Mini Review

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Mechanisms of Growth Plate Maturation and Epiphyseal Fusion

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Epiphyseal fusion · Growth plate maturation · Cartilage disorder · Growth disorder

Abstract
Longitudinal growth occurs within the long bones at the growth plate. During childhood, the growth plate matures, its total width decreases and eventually it disappears at the end of puberty with complete replacement by bone along with cessation of longitudinal growth. The exact mechanism of epiphyseal fusion is still not completely understood and experimental studies are complicated by the fact that there is a species difference between humans and rabbits that do fuse their growth plates and rodents that do not. This mini review summarizes hypotheses and theories postulated in the literature regarding growth plate maturation and epiphyseal fusion. Growth factors, local regulators and hormones involved in growth plate maturation are described as well as four postulated hypotheses and theories regarding the final steps in epiphyseal fusion: apoptosis, autophagy, transdifferentiation and hypoxia. A better insight into the mechanisms of epiphyseal fusion may ultimately help to develop new strategies for the treatment of cartilage and growth disorders.

Introduction

Longitudinal growth occurs at the epiphyseal plate, a thin layer of cartilage entrapped between the epiphyseal and metaphyseal bone, at the distal ends of the long bones [1]. In the growth plate, immature cells lie toward the epiphysis, called the resting zone, with more mature chondrocytes in the proliferating zone and large chondrocytes in the hypertrophic zone adjacent to this. During childhood, the growth plate matures, its total width decreases and eventually it disappears at the end of puberty with complete replacement by bone along with cessation of longitudinal growth. In specific disorders, timing of epiphyseal fusion is advanced or delayed; for example, in patients with estrogen deficiency it is delayed and in patients with precocious puberty it is advanced [2].

Nonsurgical treatment options to increase or decrease adult height are restricted to the period before epiphyseal fusion occurs. Delaying and/or lengthening the period of epiphyseal fusion, with or without additional growth-promoting therapy, can result in an increase in adult height by allowing more time for growth-supporting treatments in short children, while promoting epiphyseal fusion may reduce adult height in extremely tall children. The exact mechanism of epiphyseal fusion is still not

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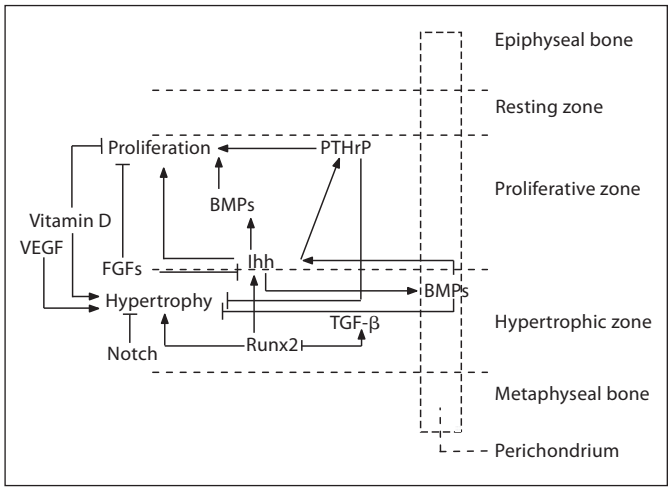

Fig. 1. Schematic picture of growth factors that play an important role in growth plate maturation.

completely understood. The fact that humans and rabbits fuse their growth plates but rodents do not complicates the interpretation of animal studies [3]. This mini review summarizes hypotheses and theories postulated in the literature regarding mechanisms of growth plate maturation and epiphyseal fusion.

Growth Factors and Local Regulators Associated with Growth Plate Maturation and/or Epiphyseal Fusion

Chondrocytes in the growth plate are influenced by various regulatory factors that together determine the rate of proliferation and maturation. These influences and interactions are depicted in figure 1 and described in this mini review. The formation of bone and cartilage begins with the migration of undifferentiated mesenchymal cells that differentiate into chondrocytes already in the embryonic stage of bone development. Postnatally bone development continues, with maturation of the growth plate influenced by multiple growth factors and hormones until late puberty when the growth plate fuses. We discuss some important growth factors, hormones and local regulators that all have an important role in growth plate regulation and thereby maturation. In addition, the adjacent perichondrium is also an important contributor to growth plate regulation. It contributes to osteoblast formation and invasion of blood vessels. Perichondrial cells send signals to chondrocytes via bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) and Wnt signaling, but vice versa also receive signals back from epiphyseal chondrocytes [1].

