

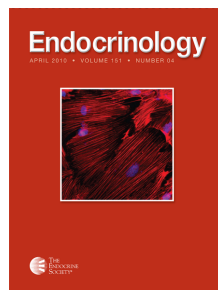
# ENDOCRINE REVIEWS

## Systemic and Local Regulation of the Growth Plate

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# Systemic and Local Regulation of the Growth Plate

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The growth plate is the final target organ for longitudinal growth and results from chondrocyte proliferation and differentiation. During the first year of life, longitudinal growth rates are high, followed by a decade of modest longitudinal growth. The age at onset of puberty and the growth rate during the pubertal growth spurt (which occurs under the influence of estrogens and GH) contribute to sex difference in final height between boys and girls. At the end of puberty, growth plates fuse, thereby ceasing longitudinal growth. It has been recognized that receptors for many hormones such as estrogen, GH, and glucocorticoids are present in or on growth plate

chondrocytes, suggesting that these hormones may influence processes in the growth plate directly. Moreover, many growth factors, *i.e.*, IGF-I, Indian hedgehog, PTHrP, fibroblast growth factors, bone morphogenetic proteins, and vascular endothelial growth factor, are now considered as crucial regulators of chondrocyte proliferation and differentiation. In this review, we present an update on the present perception of growth plate function and the regulation of chondrocyte proliferation and differentiation by systemic and local regulators of which most are now related to human growth disorders. (*Endocrine Reviews* 24: 782–801, 2003)

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Abbreviations: A, Androstenedione; AR, androgen receptor; ArKO, aromatase knockout; ARKO, AR knockout; BERKO, knockout mice for ER $\beta$ ; BMP, bone morphogenetic protein; BOCD, Blomstrand lethal osteochondrodysplasia; DERKO, knockout mice for ER $\alpha$  and ER $\beta$ ; DHEA, dihydroepiandrosterone; DHT, dihydrotestosterone; E<sub>1</sub>, estrone; E<sub>2</sub>, 17 $\beta$ -estradiol; ECM, extracellular matrix; ER, estrogen receptor; ERKO, knockout mice for ER $\alpha$ ; FGF, fibroblast growth factor; FGFR, FGF receptor; GC, glucocorticoid(s); GDF, growth and differentiation factor; GHBP, GH binding protein; GHR, GH receptor; GHS, GH secretagogues; GR, GC receptor; HIF, hypoxia inducible factor; HSD, hydroxysteroid dehydrogenase; IGFBP, IGF binding protein; Ihh, Indian hedgehog; MMP, matrix metalloproteinases; OVX, ovariectomy; Ptc, patched; T, testosterone; TR, thyroid hormone receptor; VEGF, vascular endothelial growth factor.

## I. The Growth Plate

THE GROWTH PLATE is a highly organized cartilage structure entrapped between the epiphyseal and metaphyseal bone at the distal ends of the long bones. Longitudinal growth takes place by a process called endochondral ossification, in which a cartilaginous scaffold is replaced by bone in a coordinated fashion. The growth plate can be divided into horizontal zones of chondrocytes at different stages of differentiation (Fig. 1) (Ref. 1).

### A. Structural organization

The process of chondrocyte proliferation and differentiation has been reviewed in detail previously (1–3). Of particular interest are the early studies in human femoral growth plates, describing the relationship between growth velocity, chondrocyte proliferation rate, and hypertrophic cell height, being profoundly different from that of rodents and rabbits (4, 5).

At the epiphyseal end of the growth plate, the reserve zone, also called germinal or stem cell zone, contains the resting chondrocytes. These cells have recently been shown to be crucial for orientation of the underlying columns of chondrocytes and therefore unidirectional bone growth, probably by secreting a growth plate-orienting factor (6). Upon some unknown trigger, the stem cells enter into the proliferating zone. In this matrix-rich zone, the flattened chondrocytes undergo cell divisions in a longitudinal direction and organize in a typical columnwise orientation. They synthesize substantial amounts of extracellular matrix (ECM) proteins, which are essential for the structure of the growth plate matrix. At a given moment, either by a finite number of cell divisions or by changes in exposure to a local growth factor (for example PTHrP; see Section IV.B) (7, 8), proliferating chondrocytes lose their capacity to divide and start to differentiate and become prehypertrophic, coinciding with an increase in size. Their location is called the transition

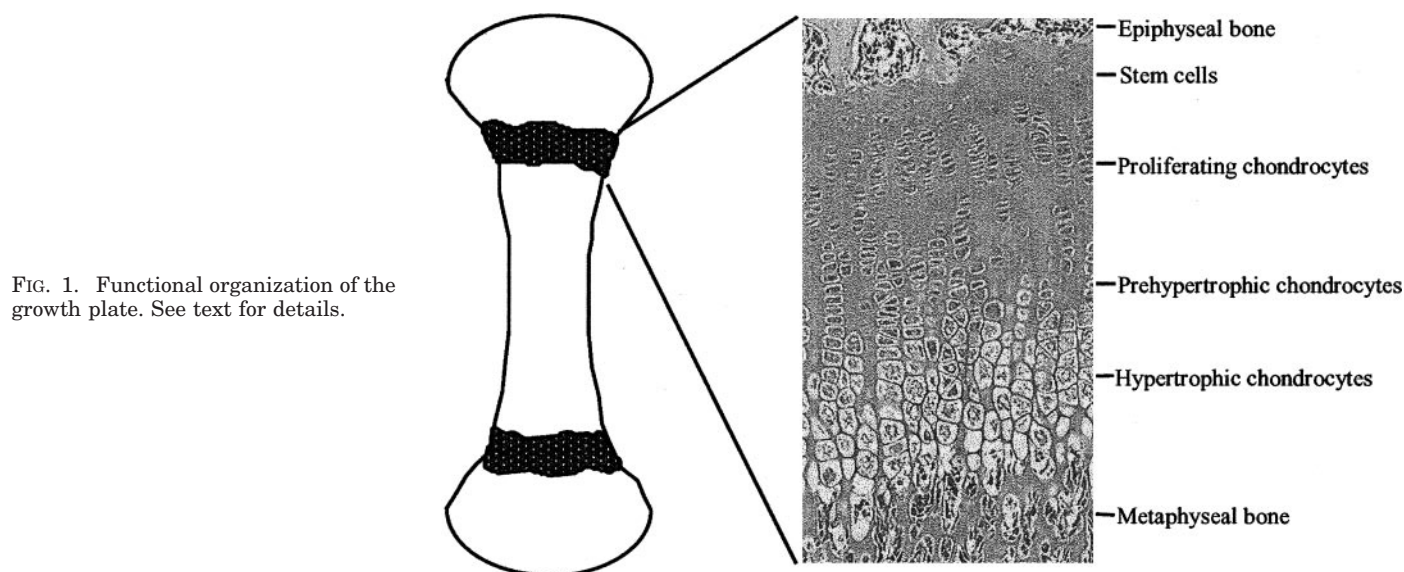


FIG. 1. Functional organization of the growth plate. See text for details.

zone. They then further progress in the differentiation pathway to become hypertrophic chondrocytes, which have a round appearance and secrete large amounts of matrix proteins. This stage is characterized by an increase in intracellular calcium concentration. This is essential for the production of matrix vesicles, which are small membrane-enclosed particles that are released from chondrocytes (9, 10). They contain large amounts of annexins, which mediate calcium uptake into the matrix vesicles (11, 12). The vesicles secrete calcium-phosphates, hydroxyapatite, and matrix metalloproteinases (MMPs), resulting in mineralization of the vesicles and their surrounding matrix. The mineralization process, in combination with low oxygen tension, attracts blood vessels from the underlying primary spongiosum (13). Subsequently, the mineralized chondrocytes undergo programmed cell death (apoptosis), leaving a scaffold for new bone formation. The apoptotic process is, among other factors, regulated by elevated intracellular calcium levels (leading to activation of proteases, lipases, and nucleases), retinoic acids, and vitamin D (12, 14–17). In particular, vitamin D deficiency resulting in childhood rickets is associated with failure of growth plate calcification, vascularization, and subsequent decrease in bone formation, phenomena that have also been observed in rachitic mice (18, 19). Longitudinal and transverse septae, which keep the chondrocytes in a columnwise orientation, are resorbed by chondroclasts or osteoclasts from the underlying primary spongiosum (20, 21). At the same time, osteoblasts enter the area to lay down new metaphyseal trabecular bone. The combination of chondrocyte proliferation, the enlargement of maturing chondrocytes in the hypertrophic zone, and the production of ECM are the major contributors to longitudinal bone growth.

