this experimental approach. For example, it has been demonstrated that locally produced estrogen increases metatarsal growth by stimulating chondrocyte proliferation and inhibition of apoptosis (118). Inhibition of estrogen activity by the SERM tamoxifen results in growth inhibition (91). Modulation of the androgen receptor does not have an effect on growth (119). Metatarsal cultures have also made clear that glucocorticoids, pro-inflammatory cytokines and parathyroid hormone related protein (PTHrP) inhibit bone growth, possibly by interfering with the phosphoinositide-3-kinase (PI3K) pathway (120-124).
While there are clear advantages of this model, such as the easy accessibility of the explanted whole bone tissue culture, there are also disadvantages. When aiming at the analysis of chondrocyte differentiation, it has to be taken into account that other tissues such as bone and

hematopoietic tissue are also present in this culture system which may influence the behavior of the chondrocytes as well. In addition, species differences may hamper the translation of experimental data obtained in rodents to the regulation of growth in humans.

Growth plate studies

Rats and mice do not display epiphyseal fusion after completion of the sexual maturation phase. In contrast, rabbit growth plates do undergo fusion at the end of sexual development and important knowledge on this phenomenon typically observed in humans has emerged from studies in rabbits. It is hypothesized that epiphyseal fusion is a consequence of senescent changes within the growth plate, a process that is accelerated by estrogen (125). Catch-up growth, that is often seen in children after removal of a growth-inhibiting condition, was also demonstrated in rabbits after termination of growth-inhibiting dexamethasone treatment and was found to be associated with delayed growth plate senescence (126).
Theoretically, human growth plate specimens may also be used for analysis of growth plate architecture at different ages and phases of development, under the condition that a sufficient number of growth plates is available for such studies. However, normal human growth plate specimens are difficult to obtain, as they cannot be biopsied or removed from healthy children. Moreover, single growth plates have limited value in growth studies, as they only represent a specific growth stage corresponding with the donor's age and gender.

Chondrocyte culture

Various animal-derived and human cellular model systems have been developed to study the biomechanics of chondrocyte differentiation. The focus of this paragraph will be on human culture models. Amongst the most often used models are the primary chondrocyte culture, clonal normal cell lines (e.g. HSC-2/8), and transformed clonal cell lines (e.g. C-28/12, T/C-28a2) (127). Although applied for many years, there are many disadvantages to these models. Primary chondrocyte cultures have a low proliferative capacity, that is directly correlated with the age of the donor. Due to ethical considerations, it is difficult to obtain sufficient chondrocytes. Moreover, primary chondrocytes often show a tendency to dedifferentiation.
Clonal normal cell lines descend from one common ancestor and are non-transformed. Many cell-lines are derived from tumors, such as HSC-2/8 that was derived from an aggressive chondrosarcoma. The malignant origin of such cell lines may affect their biological characteristics. In addition, cell lines may display genomic instability or the frequent occasion of random mutations.

Transformed clonal cell lines are genetically modified resulting in immortalization of the cell line. These cell lines may display continuous proliferation in monolayer culture, but the loss of chondrocyte phenotypical characteristics has also been reported, as well as gradual loss of proliferative capacity.
Many of these cell lines have been obtained from articular cartilage, which is not identical to growth plate cartilage, and it is therefore unlikely that such models are representative for processes taking place in the growth plate during endochondral ossification. Some cell lines originate from diseased cartilage, such as osteoarthritic or trauma-damaged cartilage, which has been reported to alter the characteristics of the chondrocytes (127).

Stem cells

Alternatively, much effort is currently put into development of a human model for chondrogenesis using human mesenchymal stem cells (hMSC). hMSC are multipotent cells that can differentiate into a variety of cell types, such as chondrocytes, adipocytes, myocytes, and osteocytes, when exposed to the proper stimuli. hMSCs driven towards chondrogenic differentiation would provide a model that might facilitate a detailed analysis of molecular and biochemical processes underlying growth as it occurs within the human growth plate. It would allow a detailed analysis of even the very first stages of chondrogenic development, when the stem cells are triggered to undergo chondrogenic differentiation.
Some studies have addressed the potential use of hMSC-derived chondrocytes as a model for chondrogenic development and have characterized this model by means of large-scale expression profiling (128-130). Data emerging from these studies are promising, with respect to the aspect that cartilage is indeed formed after appropriate stimulation of hMSCs. However, before cartilage derived from hMSCs can be used as a model for studying chondrogenesis as it occurs in the human growth plate during endochondral ossification, it still remains to be determined whether hMSC-derived chondrocytes adopt a phenotype similar to growth plate or to articular cartilage.

D. Structure and scope of this thesis

In this thesis, we aimed to answer three questions related to the regulation and manipulation of growth. After the introductory ***Chapter 1***, the main body of the thesis is divided into three parts addressing these questions.

The first question we aimed to resolve in part A of this thesis was: what are the long-term results of two novel treatment modalities of children with ISS? Here we report the results of two randomized controlled clinical trials in children with ISS. ***Chapter 2*** describes adult height outcome after high dose GH treatment restricted to the prepubertal period. ***Chapter 3*** presents the effects of combined GH and GnRHa treatment on growth in adolescents with a relatively early pubertal onset.

In part B, we addressed the question: what is the effect of the administration of aromatase inhibitors in the rat? Aromatase inhibitor treatment has been suggested as a possible alternative to currently available growth-enhancing therapeutic regimens for children with ISS, but their efficacy and potential adverse effects have not been studied in a growing and developing model system. The answers to these questions would enable a better estimation of the potential efficacy and adverse effects of aromatase inhibition when considering to apply this type of medication to children. In ***Chapter 4*** the effects are shown of 3 weeks treatment with the steroidal aromatase inhibitor exemestane on longitudinal growth parameters, bone quality and gonadal morphology in female prepubertal rats. ***Chapter 5*** summarizes the results of 6 weeks of exemestane treatment on growth and bone quality in male prepubertal rats.

Part C provides an answer to the question: can mesenchymal stem cells differentiate into chondrocytes with a phenotype resembling the epiphyseal growth plate? ***Chapter 6*** illustrates how human fetal mesenchymal stem cells can be stimulated to undergo chondrogenic differentiation and may serve in future studies as a model for investigating processes taking place in the epiphyseal plate during growth.

Finally, ***Chapter 7*** provides a general discussion on the various elements of this thesis and is followed by a summary in ***Chapter 8***.

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