Paracrine regulators like parathyroid hormone-related protein (PTHrP) and Indian hedgehog (Ihh) are considered key factors in the regulation of the growth plate. These secreted growth factors coordinate endochondral ossification by regulating chondrocyte proliferation and differentiation as well as osteoblast differentiation [4, 5]. Both factors have been identified in the postnatal human growth plate and have been postulated to play a role in growth plate fusion since the expression levels change in puberty [6, 7].

In humans, mutations in the Ihh gene can lead to growth disorders. For example, acrocapitofemoral dysplasia, which is characterized by disproportional short stature, brachydactyly with cone-shaped epiphysis and premature fusion of the growth plates, is caused by a homozygous mutation of Ihh [8]. Postnatal ablation of Ihh in inducible and conditional knockout mice results in loss of the columnar structure in the growth plate, formation of ectopic hypertrophic chondrocytes, and premature vascular invasion. This causes advancement of growth plate maturation and induces early fusion of the growth plate [9]. In mammals, there are homologous proteins to Ihh in the hedgehog family, i.e. Sonic hedgehog and Desert hedgehog. Sonic hedgehog is very important during early embryonic development for patterning of many systems including the axial skeleton [10]. Interestingly, overexpression of Sonic hedgehog in chondrocytes interferes with growth plate organization and abrogates chondrocyte hypertrophy [11].

Modulation of parathyroid hormone and PTHrP signaling in the growth plate of mice also leads to abrupt closure of the growth plate, associated with decreased chondrocyte proliferation, accelerated differentiation and cell death [12]. This is in line with observations in Blomstrand chondrodysplasia patients who have an inactivating mutation of the parathyroid hormone receptor resulting in chondrodysplasia with advanced bone maturation [13]. In contrast, patients with Jansen chondrodysplasia who have an activating mutation of the parathyroid hormone receptor show a delay in bone maturation and are extremely short. Many of these features are recapitulated in the Jansen mouse model. Remarkably these mice show growth plate fusion early in life, suggesting that premature fusion may contribute to the extremely short stature of Jansen patients [12].

The transcription factor Runx2 plays an important role in the regulation of chondrocyte hypertrophy and

associated changes in the extracellular matrix [14]. In vitro studies showed that the expression and activation of this transcription factor is in part regulated by PTHrP and Ihh [15]. In addition, Runx2 interacts with TGF-β signaling via Smads in order to control chondrocyte maturation [16]. TGF-β is stimulatory in early stages of cartilage formation but in later stages it inhibits chondrocyte terminal differentiation and it has been hypothesized that it stabilizes the phenotype of the prehypertrophic chondrocyte [17].

A critical step in endochondral ossification is when blood vessels enter from the primary spongiosum, and osteoblasts invade from the bone marrow to lay down trabecular bone. Vascular endothelial growth factor (VEGF) is a potent mediator of angiogenesis and shown to be important in chondrocyte and osteoblast differentiation. Recently it was suggested that VEGF might play a role in growth plate fusion. Estrogen increases VEGF expression in rat growth plate chondrocytes in vivo and in vitro [18]. In addition, in pubertal human growth plate samples the expression of VEGF was upregulated with progression of puberty [18]. This suggests that VEGF might play an important role in estrogen-induced growth plate fusion. However, when in the adult mouse VEGF was specifically overexpressed in the growth plate, no fusion was observed [19].

Vitamin D deficiency in mammals leads to disturbances of the growth plate structure including increased width of the hypertrophic zone, decreased programmed cell death in hypertrophic chondrocytes, delayed invasion of blood vessels and bone cells, and lack of mineralization [20, 21]. Vitamin D metabolites (24,25-dihydroxyvitamin D) can be produced locally in the growth plate and these metabolites have been shown to stimulate differentiation and decrease proliferation of chondrocytes [22, 23]. The vitamin D receptor is expressed in the resting, proliferative and early hypertrophic zone of the rat growth plate [24]. By what mechanism vitamin D and its metabolites have an effect on the growth plate is not precisely known, although one suggested mechanism is through Ihh and PTHrP [25].