### B. Extracellular matrix proteins

The chondrocytes are embedded in a surrounding matrix (ECM), which provides support to the chondrocytes and consists of ECM molecules, ECM remodeling enzymes, and various growth factors. The first group of ECM molecules consists of the collagens, of which types II, IX, and X are

expressed predominantly in the proliferating, prehypertrophic, and hypertrophic zones, respectively, and are pivotal for the integrity of the ECM (22). Additionally, they play an essential role in sequestering various growth factors involved in the regulation of chondrocyte proliferation and differentiation. Gene mutations in type II, IX, or X collagens have been associated with disturbances of the cartilage matrix causing spondyloepiphyseal dysplasia and hypochondriasis, multiple epiphyseal dysplasia, or Schmid metaphyseal chondrodysplasia, respectively (23–26). These dysplasias are all associated with short stature (Table 1).

Another group of ECM molecules comprises the proteoglycans, including aggrecan, biglycan, glypican, and chondroitin, which all require free sulfate groups for their activation and cross-linking of the ECM. Synthesis of undersulfated proteoglycans, for example by mutations in the diastrophic dysplasia sulfate transporter (DTSD) gene, causes several forms of autosomal recessive chondrodysplasias, including diastrophic dysplasia, atelosteogenesis type II, and achondrogenesis type 1B (27).

Communication exists between the ECM and cellular responses within the chondrocyte through cell surface adhesion receptors, known as integrins. They mediate the attachment of the chondrocytes to the surrounding ECM macromolecules, thereby increasing the integrity of the growth plate (28).

Furthermore, there is a group of ECM-remodeling enzymes, known as MMPs and their inhibitors (tissue inhibitor of MMP). These play a crucial role in the remodeling and degradation of the ECM and are involved in the preservation of the ECM integrity and the initiation of angiogenesis (29, 30). Mice lacking MMP-9, for instance, display abnormal growth plate vascularization and bone formation (31), whereas disruption of tissue inhibitor of MMP-1 in mice increases basement membrane invasiveness of primitive mesenchyme (precursor of chondrocyte) cells *in vitro* (32). Moreover, MMP-13 (collagenase-3) has been shown to be crucial for remodeling of the matrix in the transition zone of the growth plate (33). Inhibition of MMP-13 inhibits degra-

TABLE 1. Examples of causes of short and tall stature in man

Short stature	Tall stature
<b>Primary disorders</b> Chromosomal disorders Down syndrome, Turner syndrome Genetic syndromes with dwarfism Achondroplasia, hypochondroplasia, and thanatophoric dysplasia <sup>a</sup> (FGFR3) Jansen's metaphyseal chondrodysplasia and Blomstrand osteochondrodysplasia <sup>a</sup> (type 1 PTH/PTHrP receptor) Hereditary multiple exostoses (EXT) Spondyloepiphyseal dysplasia and hypochondriasis, multiple epiphyseal dysplasia, or Schmid metaphyseal chondrodysplasia (type II, IX, and X collagen) Noonan syndrome Intrauterine growth retardation  <b>Secondary disorders</b> GH deficiency Pit-1, Prop-1, GHRHR, or GH deficiency GH resistance Laron syndrome (GHR), IGF-I deficiency Hypothyroidism Malnutrition Celiac disease, food deprivation, anorexia nervosa Renal failure Glucocorticoid excess Hypercortisolism, GC-treatment regimens	Chromosomal disorders 47,XXY (Klinefelter syndrome) and variants Genetic syndromes associated with overgrowth Sotos, Marfan, Weaver syndrome, homocystinuria Genetic syndromes with neonatal macrosomia Beckwith-Wiedemann, Simpson-Golabi-Behmel  GH excess, pituitary gigantism GH-secreting tumors (pituitary adenoma, McCune Albright syndrome, multiple endocrine adenomatosis type 1), GHRH secreting tumors Hyperthyroidism Familial glucocorticoid deficiency Hyperinsulinism Lipodystrophy syndromes Sex hormone deficiency or resistance Androgen insensitivity syndrome (AR), testosterone synthesis disorders, estrogen resistance (ER), aromatase deficiency (Pseudo)precocious puberty Central and pseudo-precocious puberty
<b>Idiopathic short stature</b>	<b>Familial tall stature</b>

Currently known genes responsible for the particular disorder are mentioned in parentheses.

<sup>a</sup> Growth disorders that are not compatible with life.

dation of collagen II, which is predominant in the proliferating zone and suppresses the expression of collagen X, which is the major collagen of the hypertrophic zone (33). The ECM also functions as a reservoir of various growth factors that may be released and may influence chondrocyte function when the ECM is degraded. Moreover, the ECM may control the diffusion capacity of growth factors, including fibroblast growth factors (FGFs) and hedgehogs, which will be discussed later.

The role of the ECM is crucial for the integrity of cartilage and for normal longitudinal growth, but the interaction between collagens, MMPs, integrins, and the multitude of growth factors within the ECM is still far from understood.

## II. Longitudinal Growth

Longitudinal bone growth is the result of chondrocyte proliferation and subsequent differentiation in the epiphyseal growth plates of the long bones. It is regulated by a multitude of genetic and hormonal factors, growth factors, environment, and nutrition (34–38). All of these contribute to establishing the final height of an individual. There are at least three distinct endocrine phases of linear growth during postnatal life in man. A high growth rate is observed from fetal life, with a rapid deceleration up to about 3 yr of age. The second phase is characterized by a period of lower, slowly decelerating growth velocity up to puberty. The last phase, puberty, is characterized by an increased rate of lon-

gitudinal growth until the age of peak height velocity has been reached. Then, growth velocity rapidly decreases due to growth plate maturation in long bones and spine, leading to growth plate fusion and cessation of longitudinal growth (39, 40). Recently, the process and moment of growth plate fusion has been elegantly studied by Turner and co-workers (41), who determined the number of bony bridges between the epiphysis and metaphysis by microcomputed tomography in rats between 2 and 25 months of age. Although it is generally believed that cessation of growth succeeds growth plate fusion, this has recently been disputed by Parfitt (42). He observed cessation of growth of a metacarpal in a patient with pseudohypoparathyroidism, which was followed later by fusion of the growth plate. In support of this, a recent study in aged rats has shown that, despite cessation of growth, growth plates still exist with sporadic chondrocyte proliferation (43). Many studies, including those with transgenic mice models, have provided useful information concerning growth velocity and timing of puberty in humans and sexual maturation in rodents, which will be mentioned in the sections dealing with the various hormones and growth factors influencing these processes.

### A. Growth disorders

Disturbances of longitudinal bone growth occur quite frequently with a high diversity in etiology. In Table 1, causes of short and tall stature are summarized, some of which are



discussed here. Both short and tall stature disorders are divided into primary (defect presumed in bone/cartilage), secondary (defect located outside bone/cartilage), or idiopathic (cause unknown) growth disorders (44).

Short or tall stature does not necessarily lead to clinical problems during childhood and puberty, but psychosocial problems may occur in this vulnerable period of life. Examples are social withdrawal, practical problems relating to clothing and shoes, fear about future compatible partners and career planning, and (only in case of tall stature) a kyphotic posture (45).

### B. Catch-up growth

Many systemic diseases impair longitudinal bone growth. Interestingly, after remission, growth often accelerates beyond the normal growth rate for that particular age, a phenomenon called catch-up growth (reviewed in Ref. 46). This has been observed in many growth-retarding conditions such as Cushing syndrome (47), hypothyroidism (48), celiac disease (49), anorexia nervosa/malnutrition (47), and GH deficiency (46).

To explain catch-up growth, it was originally believed that a mechanism exists in the brain that compares the actual body size with an age-appropriate set point and adjusts the growth rate accordingly, and this is termed "size-stat" (50). This neuroendocrine hypothesis was challenged by an experimental study in the rabbit. In this experiment, dexamethasone was infused by an osmotic minipump directly in the tibial growth plate, which slowed bone growth of the treated leg but not of the contralateral vehicle-treated leg (51). When dexamethasone infusion was abrogated, tibial bone growth was not just normalized but even increased compared with the contralateral leg, thereby demonstrating catch-up growth (51). Based on these findings, Gafni and Baron (52) proposed that the underlying mechanism for catch-up growth was intrinsic to the growth plate, also termed growth plate hypothesis. A mechanism explaining catch-up growth may be that a maximum number of cell divisions exist for growth plate chondrocytes and that at each cell division the proliferation rate decreases, a process termed senescence. Growth retardation reduces chondrocyte proliferation, leaving them less senescent; when remission takes place, these cells have a greater proliferating potential, explaining the increased growth rate compared with the unaffected growth plate. This was recently supported by im estrogen injections in rabbits resulting in a more rapid senescence of growth plate chondrocytes, causing proliferative exhaustion and earlier growth plate fusion compared with nontreated rabbits (53). Still, these studies have all been performed in animals, and their pattern of catch-up growth is quite different from that of humans. For example, in a child who catches up, height velocity can be four times that of normal growth, whereas in rats and rabbits the growth velocity increment is minimal. To date, additional studies are required in humans to generate a more solid and satisfactory hypothesis for the process of catch-up growth (46, 54).