Other suggested factors important in chondrocyte differentiation and thereby growth plate maturation are the BMPs that promote chondrocyte differentiation. BMPs are differentially expressed across the rat growth plate and perichondrium with BMP agonists primarily expressed in the hypertrophic zone and BMP antagonists in the resting and proliferative zones [26]. This pattern might suggest evidence for a substantial role for BMP signaling in chondrocyte differentiation and thereby also growth plate maturation. Mice with a mutation in the BMP signaling pathway show deformities in bone, limb and digit development [27]. Minina et al. [28] published evidence for an interaction between BMP signaling and the Ihh-PTHrP feedback loop in the mouse. Ihh induces the expression of various BMPs and proliferating chondrocytes react to BMP signals with the upregulation of Ihh expression.

Another important pathway in chondrocyte development is the Wnt signaling pathway. Wnt signaling is involved in all stages of chondrocyte development since activation of the canonical Wnt pathway with β-catenin prevents differentiation of progenitor cells into chondrocytes and instead induces formation of osteoblasts [29]. In chondrocytes of the growth plate, canonical Wnt signaling stimulates hypertrophic chondrocyte differentiation. From in vitro studies it has been hypothesized that an alternative route of Wnt signaling through calcium-dependent kinases is predominant in chondrocyte differentiation [29].

An evolutionarily conserved pathway downstream of many developmental processes is Notch signaling, which has also shown to be important in cartilage development. Notch signaling suppresses chondrocyte hypertrophy [30]. For example, Delta-Notch2 signaling that occurs downstream of the Ihh, BMP and PTHrP pathways inhibits the differentiation of prehypertrophic to hypertrophic chondrocytes. Overexpression of these pathways results in stunted limbs with reduced ossification in the chicken [30].

Finally, the group of FGFs can act as antagonists of BMP signaling and negatively regulate Ihh expression as shown in mice [31]. FGF signaling inhibits chondrocyte proliferation. Temporal changes in FGF and FGF receptor expression were found in the growth plate of rats and it was speculated that this might contribute to growth plate senescence and thereby longitudinal growth [32]. Activating mutations in one of the receptors for FGF (FGFR3) result in achondroplasia or hypochondroplasia, and in delayed growth plate maturation early in human life which normalizes in adolescence [33, 34]. Loss-of-function mutations of the FGFR3 gene result in tall stature in humans [35].

Hormones Involved in Growth Plate Maturation and Epiphyseal Fusion

Longitudinal bone growth is not only influenced by a variety of growth factors, but also by various hormones acting directly or indirectly on the growth plate. Estro-

gens are known to play a key role in longitudinal bone growth by stimulating growth plate maturation, epiphyseal fusion and bone mineral accrual. Premature estrogen exposure in, for example, precocious puberty accelerates skeletal maturation, whereas on the contrary hypogonadism results in delay in skeletal maturation [36, 37]. Smith et al. [38] in 1994 described a male with an inactivating mutation in the estrogen receptor alpha (ERα) that showed no pubertal growth spurt and continued growth into adulthood associated with absence of growth plate fusion, resulting in tall stature (210 cm) and osteoporosis suggesting a role for ERα in growth plate fusion. To determine the role of ERα in growth plate cartilage for skeletal growth, a mouse model with cartilage-specific inactivation of ERα was recently developed [39]. Using these animals, it was found that ERα in growth plate cartilage is not important for skeletal growth during early sexual maturation. In contrast, it is essential for high-dose 17β-estradiol to reduce the growth plate height in adult mice and for reduction of longitudinal bone growth in elderly mice. Any functional role of ERβ has not yet been defined in the human growth plate. Interestingly, the membranous G-protein-coupled estrogen receptor 30 has been found to be widely expressed in the human growth plate and, moreover, to decline during the progression of puberty [40]. Furthermore, in genetically manipulated female mice G-protein-coupled estrogen receptor 30 was recently found to be involved in mediating estrogen effects on bone growth [41]. Altogether these findings suggest that G-protein-coupled estrogen receptor 30 may play a role in mediating estrogen effects in the growth plate.