## III. Hormonal Regulation

The major systemic hormones that regulate longitudinal bone growth during childhood are GH and IGF-I, thyroid hormone ( $T_3$  and  $T_4$ ), and glucocorticoids (GC), whereas during puberty the sex steroids (androgens and estrogens) contribute a great deal to this process. For most hormones the effects on longitudinal growth and final height have been accurately described, but many of the molecular mechanisms underlying these effects have remained unclarified to date. In this review, the focus lies on the hormonal effects on longitudinal growth during childhood and puberty. For each hormone, important clinical data will be presented, followed by crucial *in vitro* and *in vivo* data from animal studies. The role of genetic factors, environmental influences, and nutrition on longitudinal bone growth should not be underestimated, but they will not be discussed here.

### A. GH-IGF-I system

Before birth, IGF-I and -II are believed to be the key regulators of growth, and largely independent of GH. This is based on findings in knockout mice, as well as on experiments of nature in the human; in congenital GH deficiency, birth length is only mildly diminished, whereas in the boy with congenital IGF deficiency birth size was severely diminished (55). After birth, GH is an important modulator of longitudinal bone growth and appears, together with IGF-I, the central player of the hypothalamus-pituitary-growth plate axis. GH secretion from the pituitary is tightly controlled by the activity of GHRH (stimulator) and somatostatin (inhibitor), which are released by the hypothalamus. With the discovery of ghrelin, the endogenous ligand with similar actions as the synthetic GH secretagogues (GHS) developed earlier, and the GHS receptor, a new physiological dimension in the regulation of GH secretion has arisen (56). Despite the increased complexity, it has become apparent that GHS acts synergistically with GHRH on GH release, making it a promising therapeutic strategy for GH-deficiency disorders (57). GH is secreted in a pulsatile fashion, which is more regular with higher peak levels in boys, whereas in girls GH secretion is more irregular (58). A pituitary adenoma in childhood or adulthood causes enhanced GH secretion, leading to pituitary gigantism or acromegaly, respectively (Table 1) (59, 60). Conversely, defects in the formation of GH-secretory cells (e.g., by Prop-1 or Pit-1 mutations), synthesis or release of GH (e.g., by GHRH-receptor or Pit-1 mutations, GH-gene deletions, and other forms of GH deficiency), or GH insensitivity, including defects in the GH receptor (GHR) and IGF-I deletion, all result in severe dwarfism (Table 1) (61–65).

*1. Hypotheses for the action of GH and IGF-I on longitudinal growth.* GH acts on its target tissue either directly or through two intermediates: IGF-I and IGF-II. They were called insulin-like due to their similarity in structure and activity compared with insulin. IGF-II is essential for normal embryonic growth (66), whereas IGF-I is a ligand that has a continuous function throughout development and adulthood (67). The role of IGF-II after birth remains less clear, especially because there are clear species differences in IGF-II expression after birth in bone. There is now substantial evidence that both

IGFs have a unique and complementary role in regulating bone growth (68). According to the somatomedin-hypothesis by Salmon and Daughaday (69) in 1957, GH stimulates somatomedin (IGF-I) synthesis in the liver, which in turn activates chondrocyte proliferation in the growth plate, thereby achieving longitudinal growth. It was not until the mid-1980s that several studies challenged this view, including the finding that local GH injection stimulates tibial bone growth significantly, whereas the contralateral tibia did not show this increase (70). The findings by Isaksson *et al.* (70) were corroborated by two additional studies in rats, demonstrating increased tibial bone growth after local GH or IGF-I injection compared with the contralateral vehicle-injected tibia (71, 72). Interestingly, it was demonstrated that local injection of GH regulates the number of chondrocytes expressing IGF-I in rats (73).

In analogy to the proposed dual-effector theory in adipocytes by Green *et al.* (74), Isaksson and co-workers used cultured growth plate chondrocytes to show that GH acts on resting zone chondrocytes and is responsible for local IGF-I production, which stimulates clonal expansion of proliferating chondrocytes in an autocrine/paracrine manner (75). Partly in agreement with the dual-effector theory but also contradictory to it, Hunziker *et al.* (76) showed that in hypophysectomized rats, stem cell cycle times reduced with either GH or IGF-I administration. In addition, proliferating cell cycle time and the duration of the hypertrophic phase were reduced. It was concluded that besides GH, IGF-I was also capable of stimulating growth plate stem cells, albeit to a lesser extent (76, 77).

In support of direct effects of GH on the growth plate, GHR was demonstrated on chondrocytes in rabbit and human growth plates (78, 79). Only very recently, both GHR and GH binding protein (GHBP) were also found in rat growth plate chondrocytes during postnatal development (80). Interestingly, the expression of GHR and GHBP in the growth plate was regulated by hypophysectomy, GH, T<sub>3</sub>, T<sub>4</sub>, and dexamethasone, which all affect longitudinal growth (80). Another interesting finding was that administration of GH increased the width of the germinal zone in mice deficient for IGF-I (*igf-1* null mice), further substantiating a direct role for GH on the growth plate (81).

Besides GH, IGF-I plays an important role in longitudinal bone growth during prenatal and postnatal life because knockout mice for IGF-I show, among other phenotypes, severe dwarfism (67, 82), and a child with a homozygous IGF-I deletion had extremely short stature (Table 1) (55). GH treatment had no effect on longitudinal growth and body weight in IGF-I deficiency in both humans and mice (55, 83). In addition, mice with an inactivated GHR gene, a potential model for Laron syndrome (Table 1), and a double knockout mouse for GHR and IGF-I were generated (84, 85). The GHR/IGF-I double mutants were smaller than either GHR or IGF-I single mutant, indicating that both GH and IGF-I contribute significantly to longitudinal growth. Further analysis of these mice demonstrated that IGF-I is the major determinant of both embryonic and postnatal growth and that IGF-I expression is modulated by GH in the postnatal period (83–85). In support of this, using IGF-I and IGF-II knockout as well as GH-deficient *lit/lit* mice, Mohan *et al.* (86) elegantly

showed that GH/IGF-I but not IGF-II was critical for puberty-induced bone growth. In addition, it was demonstrated that during prepuberty the effect of IGF-I on bone accretion was mediated via GH-dependent mechanisms, whereas during puberty IGF-I exerts both GH-dependent and independent effects on bone accretion.

## 2. Contribution of systemic vs. local IGF-I to longitudinal growth.

Recent molecular approaches have been used to further examine the relative contribution of systemically and locally produced IGF-I on longitudinal growth. Using the LoxP-Cre recombination system, liver-specific *igf-1* gene-deleted mice were generated, resulting in mRNA levels for IGF-I less than 1% of the levels in wild-type animals (87). These mice had greatly reduced serum IGF-levels as expected (25% of normal), but surprisingly they demonstrated similar growth rates compared with wild-type mice, despite elevated GH levels (88, 89). Based on these findings, the “Somatomedin hypothesis 2000” was proposed in which the liver was excluded from the somatotrophic axis (68). Still, challenges can be made against this model, because 25% of normal serum IGF-I, unaltered free IGF-I, and elevated GH serum levels may be sufficient to maintain normal growth (90). In addition, the liver-specific knockout of IGF-I may have been incomplete during the earliest time period when growth rates were still high (4 wk of age), whereas body growth had almost come to an end by the time the knockout was complete (89). In a recent study, double gene disrupted mice for liver IGF-I and the acid-labile subunit were generated, resulting in even lower serum IGF-I levels (91). These mice revealed reduced linear growth and decreased bone mineral density, suggesting that a threshold concentration of IGF-I is necessary for normal bone growth and bone density (91).