Sex steroids acting on the growth plate are mainly produced by the gonads, which secrete sex steroids into the circulation in a classical endocrine way. In addition to this endocrine route, estrogens can also be produced locally by aromatase in the growth plate ('intracrinology') [42]. Also other enzymes essential for estrogen production, including 17β-hydroxysteroid dehydrogenase, steroid sulfatase and type 1 5α-reductase, have been detected in epiphyseal chondrocytes and shown to be upregulated during sexual maturation in the rat growth plate suggesting a role for these enzymes and the steroids they produce during pubertal growth and growth plate maturation [43].

Androgens can stimulate longitudinal bone growth also without conversion to estrogenic compounds [44]. This growth-increasing effect is not associated with increased circulating growth hormone (GH) or insulin-like growth factor 1 (IGF-1), but might be a direct effect since androgen receptors are expressed in the human growth plate [45] and local administration of testosterone can increase unilateral tibial epiphyseal growth plate width in the rat [46]. Another route of action is that the androgenic effect is mediated by local IGF-1 expression [47, 48].

The mechanism by which estrogens and other hormones exert their effect on longitudinal growth and finally growth plate fusion is not fully understood. Besides direct effects on estrogen receptors in the growth plate, indirect estrogenic effects through other hormones like IGF-1, GH and PTHrP have also been proposed [49–51], since levels of IGF-1 and GH change in line with estrogen during puberty [52]. It is well known that GH and IGF-1 can increase growth velocity as well as accelerate bone maturation measured as a decrease in growth plate height in children [53, 54]. GH receptors and the IGF-1 receptor IGF1R are expressed on human growth plate chondrocytes [55]. The exact contributions of these hormones in growth plate maturation and epiphyseal fusion still need to be clarified.

Senescence

Senescence is a term for the structural and functional changes over time in the growth plate, such as a gradual decline in the overall growth plate height, proliferative zone height, hypertrophic zone height, size of hypertrophic chondrocytes and column density [56]. Growth plate transplantation experiments in rabbits showed that the growth rate of a transplanted growth plate depends on the age of the donor and not on the age of the recipient, suggesting that growth velocity is regulated by a local mechanism intrinsic to the growth plate [57]. With progression of puberty, senescence in the growth plate increases and it is believed that when senescence has progressed to a certain point the growth plate fuses. Recent evidence from rabbit studies indicates that senescence might occur because stem-like cells in the resting zone have a finite proliferative capacity, which is gradually exhausted [58]. A new hypothesis is that proliferation is influenced by a multiorgan genetic program and that proliferation declines when this genetic program has reached a critical point [59]. While authors of this study believe that growth of organs like the liver and kidney of mammals can be explained by this theory, the hypothesis was not tested in the growth plate.

Estrogen is thought to advance growth plate senescence, causing earlier proliferative exhaustion, and thus

earlier fusion [56]. This might explain why estrogen treatment in girls does not induce growth plate fusion rapidly, but must act for years before fusion occurs. In line with this observation, the period of estrogen treatment required for growth plate fusion is longer in younger patients (e.g. in cases of precocious puberty) and shorter in older patients, like for example adults with a deficiency in aromatase compared to normal individuals [60].

Growth Plate Maturation and Epiphyseal Fusion at the Cellular Level

While there is no doubt that hormones and growth factors play a role in epiphyseal fusion, the final step at the cellular level is not completely understood. There are 4 mechanisms described in the literature which we would like to discuss in this review: apoptosis, autophagy, hypoxia and transdifferentiation.

Apoptosis

A most widely held hypothesis is that at the chondro-osseous junction site of the growth plate, terminally hypertrophic chondrocytes die by undergoing apoptosis leaving behind a scaffold of cartilage matrix for osteoblasts that invade and lay down bone [61]. It is assumed that the same mechanism eventually also results in epiphyseal fusion. Studies in the rat showed that apoptosis-regulating proteins (the so-called caspases, which are cysteine proteases) are expressed in the growth plate and that there is an increased expression of proapoptotic factors with age [62]. Typical morphological changes when cells undergo apoptosis include cell shrinkage with intact organelles and integrity of membranes, pyknotic nuclei by aggregation of chromatin, fragmented DNA, partitioning of the cytoplasm and nucleus into membrane-bound vesicles (apoptotic bodies) and absence of an inflammatory response [63, 64]. Interestingly, several recent studies failed to demonstrate a typical apoptotic appearance in the terminal hypertrophic chondrocytes and, therefore, these studies have questioned whether apoptosis is the final mechanism through which chondrocytes die in the terminal hypertrophic zone [63, 65]. Furthermore, we recently analyzed a unique piece of fusing human growth plate tissue during epiphyseal fusion and were not able to find signs of classical apoptosis [66].