## 3. Localization of the IGF system in the growth plate.

It remains unclear to what extent local IGF-I contributes to longitudinal growth. A few reports have demonstrated IGF-II, but not IGF-I in growth plate chondrocytes (92, 93). Interestingly, *igf-1* null mice show a 30% decrease in linear dimension of the terminal hypertrophic chondrocytes, suggesting a role for IGF-I in the regulation of chondrocyte hypertrophy but not in proliferation (81). From these findings, Le Roith *et al.* (68) proposed a working mechanism in which IGF-II, controlled by GH, was involved in the regulation of chondrocyte proliferation, whereas IGF-I was responsible for chondrocyte differentiation. However, there is still debate about IGF-I localization in the growth plate. IGF-I and -II mRNA have been demonstrated in hypertrophic chondrocytes in mice postnatally (94), but in growth plates of fetal cows both mRNAs were detected in proliferating chondrocytes 5- to 32-fold higher than in hypertrophic chondrocytes (95). In another report, IGF-I mRNA has been demonstrated in proliferating chondrocytes of mouse growth plates (85). Finally, in rats, IGF-I was detected in all zones of the growth plate, with the highest expression levels in the proliferating and the prehypertrophic chondrocytes (73, 96). Adding to the complexity of IGF action in the growth plate, the expression of IGF-IR and -IIR as well as IGF binding proteins (IGFBP)-1 to -6 have also been studied in chondrocytes. In fetal cows, transcripts were detected for IGF-IR and IGFBP-2 to -5, with

reduced expression of IGFBP-3 to -5 in hypertrophic chondrocytes (95). Furthermore, IGF-II, both IGF receptors, and IGFBP-5 and -6 are coexpressed during early murine chondrogenesis, suggesting functional interactions between them (93). Moreover, IGFBP-3 and -5 have been demonstrated in rabbit costal chondrocytes (97). In another study, IGFBP-2 and -5 were present in developing chicken limbs, of which IGFBP-2 was proposed to be an inducer of chondrocyte maturation *in vitro* (98).

These findings in combination with the data from the knockout studies necessitate further research to delineate the exact working mechanism of the somatotrophic axis in the regulation of longitudinal growth.

### B. Thyroid hormone

Besides GH,  $T_3$  and, to a lesser extent, its precursor  $T_4$  are crucial for normal bone maturation (36, 99). Both congenital hypothyroidism and  $T_3$  deficiency are associated with severe growth retardation in rodents and humans (Table 1) (100–103). Hyperthyroidism causes an increased growth velocity in children but also leads to premature growth plate fusion and short stature (104, 105). These findings were recently underscored in thyroid hormone receptor (TR) $\beta$  mutant mice harboring a targeted resistance to thyroid hormone (106). In agreement with these observations, double knockout mice for both TRs (TR $\alpha$ 1 and  $\beta$ ) display retarded growth and bone maturation (107). However, the single knockouts for TR $\alpha$ 1 or  $\beta$  do not show this phenotype, indicating rescue effects in the single knockout mice, in which loss of one receptor can be compensated by the other (108, 109). Despite affected longitudinal growth, the phenotype of the double knockout is much milder compared with that in mice with severe hypothyroidism. This might be explained by the ability of TRs to bind thyroid hormone response elements and modulate gene transcription, even in the absence of ligand (107).

Besides indirect effects of thyroid hormones on longitudinal growth, for example by influencing GH secretion (100, 107), actions of  $T_3$  are at least partially direct, because TR $\alpha$ 1 and  $\beta$  proteins have both been demonstrated in stem cells and early proliferating chondrocytes of rat and human growth plates (110, 111).  $T_3$  seems to stimulate the recruitment of cells to the proliferating zone from the germinal zone and facilitate the differentiation of growth plate chondrocytes, in chicken chondrocytes, and in rat mandibular condyle and femur organ cultures (112–114). Its precursor,  $T_4$ , has been shown to increase the number of [ $^3H$ ]methylthymidine-labeled chondrocyte nuclei and [ $^{35}S$ ] incorporation in Snell dwarf mice growth plates, suggesting a stimulatory role on both chondrocyte proliferation and differentiation (115). Recent data have confirmed that  $T_4$  induces the expression of both type II and X collagen, the activity of the differentiation marker alkaline phosphatase, and chondrocyte hypertrophy (116). Furthermore,  $T_4$  but not GH is capable of completely reverting reduced widths of the proliferating and hypertrophic zone, as well as a disturbed growth plate architecture and vascular invasion of the growth plate in hypothyroid rats (117), further establishing a unique role for thyroid hormones in the regulation of bone growth and maturation.

Besides influencing GH secretion, thyroid hormones have been shown to interact with the GH-IGF-I pathway at the level of the growth plate. In the 1980s,  $T_3$  was shown to promote proliferation of embryonic chicken chondrocytes by enhancing IGF-I mRNA expression. Also in cultured rat epiphyseal chondrocytes,  $T_3$  stimulated IGF-I mRNA expression (112, 118). In addition, we have recently shown that  $T_3/T_4$  can regulate GHR expression, *in vivo* in rat growth plates (80). In summary, thyroid hormones act through chondrocytes bearing TRs to modulate growth plate proliferation, differentiation, and vascular invasion. Part of these effects appear to be mediated by modulating local GH and/or IGF-I actions.

### C. Glucocorticoids

Various clinical conditions, such as juvenile rheumatoid arthritis, chronic asthma, and post renal transplantation, require prolonged GC therapy, leading among other phenomena to decreased bone volume and growth retardation (119). In contrast, familial GC deficiency is associated with tall stature (Table 1) (120).

Dexamethasone treatment inhibited chondrocyte proliferation and matrix synthesis, suggesting that GC is a potent negative regulator of chondrogenesis (121). Early evidence for a direct effect of GC in the growth plate came from a study in which local dexamethasone infusion considerably reduced tibial growth compared with the contralateral vehicle-injected leg (51).

A few studies have now localized the GC receptor (GR) in rat bone cells, including chondrocytes (122), and in human growth plates, especially in hypertrophic chondrocytes, suggesting direct effects of GC on the growth plate (123).

Recently, GR was demonstrated in the proliferating and hypertrophic zone of rat growth plates, and treatment with high doses of corticosterone in rats caused a reduced growth plate width in long bones, concomitant with growth retardation (124). These findings are most likely explained by reduced chondrocyte proliferation, already demonstrated by Anfeldt (121) but recently also in prepubertal mice (125), in combination with increased apoptosis in terminal hypertrophic chondrocytes (126, 127).

GC have been shown to suppress growth by modifying the GH-IGF-I pathway at different levels. Besides inhibiting the release of GH from the pituitary, GC reduce IGF-I, GHR, and IGF-IR mRNA in growth plates but also inhibit basal and IGF-I induced DNA synthesis (125, 128–130). In addition, dexamethasone induced an increase in the number of IGF-IR expressing chondrocytes in mice growth plates (131). In rabbit costal chondrocytes, dexamethasone suppressed IGFBP-5 expression, a mediator of mitogenic activity of IGF-I, but up-regulated IGFBP-3, a growth inhibitor, at the same time, suggesting that differential regulation of IGFBPs could account in part for dexamethasone-induced growth arrest (97). Only very recently was it shown in rats that dexamethasone down-regulates GHR and GHBP expression in the tibial growth plate (80). GC are also capable of modulating (local) thyroid hormone levels. It has been shown that GC regulate deiodinase activity in rat kidney and liver (132, 133), and based on the expression of deiodinase in the growth plate



(134), GC may contribute to the control of  $T_3$  levels within the growth plate. Apparently, GC cause growth retardation, not only through direct effects via the GR but also by interference with other growth-modulating pathways.

#### D. Estrogens

It has long been established that sex steroids are important for longitudinal growth, especially during puberty. It was generally assumed that in girls, estrogen was the primary sex steroid regulating pubertal growth, whereas in boys this was achieved primarily by androgen. The finding of a unique male patient with an inactivating mutation in the classical estrogen receptor ( $ER\alpha$ ) fundamentally changed this view (135). This patient, who was resistant to the actions of estrogens, demonstrated longitudinal growth into adulthood resulting in tall stature due to absence of growth plate fusion as well as severe osteoporosis, despite high levels of testosterone (T) (Table 1) (135). In support of these findings, a similar phenotype was found in two male patients with an aromatase p450 deficiency (Table 1) (136, 137). Aromatase, encoded by the CYP19 gene, catalyzes the conversion of androgens into estrogens. Moreover, although data are limited, the pubertal growth spurt seemed to be absent in all three patients (135–137). For the estrogen-resistant patient, no suitable treatment was available, but administration of conjugated estrogen (Premarin) to the patients with aromatase deficiency led to growth plate fusion and enhanced accrual of bone mass within 6 months (138). These findings have led to the assumption that in both boys and girls, estrogen is the main determinant for the puberty-associated phenomena related to longitudinal growth and bone quality (139, 140).

**1. *In vivo* studies.** Whereas in humans an obvious growth spurt occurs during puberty and growth plate fusion at the end of it, rodents do not clearly demonstrate these phenomena during sexual maturation. Still, after sexual maturation in rats and mice, longitudinal growth diminishes resulting in growth rates approaching zero. In humans, the growth spurt is probably caused by low estrogen levels (in combination with elevated GH secretion), whereas growth plate fusion is mediated by the exclusive action of higher levels of estrogen, demonstrating that estrogen (above a certain concentration) is an inhibitor of longitudinal growth. This became evident from findings in patients with a mutation in the  $ER\alpha$  and aromatase genes (135–137).