Autophagy

Roach and Clarke [67, 68] studied rabbit growth plates and described chondrocytes with condensed chromatin, suggestive of apoptosis, but the 'morphology of the cytosol' was unlike that of necrotic, apoptotic, or normal cells. In 2004, these authors came up with the term chondroptosis to describe the appearance of these cells [69]. They reported autophagic vacuoles in the chondroptotic cells, suggesting a role for autophagy in the process of cell death of the terminal hypertrophic cell. Autophagic cell death is a different form of programmed cell death that involves a catabolic process in which the cell degrades its own components through autophagosomes. Signs of autophagy (like condensed chromatin, double-membraned structures and autophagosomes) were also observed in avian hypertrophic chondrocytes and in chondrocytes of newborn mice [70, 71]. Roach et al. [72] reported autophagic vacuoles in terminal hypertrophic cells suggesting a role for autophagy in the final step of endochondral ossification. However, no autophagosomes or signs of autophagy have ever been described in the human growth plate.

Transdifferentiation

The oldest hypothesis is that at the chondro-osseous junction site of the growth plate terminal hypertrophic chondrocytes can transdifferentiate into osteoblasts [73]. This theory is based on mostly organ and cell culture models, like for example chondrocytes in mice and murine metatarsal bone cultures that were able to transdifferentiate into osteoblasts producing bone matrix [74]. Adams and Shapiro [75] discussed that evidence in support of transdifferentiation is mostly circumstantial. It is based on microscopic examination of chondrocyte and osteoblast populations at the chondro-osseous junction and results from different studies are inconsistent. Although direct evidence is lacking, others speculate that transdifferentiation is present at the chondro-osseous junction because terminally differentiated cells are producing collagen type 1 together with extracellular matrix factors [76]. In addition, to our knowledge human studies on transdifferentiation at the chondro-osseous junction in the growth plate have not been described.

Hypoxia

In a unique human growth plate tissue specimen in the process of undergoing epiphyseal fusion, we observed a dense border of thick bone surrounding growth plate remnants at the site where normally the growth plate is located (fig. 2). In addition, signs of hypoxia and early necrosis were found [66]. We postulated that the border of dense bone might function as a physical barrier for