Rats tend to respond in a similar fashion to estrogen: removal of estrogen by ovariectomy (OVX) stimulates longitudinal growth, whereas estrogen treatment inhibits this, although rats do not close their growth plate until late in life (141–143). In support of this role for estrogen, ovariectomized immature rabbits treated with either estrogen or the selective ER modulator raloxifene showed reduced chondrocyte proliferation and growth plate height and accelerated growth plate senescence (144). This study also showed that raloxifene acts as an estrogen antagonist on the growth plate in rabbits. In contrast, OVX in mice causes no change in nose-to-tail length compared with their sham-operated controls (145). Still, in both rats and mice a decrease in bone

density, cancellous bone area, and bone strength is observed, whereas increases are observed in bone turnover and radial bone growth (141, 145). Interestingly, several studies in hypophysectomized rats have shown that estrogen can inhibit longitudinal growth in the absence of GH (142, 146, 147). This suggests that in rats estrogen can inhibit growth independently of GH, possibly by directly influencing the activity of growth plate chondrocytes. In agreement with these data, patients with Laron syndrome demonstrate a pubertal growth spurt and epiphyseal fusion, despite having a defect in the GHR (148).

A number of studies have now demonstrated both  $ER\alpha$  and  $ER\beta$  in growth plate chondrocytes during development and in several species, indicating that estrogens can have direct effects on chondrocytes (149–157). Slight species differences in  $ER\alpha/\beta$  distribution in the growth plate might explain the observed discrepancies in growth responses and growth plate fusion to estrogen.

The actual longitudinal growth of an individual is achieved through activity of chondrocyte proliferation and differentiation within the growth plate. Indeed, after OVX in rats, bone length and growth plate thickness increased (143, 158), which was associated with an increased immunostaining for proliferating cell nuclear antigen, a marker for dividing cells (143). The opposite occurred in orchidectomized mice and rats (143, 159, 160). There is conflicting evidence on the role of estrogen during chondrocyte differentiation. In one study, it was reported that estrogen decreased matrix synthesis, contributing to the age-related decline in longitudinal growth (161). On the other hand, chondrocyte differentiation was shown to be increased by estrogen, as determined by [ $^{35}$ S] uptake in rabbit and human cultured chondrocytes (162, 163). In summary, besides indirect effects, estrogen exerts direct effects on growth plate morphology and physiology through  $ER\alpha$  and  $ER\beta$ , but their exact role in chondrocyte proliferation and (terminal) differentiation necessitates further clarification.

**2. *Knockout models.*** In the last decade, a number of animal models have been developed to study the regulation of longitudinal growth by sex steroids, including the ovariectomized rat, knockout mice for  $ER\alpha$  (ERKO),  $ER\beta$  (BERKO), and both ERs (DERKO), and the aromatase knockout mouse (141, 164–166). However, in ovariectomized as well as female estrogen-resistant ERKO mice, longitudinal growth is either unaffected or inhibited, which is contrary to the patient with the ER mutation (145, 167). Similar to the ERKO mouse, the male aromatase knockout mouse is also stunted in its longitudinal growth, which does not fit with the increased longitudinal growth in patients with aromatase deficiency (136, 137, 168). Vidal *et al.* (165) have extensively studied skeletal growth and maturation in male ERKO, BERKO, and DERKO mice. In this study, all parameters studied in the various ER knockouts, *i.e.*, longitudinal growth, growth plate width, radial skeletal growth, serum levels of osteocalcin and IGF-I were decreased in the ERKO and DERKO mice but were unaltered in the BERKO mice (165). In two other studies (169, 170), however, female BERKO mice demonstrated increased femur lengths compared with wild type, which correlated with increased serum IGF-I levels. Apparently,  $ER\beta$  is in-



volved in abrogating the effects on longitudinal growth mediated by ER $\alpha$ . Furthermore, species differences may account for discrepancies between findings in the human and rat and those in mice.

### E. Androgens

Despite the established role for estrogen as the primary modulator of pubertal growth, androgens also play a specific role. A few studies have assessed the effect of the administration of nonaromatizable androgens [dihydrotestosterone (DHT), a metabolite of T and the synthetic androgens fluoxymesterone and oxandrolone] on longitudinal growth in boys with constitutionally delayed growth. Longitudinal growth and ulnar length significantly increased without advancement of bone age, suggesting that androgen specifically stimulated longitudinal growth (171, 174). Moreover, DHT has been shown to increase longitudinal bone growth in 3-month-old rats (173) and increased [ $^{35}\text{S}$ ] incorporation in cartilage of growing rabbits during sexual maturation (162). T has also been described to increase body weight gain, body length, and growth plate width in mice, rats, and humans but it remains difficult to distinguish between the androgenic and estrogenic (after conversion) effect of T on these parameters (145, 174). However, local injection of supraphysiological amounts of T increased growth plate width compared with the vehicle-injected contralateral leg (175). These findings were corroborated in the mandibular condyle *in vitro* and in hypophysectomized and castrated rats, in which administration of T caused increased IGF and IGF-IR expression but also tibial growth plate width (176, 177). Thus, androgen appears to have stimulatory effects on longitudinal growth, independently of estrogen action and by influencing local mechanisms in chondrocytes.

Using a cre-lox conditional knockout strategy, Yeh *et al.* (178) reported the generation of androgen-receptor (AR) knockout (ARKO) mice. Besides other androgen-dependent phenotypes, the osteopenic phenotype of male ARKO mice strongly supports an important role of AR signaling in bone metabolism. By generating a bone-specific ARKO mouse, valuable information can be gathered about the precise role of androgens in bone.

Androgens are thought to play a pivotal role in the mas-

culinization of the skeleton and may do so by influencing the somatotrophic axis (58, 179). However, direct effects on cartilage are likely, because a high-affinity AR has been detected in rat costochondral chondrocytes (180). Furthermore, AR has been localized in hypertrophic chondrocytes of the human tibiae derived from surgery for corrective osteotomy (181, 182). AR mRNA and protein were recently demonstrated in the tibial growth plate of the rat (183). The differences in subcellular AR expression between males and females around sexual maturation suggests a role for T in establishing sex differences in longitudinal growth during sexual maturation (183). Taken together, these studies suggest that, besides estrogens, androgens also can directly influence processes in the growth plate and that they may account for some skeletal differences between males and females.

### F. Intracrinology

Originally, it was thought that peripheral organs obtained their sulfated and nonsulfated sex steroids from the circulation. Both androgens and estrogens are derived from cholesterol by a series of enzymatic steps taking place in the gonads and to a lesser extent in the adrenals (Fig. 2) (184, 185). Among the steroidogenic enzymes are steroid sulfatase, which catalyzes the formation of dihydroepiandrosterone (DHEA) and estrone ( $\text{E}_1$ ) from the sulfated precursors, DHEA sulfate and  $\text{E}_1\text{-S}$ , and sulfotransferase, which catalyzes the opposite direction. DHEA is converted into androstenedione (A) by  $3\beta$ -hydroxysteroid dehydrogenase (HSD). Furthermore, type I  $17\beta$ -HSD converts A into T and  $\text{E}_1$  into  $17\beta$ -estradiol ( $\text{E}_2$ ), whereas type II catalyzes the conversion in the opposite direction. Aromatase mediates the conversion of the androgens A and T into the estrogens,  $\text{E}_1$  and  $\text{E}_2$ . Finally, type I  $5\alpha$ -reductase irreversibly converts T into DHT (Fig. 2).

In the 1980s, the question arose as to what the source is of sex steroids in the peripheral organs, such as breast and fat tissue. It appeared that besides deriving nonsulfated sex steroids from the circulation, these organs were capable of locally synthesizing sex steroids from sulfated precursors, which are present in high amounts in the circulation. As a

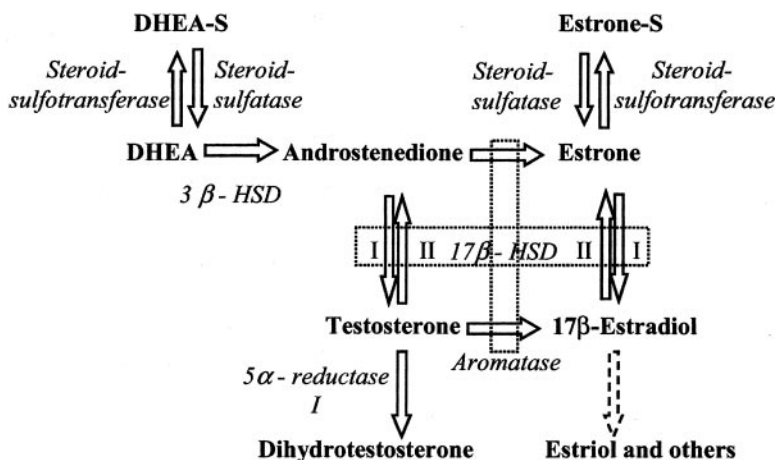


FIG. 2. Enzymes involved in the synthesis of sex steroids. See text for details.

consequence, the term intracrinology was introduced, stating that in some tissues sex steroids can be synthesized and act in the same cell without being released in the extracellular space or in the general circulation (184, 186, 187).