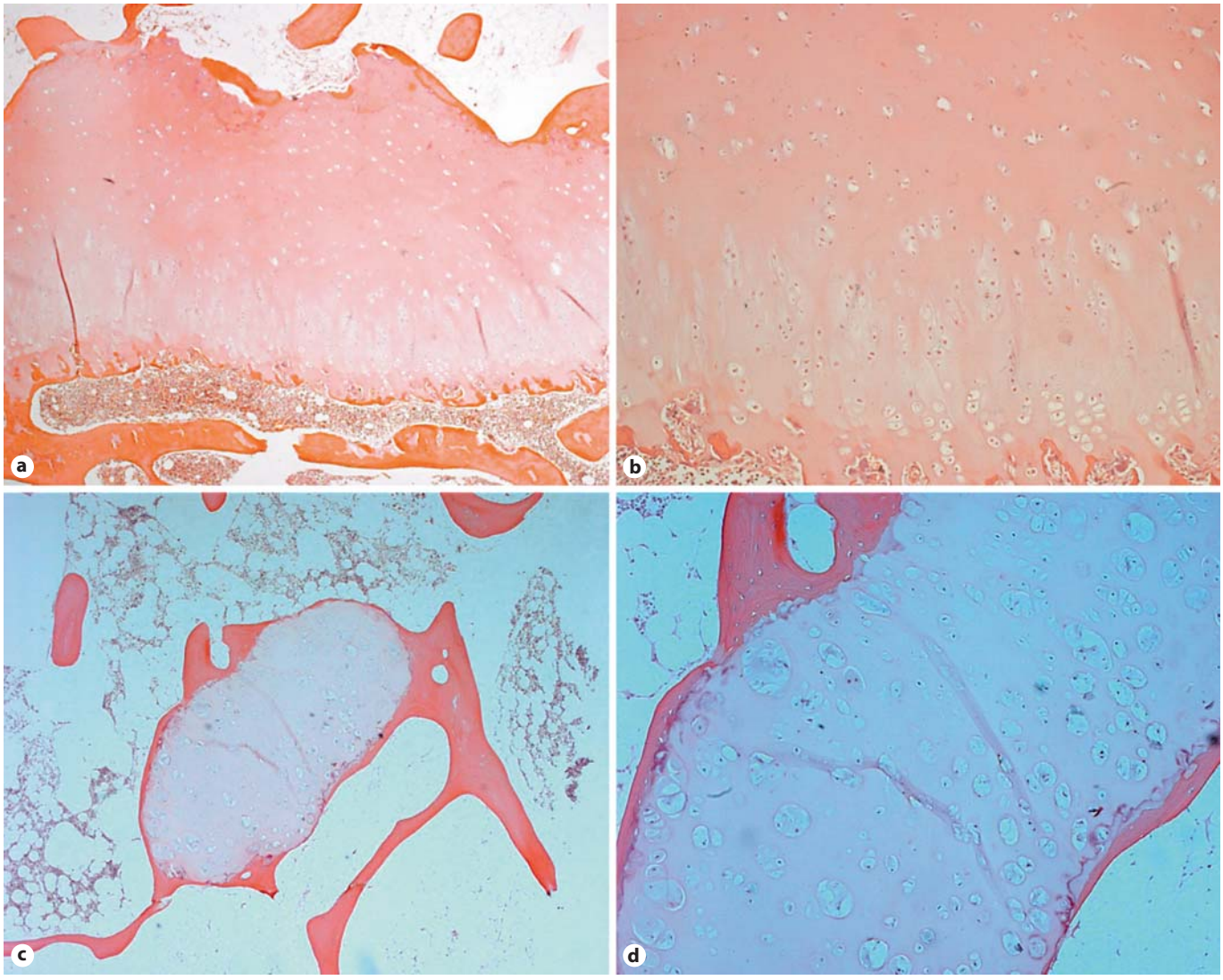

Fig. 2. Hematoxylin and eosin staining of sectioned human growth plates. In early pubertal patients, growth plate chondrocytes were organized in parallel columns. **a** ×40 magnification. **b** ×100 magnification. In a late pubertal patient, the growth plate was diminished to a small remnant surrounded by dense cortical-like bone. **c** ×40 magnification. **d** ×100 magnification. Reproduced from Emons et al. [66], with permission.

oxygen and nutrients to reach the fusing growth plate resulting in hypoxia and eventually cell death in a nonclassical apoptotic way through necrosis or a mixture of apoptosis and necrosis. In line with this new hypothesis, White et al. [77] recently demonstrated bridging bone in the center of a distal human tibial growth plate obtained from a 12.9-year-old girl, which might be an early sign of this shelling process. Signs of a hypoxia-related process were also reported by Stewart et al. [78] who observed an upregulated expression of hypoxia-inducible factor 2α mRNA during chick and murine chondrocyte differentiation in vitro. Hypoxia-inducible factor 2α knockout mice are small, which might indicate that this gene has an important role in the growth plate and subsequently in the regulation of longitudinal growth [79]. Thus, epiphyseal fusion might be a hypoxia-related process leading eventually to cell death of growth plate chondrocytes.

Conclusion

The exact mechanism by which physiological epiphyseal fusion occurs in humans is still not yet completely understood. Most of our knowledge regarding the regulation of growth plate fusion is based on animal studies. However, most animal models only partially correspond to the human situation and rodents do not fuse their growth plates at the end of puberty in normal physiological situations. More studies and better models are needed to reveal the mechanisms involved in epiphyseal fusion. Ultimately, this may help to develop new strategies for the treatment of cartilage and growth disorders.

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