There is some evidence that steroidogenic enzymes are also present in bone cells. In primary cultures of rat osteoblasts, rat and human osteoblastic cell lines, and spongiosa obtained from patients undergoing orthopedic surgery, mRNA expression and bioactivity of the enzymes aromatase, type I and II 17 $\beta$ -HSD, steroid sulfatase, and type I 5 $\alpha$ -reductase have been demonstrated (188–193). Moreover, using *in situ* hybridization and immunohistochemistry, Sasano *et al.* (194) have localized aromatase mRNA and protein and type I 17 $\beta$ -HSD immunoreactivity in lining cells and osteoblasts in sections of 16 human tibiae. This finding was confirmed for aromatase in a study by Oz *et al.* (195). In the same study, aromatase immunopositive chondrocytes were detected in the femoral growth plate. In a recent study, aromatase, type I 17 $\beta$ -HSD, steroid sulfatase, type II 17 $\beta$ -HSD, and 5 $\alpha$ -reductase mRNA and bioactivity of the former three enzymes were detected in rat tibial growth plates (196). The data indicated that sex steroid metabolism occurs in the growth plate, especially around sexual maturation and thereafter. Perhaps during puberty in humans aromatase is up-regulated within the growth plate, thereby providing a mechanism to increase local estrogen levels, which may contribute to growth plate fusion at the end of puberty. Summarizing, these studies indicate that intracrinology may occur in the growth plate, thereby providing an additional mechanism to modulate local estrogen levels within the growth plate.

#### IV. Local Regulation

New insights into local regulation of chondrocyte activity in the growth plate mostly come from studies in transgenic mice revealing crucial roles of various growth factors in growth regulation in the embryonic growth plate (reviewed in Ref. 38). Besides IGF-I, the most important locally acting growth factors influencing longitudinal growth that have been identified are Indian hedgehog (Ihh), PTHrP, FGFs, bone morphogenetic proteins (BMPs), and vascular endothelial growth factor (VEGF). Many other factors such as vitamin D metabolites (197–199), retinoids (200), leptin (201–205), chondromodulin (206), C-type natriuretic protein (207), prostaglandins (198), hepatocyte growth factor (208), and Wnt proteins (209) contribute to local processes in the growth plate but are not discussed here. It has become increasingly clear that the same factors are also operational in growth plate regulation after birth with slight modifications. As such, these mechanisms are prime targets for regulation by hormones involved in longitudinal growth regulation, which will be discussed in Section V.

##### A. Indian hedgehog

Ihh belongs to the family of hedgehog proteins, which are morphogens that play crucial roles in embryonic patterning and development. Hedgehogs bind to a receptor called patched (Ptc), thereby releasing smoothened (Smo), a mem-

brane protein with an intrinsic intracellular activity that is abrogated by Ptc in the absence of hedgehogs. Releasing Smo results in its conformational change and a downstream signal to activate its intracellular targets (210–212). Ihh was found to be expressed in prehypertrophic chondrocytes of chicken and mouse fetal long bones (213, 214). Ihh has been recognized as a regulator of the pace of chondrocyte differentiation. Misexpression of Ihh in chicken long bones blocked chondrocyte differentiation (213). In a more recent study using Ihh-null mutant mice, apart from reduced chondrocyte differentiation as judged by histology and reduced collagen X expression, two additional roles for Ihh in endochondral ossification were revealed (215). The knockout mice showed dwarfism and exhibited markedly reduced chondrocyte proliferation but also failure of osteoblast development (215). Although Ihh regulated the pace of chondrocyte differentiation through the activity of PTHrP signaling (see Section IV.B), PTHrP was not able to rescue the effect on chondrocyte proliferation in mice with an Ihh null mutation. This indicated that Ihh regulation of chondrocyte proliferation occurs independently of PTHrP signaling (216). These data identify Ihh as a coordinator of endochondral ossification, regulating chondrocyte proliferation and differentiation and osteoblast differentiation, and coupling chondrogenesis to osteogenesis (Fig. 3) (215, 216).

##### B. PTH-related peptide

PTHrP was first associated with the pathogenesis of humoral hypercalcemia of malignancy (217). PTHrP and PTH share a common receptor, the type I PTH/PTHrP receptor (218). The crucial role of PTHrP and the PTH/PTHrP receptor in embryonic bone formation and longitudinal growth was recognized by a number of studies. Knockout mice for PTHrP showed accelerated chondrocyte differentiation leading to dwarfism (219), whereas ectopic expression of PTHrP in the growth plate caused inhibition of chondrocyte differentiation leading to a smaller cartilaginous skeleton compared with wild-type mice (220). The PTH/PTHrP receptor knockout mice demonstrated a similar, although more severe, phenotype compared with the PTHrP knockouts (221). Two human conditions, in which the function of the PTH/PTHrP receptor is disturbed, underscored the mouse studies (Table 1). Jansen-type metaphyseal chondrodysplasia was first described by Jansen in 1934 (222) and is characterized by abrogated chondrocyte differentiation resulting in severe dwarfism due to a constitutively activated PTH/PTHrP receptor (223). Conversely, homozygous inactivation of this receptor causes Blomstrand lethal osteochondrodysplasia (BOCD), which is also associated with dwarfism (224–226). BOCD presents as a severe form (type I) and a milder form (type II) and is characterized by accelerated endochondral ossification, the mirror image of the Jansen-type metaphyseal chondrodysplasia. Moreover, the phenotype of BOCD corresponds with the findings in the PTHrP and PTH/PTHrP knockout mice (219, 221).

PTHrP is expressed abundantly in the embryonic periaricular perichondrium in mouse, rat, and chicken (213, 227, 228). Its receptor at that stage is detected in late proliferating and early hypertrophic chondrocytes (213, 229). As men-

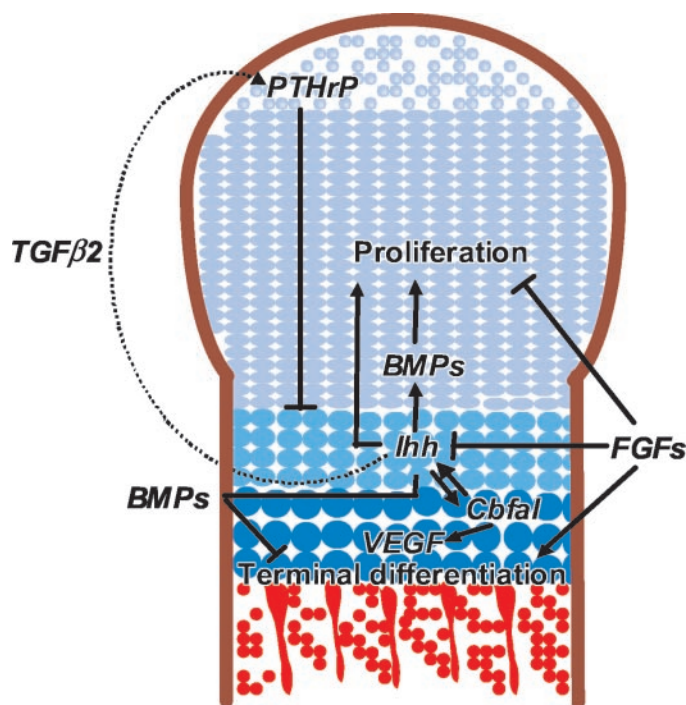


FIG. 3. Interaction of Ihh, PTHrP, BMP, and FGF signaling in modulating chondrocyte proliferation and differentiation during prenatal endochondral bone formation. Ihh is expressed by chondrocytes making the transition from a proliferating into a hypertrophic phenotype. Expression of Ihh at this stage is up-regulated by BMPs but inhibited by FGFs. Ihh activates adjacent chondrocytes and diffuses toward the lateral perichondrium, where it can bind to its receptor Ptc. Via an as yet unknown mechanism, PTHrP production is stimulated in the periarticular perichondrium. Then, PTHrP diffuses toward the prehypertrophic zone, which expresses high levels of PTH/PTHrP receptors and inhibits the differentiation of proliferating chondrocytes to cells capable of synthesizing Ihh. Besides modulating chondrocyte differentiation, Ihh also stimulates chondrocyte proliferation, both directly and indirectly (through BMP signaling). FGFs are able to inhibit chondrocyte proliferation independently of the two stimulatory pathways. BMP signaling inhibits terminal differentiation of chondrocytes, a process that FGFs can promote. The balance between BMP and FGF signaling seems to be crucial in regulating proliferation, Ihh expression, and terminal differentiation of chondrocytes.

tioned before, Ihh misexpression causes inhibition of chondrocyte differentiation but also induces PTHrP expression in the perichondrium, suggesting that Ihh is an upstream positive regulator of PTHrP (216). Indeed, in Ihh knockout mice, PTHrP expression in cartilage is virtually absent (215). Based on these findings, Vortkamp *et al.* and Lanske *et al.* (213, 221) proposed a model for a locally acting growth-restraining feedback loop, which controls the level of PTHrP in the growth plate. In this model, Ihh is expressed by chondrocytes making the transition from a proliferating into a hypertrophic phenotype. Ihh activates adjacent chondrocytes and diffuses toward the lateral perichondrium, where it can bind to its receptor Ptc. Via an as yet unknown mechanism, PTHrP production is stimulated in the periarticular perichondrium. Then, PTHrP diffuses toward the prehypertrophic zone, which expresses high levels of PTH/PTHrP receptors and inhibits differentiation of proliferating chondrocytes to cells capable of synthesizing Ihh (Fig. 3). Because Ihh and PTHrP are not expressed in close proximity to each other and the

ECM surrounding chondrocytes allows only limited diffusion of growth factors, intermediates may play a role between Ihh and PTHrP signaling and vice versa.

### C. Fibroblast growth factors

The family of FGFs constitutes at least 22 members that interact with at least four receptors (FGFR) and are major regulators of embryonic bone development (230, 231). Both FGF1 and -2 as well as FGFR1, -2, and -3 are expressed in chondrocytes (232–235). In humans, activating mutations in the FGFR3 cause achondroplasia (Table 1), the most common type of human dwarfism (97% of mutations have a Gly to Arg mutation in codon 380) (236–238). Other forms of chondrodysplasia due to mutations in the FGFR3 gene include hypochondroplasia, a milder form of dwarfism and two severe types, SADDAN (severe achondroplasia with developmental delay and acanthosis nigricans), and thanatophoric dysplasia (Table 1) (239, 240). Conversely, mice with an inactivating mutation in the FGFR3 gene demonstrate increased longitudinal growth (241, 242). In addition, overexpression of FGF2 slows longitudinal growth (243). Only very recently, mice lacking FGF18 have been generated. These mice demonstrated a phenotype similar to that observed in mice lacking FGFR3, including expanded proliferating and hypertrophic zones, increased proliferation, differentiation, and Ihh signaling (244). In addition, FGF18 deficiency leads to delayed ossification and decreased expression of osteogenic markers, not seen in the FGFR3 knockout phenotype, which prompted the authors to suggest that FGF18 coordinates chondrogenesis and osteogenesis through FGFR3 and -2, respectively (244). In addition, FGF18 appeared to act as a physiological ligand for FGFR3 in the growth plate. These studies indicate that FGFR signaling reduces growth by inhibiting proliferation and differentiation (Fig. 3).

Mancilla *et al.* (245) studied the effects of FGF2 on chondrocyte differentiation in a metatarsal organ culture system and found three growth-inhibiting mechanisms for FGF2: decreased growth plate chondrocyte proliferation, decreased cellular hypertrophy, and at high concentrations, decreased synthesis of cartilage matrix. Recently, a mouse model for thanatophoric dysplasia characterized by severe dwarfism was used to study the relationship between FGF signaling and the Ihh/PTHrP feedback loop (246). In these newborn mice with an activated FGFR3, Ihh and PTHrP mRNA expression were both down-regulated. In the same study, embryonic metatarsals from wild-type mice were cultured in the presence of FGF2, and similar results were found. Interestingly, FGF inhibited chondrocyte proliferation by down-regulating Ihh expression. Moreover, FGF and PTHrP signals independently inhibited chondrocyte differentiation. It was concluded that FGFR3 and PTHrP/Ihh signals act through two integrated parallel pathways that mediate both overlapping and distinct functions during longitudinal bone growth (246). In a recent study by Minina *et al.* (8), using a limb culture system, it was found that FGF and BMP signaling are antagonistic in the regulation of chondrocyte proliferation and in Ihh expression and the process of hypertrophic differentiation. The balance between the two adjusts



the pace of the differentiation process to the proliferation rate (Fig. 3) (8).

#### *D. Bone morphogenetic proteins/transforming growth factor $\beta$*

The family of BMPs is comprised of at least 15 members, which are all part of the TGF $\beta$  superfamily. BMPs were originally identified as stimulators of bone formation but are now recognized as important regulators of growth, differentiation, and morphogenesis during embryology (247). Within the developing limb cartilage elements, BMP2, -4, and -7 have been detected in the perichondrium, whereas BMP6 was found in prehypertrophic and hypertrophic chondrocytes (248–253). In addition, BMP7 was detected in chick sternal prehypertrophic and mice metatarsal proliferating chondrocytes (252, 253).

The effects of BMPs are mediated by two type I receptors, BMPRIA and -IB, which heterodimerize with the type II receptor, BMPRII. The type I receptors are differentially localized in embryonic limbs; BMPRIIB is detected in early mesenchymal condensations and is involved in early cartilage formation, whereas BMPRIA expression is confined to prehypertrophic chondrocytes (254). Constitutive active and/or dominant negative forms of BMPRIA and -IB revealed that the type IA receptor controls the pace of chondrocyte differentiation, whereas the type IB receptor is involved in cartilage formation and cell death (apoptosis) (254).

Because various BMPs are expressed in chondrocytes, cartilage defects may be anticipated in BMP-related disorders in mice (255). Mice bred with homozygous null mutations in BMP2 and -4 are not compatible with life (256, 257), whereas other family members such as growth and differentiation factor 5 (GDF5) and BMP5 are important mediators of chondrocyte differentiation in mesenchymal condensations at various sites (258–261). In addition, mice carrying a targeted disruption of BMPRIIB show defects in proliferation of prechondrogenic cells and chondrocyte differentiation in the phalangeal region (262). Additional BMPRIIB/GDF5 and BMPRIIB/BMP7 double knockout studies revealed that GDF5 is a ligand for BMPRIIB and that in the absence of BMPRIIB, BMP7 plays an essential role in appendicular skeletal development (262). In humans, only a few mutations in members of the TGF $\beta$  superfamily cause cartilage disorders. Genomic mutations in the human GDF5 gene have been shown to cause chondrodysplasia Grebe type, acromesomelic chondrodysplasia Hunter Thompson type, and brachydactyly type C, all of which are mainly characterized by defects of the limbs, with increasing severity toward the distal regions (263–265). Several mutations in the BMP antagonist noggin result in proximal symphalangism and multiple synostoses syndrome (266).

Recently, BMP6 was introduced as a possible mediator in the growth-restraining feedback loop involving Ihh and PTHrP (252). The fact that BMPRIA is expressed in the same region and that it has been shown to be critical for chondrocyte hypertrophy further strengthens an autocrine/paracrine role for BMP6 in prehypertrophic chondrocytes (254, 267). Still, the BMP6 knockout mouse hardly has any phenotype, leaving little evidence for an important physiological

role for BMP6 in chondrocyte differentiation (268). This was underscored by Minina *et al.* (269), who elegantly showed that BMPs do not act as a secondary signal of Ihh to induce PTHrP expression or to delay the onset of hypertrophic differentiation (Fig. 3). Despite this, they showed that normal chondrocyte proliferation requires parallel signaling of both Ihh and BMPs and that BMPs are capable of inhibiting chondrocyte differentiation independently of the Ihh/PTHrP pathway (269).

In another study, inhibition of chondrocyte differentiation by TGF $\beta$  was shown to be at least partly mediated by induction of PTHrP expression (270). In a second study by the same group, it was established that Shh, a functional substitute for Ihh, stimulates expression of TGF $\beta$ 2 and -3 in mouse metatarsals and that TGF $\beta$ 2 signaling is required for inhibition of differentiation and regulation of PTHrP expression by Shh (271). They concluded that TGF $\beta$ 2 acts as a signal relay between Ihh and PTHrP in the regulation of chondrocyte differentiation (274). These data imply that the BMPs/TGF $\beta$  and their receptors act as a signaling system, both dependently and independently of the Ihh/PTHrP feedback loop, at different levels during embryonic bone formation (Fig. 3).

#### *E. Vascular endothelial growth factor*

VEGF is a chemoattractant for endothelial cells and is one of the most important growth factors for endothelial cells (272). During chondrocyte hypertrophy, ECM surrounding the hypertrophic cells becomes calcified, which triggers the invasion of blood vessels from the underlying metaphyseal bone. This is preceded by the expression of VEGF in hypertrophic chondrocytes (31, 273). Inactivation of VEGF by systemic administration of a soluble receptor to 24-d-old mice suppressed blood vessel invasion and trabecular bone formation concomitant with an increased width of the hypertrophic zone (31). Moreover, recruitment of chondroclasts expressing MMP-9 and resorption of terminally differentiated chondrocytes were inhibited (31). In this context, MMP-9 knockout mice show a wider hypertrophic zone along with delayed chondrocyte apoptosis, vascularization, and ossification (21). These data indicate that VEGF and MMP-9 are key players in the events that take place during the endstage of endochondral bone formation such as terminal differentiation of chondrocytes, vascular invasion, chondrocyte apoptosis, and their subsequent replacement by bone (21, 31, 274). Other promoters or inhibitors of angiogenesis have been described in the literature, including transferrin (promotor) (275), chondromodulin (inhibitor) (276), and FGFs (promotor) (277).

In embryonic growth plates, Schipani *et al.* (13) described the role of hypoxia inducible factor (HIF)-1 $\alpha$ , which is a transcription factor that regulates VEGF expression (13, 278). Growth plate-specific targeted deletion of HIF-1 $\alpha$  caused increased cell death and reduced VEGF expression (13). At the same time, cells surrounding the area of increased cell death contained enhanced VEGF levels, which was secondary to their altered redox status. This suggests that VEGF expression is regulated in an HIF-1 $\alpha$ -dependent and -independent manner (13).

Recently, a member of the runt family of transcription factors, Cbfa1/Runx2, was demonstrated in hypertrophic chondrocytes (279). Constitutive expression in proliferating chondrocytes induced hypertrophic chondrocyte differentiation but also Ihh expression (280). In contrast, in primary chondrocytes from newborn mice, hedgehog signaling enhanced the expression of Cbfa1 (281). Apparently, a regulatory loop exists, in which Cbfa1 and Ihh regulate each other, involving hypertrophic chondrocytes and mesenchymal cells of the bone collar (282). Furthermore, Cbfa1 was shown to play a role in the expression of VEGF in growth plate chondrocytes (283). These data provide a link between Ihh and VEGF in regulating the pace of hypertrophic and terminal differentiation of growth plate chondrocytes (Fig. 3).

## V. Growth Plate Regulation after Birth and Interactions between Hormonal and Local Mechanisms

### A. Local growth plate regulation after birth

All growth factors mentioned above are expressed in embryonic growth plates, suggesting that they fulfill a crucial role in longitudinal growth before birth. However, evidence is gathering that they also play a role after birth. A number of papers have now localized Ihh, PTHrP, and their receptors in mammalian growth plates and fracture calluses, suggesting that these factors play a role in local regulation of longitudinal bone growth and fracture repair after birth as well (214, 228, 284–289). We were among the first to demonstrate Ihh, PTHrP, Ptc, and the PTH/PTHrP receptor in female and male rat growth plates during development (290). Interestingly, all genes studied within the postnatal growth plate were predominantly expressed in the transition zone and to a lesser extent in the growth plate stem cells. Because the distance from the growth plate chondrocytes to the periarthicular perichondrium considerably increases during embryonic bone development, due to the appearance of a secondary ossification center, diffusion of growth factors between these two areas will be seriously hampered. The localized expression of all players in the Ihh/PTHrP feedback loop predominantly in the transition zone of the growth plate suggests that diffusion over long distances is not required after birth and that the feedback loop may be in part autocrine (293).

Besides Ihh, PTHrP, and their receptors, BMP2, -4, -6, and -7 are also expressed in proliferating and maturing chondrocytes of growth plates after birth (284, 291–293). Moreover, their receptors, BMPRIA, -IB and -II, were found throughout the growth plate, albeit at a lower level in hypertrophic chondrocytes (293). Also, FGFs and VEGF are found in the postnatal growth plate (294–296), adding to the concept that the complete regulatory array of growth factors found in embryonic bones is present after birth as well and may play a similar role in the regulation of chondrocyte proliferation and differentiation.

Functional evidence for effects of growth factors in the growth plate after birth is still limited. A regulatory role for Ihh in chondrocyte differentiation comes from findings in a human condition known as hereditary multiple exostoses,

which becomes manifest after birth and in which mutations in genes encoding exostosins (EXTs) have been identified (297–299). Mutations in EXT 1 and 2 led to an absence of the proteoglycan heparan sulfate, which is an important signaling molecule involved in transport of growth factors in cartilage, including Ihh and FGFs (300–302). Absent or under-sulfated heparan sulfate may therefore result in disturbed Ihh and FGF transport through the growth plate, leading to disorganization of growth plate chondrocytes, further establishing Ihh as a central player in chondrocyte proliferation and differentiation (27, 302). In this respect, it is worthwhile mentioning that a recently identified mutation of the PTH/PTHrP receptor gene in human enchondromatosis signals abnormally *in vitro* and causes enchondroma-like lesions in transgenic mice. The mutant receptor constitutively activates hedgehog signaling, most likely causing formation of enchondroma-like lesions (303). In a more recent study, reduced growth plate Ihh and PTHrP expression was suggested to contribute to the observed growth delay and disturbed growth plate architecture in irradiated tibiae of young rats (304).

An important finding for a crucial role of PTHrP in growth plates after birth comes from a study by Schipani *et al.* (305). Targeted expression of a Jansen receptor (a constitutively active PTH/PTHrP receptor) in the growth plate of PTHrP-ablated mice rescued these mice from perinatal death and caused, among other phenomena, a reduction in longitudinal growth. Surprisingly, premature epiphyseal closure was observed in 3-wk-old mice. Growth plate closure does usually not occur until late in life. These observations suggest a role for PTHrP and its receptor in growth plate fusion (305).

### B. Interactions between hormonal and local regulation

Increasing evidence is gathering for interactions between hormonal regulation and local factors at the level of the growth plate. Receptors for GH, T<sub>3</sub>/T<sub>4</sub>, GC, estrogens, and androgens have all been detected in growth plates from various species, indicating that most, if not all, hormones can have direct effects on processes in the growth plate after birth (80, 110, 111, 122, 154, 155, 181, 183).

Stevens *et al.* (287) studied PTHrP and PTH/PTHrP receptor expression in hypothyroid, thyrotoxic, and hypothyroid-T<sub>4</sub>-treated rat growth plates. They found that thyroid status greatly influenced the expression of both proteins; hypothyroidism caused, in addition to disorganized growth plates, enhanced PTHrP expression, whereas in thyrotoxic rats the PTH/PTHrP receptor was undetectable. These data indicate that disturbed Ihh/PTHrP feedback loop activity may be a mechanism that underlies growth disorders in childhood thyroid disease (287). Only very recently, Kindblom *et al.* (306) examined the expression of Ihh and PTHrP in growth plates from patients subjected to epiphyseal surgery. It appeared that immunostaining for both Ihh and PTHrP was present predominantly in early hypertrophic chondrocytes and that its level of expression was higher during early stages and lower during later stages of puberty. These data are suggestive for the assumption that both Ihh and PTHrP are involved in the regulation of pubertal growth in humans and combined with the observations in the Jansen

mice suggesting a role for this system in growth plate closure (287, 305). Whether the Ihh/PTHrP feedback loop can be regulated by estrogens in the growth plate is, however, not clear. Interestingly, ERs are expressed in zones of the growth plate that also express Ihh and/or PTHrP, suggesting that the expression of these genes may be regulated by this sex steroid. That this might be the case comes from observations in the rat uterus, where E<sub>2</sub> induces PTHrP expression (307). Summarizing, it seems likely that systemic hormones involved in the regulation of longitudinal growth exert their effects on growth plate chondrocytes by influencing the expression and/or activity of locally acting growth factors, such as Ihh, PTHrP, BMPs, FGFs, and VEGF.

## VI. Conclusions and Perspectives

Over the last decade, much knowledge has been gained regarding local mechanisms regulating chondrocyte proliferation and differentiation in the embryonic growth plate. There is increasing evidence that these local regulatory systems are also functional after birth. The effects of hormones on longitudinal growth and final height are well known, and it is now recognized that these hormones exert a large part of their effects by acting directly on the growth plate. Still, the molecular mechanisms that underlie these effects are largely unknown. Intensive research focusing on the interaction of systemic hormones with locally acting mechanisms such as the Ihh/PTHrP feedback loop in the coming years will undoubtedly provide additional data that will shed light on the molecular regulation of longitudinal growth at the level of the epiphyseal plate.

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The National Institute on Aging (NIA) announces the annual Summer Institute on Aging Research, a weeklong workshop for investigators new to aging research, focused on current issues, research methodologies and funding opportunities. The program will also include consultations on the development of research interests. The 2004 Summer Institute will be held July 10–16 in Queenstown, MD. Support is available for travel and living expenses. Applications are due March 1, 2004. To increase the diversity of participants, minority investigators are strongly encouraged to apply. The applicant must be a U.S. citizen, non-citizen national or permanent resident. For additional information and application form contact:

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