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Review article

Morphology and physiology of the epiphyseal growth plate

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Abstract: The epiphyseal growth plate develops from the cartilaginous-orientated mesenchymal cells that express SOX family genes. This multilayer structure is formed by the proliferation and hypertrophy of cells that synthesize the extracellular matrix composed of collagen (mainly type II, IX, X, XI) and proteoglycans (aggrecan, decorin, annexin II, V and VI). The resting zone is responsible for protein synthesis and maintaining a germinal structure. In the proliferative zone, cells rapidly duplicate. The subsequent morphological changes take place in the transformation zone, divided into the upper and lower hypertrophic layers. In the degenerative zone, the mineralization process becomes intensive due to increased release of alkaline phosphate, calcium and matrix vesicles by terminally differentiated chondrocytes and some other factors *e.g.*, metaphyseal ingrowth vessels. At this level, as well as in the primary and secondary spongiosa zones, chondrocytes undergo apoptosis and are physiologically eliminated. Unlike adult cartilage, in fetal and early formed growth plates, unusual forms such as autophagal bodies, paralysis and dark chondrocytes are also observed. Their ultrastructure differs greatly from apoptotic and normal cartilage cells. Chondrocyte proliferation and differentiation are regulated by various endocrine, paracrine, and autocrine agents such as growth, thyroid and sex hormones, beta-catenin, bone morphogenetic proteins, insulin-like growth factor, iodothyronine deiodinase, leptin, nitric oxide, transforming growth factor beta and vitamin D metabolites. However, the most significant factor is parathyroid hormone-related protein (PTHrP) which is synthesized in the perichondrium by terminally differentiated chondrocytes. Secondary to activation of PTH/PTHrP receptors, PTHrP stimulates cell proliferation by G protein activation and delays their transformation into prehypertrophic and hypertrophic chondrocytes. When proliferation is completed, chondrocytes release Indian hedgehog (Ihh), which stimulates PTHrP synthesis via a feedback loop. Any disturbances of the epiphyseal development and its physiology result in various skeletal abnormalities known as dysplasia.

Key words: epiphyseal growth plate, cartilage, chondrocyte, cell differentiation, calcification, bone development, dark chondrocytes, paralysis chondrocytes

Introduction

Mammalian growth plate, also known as epiphyseal plate or physis, is highly specialized mesoderm-derived cartilaginous structure. It develops in the bone bud, secondary to presence of the primary ossification centers and is responsible for bone elongation. The plates are formed by numerous cells that rapidly divide and mature. Post puberty, the epiphyseal cartilage cell division decreases, bone completely replaces cartilage, and the epiphyseal plates fuse together with primary and secondary ossification centers [1,2].

Cartilage differentiation process

Presently, four major stages of chondrocyte differentiation are known, *i.a.*, mesenchymal precursor cells (MPCs), prechondrocytes, early chondroblasts and terminally differentiated chondrocytes [1-4].

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The initial steps in cartilage formation are secondary to condensation of mesenchymal cell from 12 to 15 per 1000 μm². MPCs initiate chondrogenesis by first migration to presumptive skeletogenic sites from the cranial neural crest, paraxial mesoderm, and lateral plate mesoderm and formation of cell mass condensations [3,5]. MPCs divide in the center of the condensations to form prechondrocytes that turn off the expression of mesenchymal and condensation markers. Instead of an elongated shape they become rounder with concomitant decrease of intercellular adhesion and intensive endothelial cell proliferation [6]. Such process is highly dependent on N-catherin and the presence of glycosaminoglycans, which synthesis stops at the end of cellular condensation [7]. Mesenchymal-endothelial junctions are stabilized by fibronectin, the synthesis of which is stimulated by transforming growth factor β (TGFβ) [8]. The following steps depend on syndecan-3 which requires transformations from mesenchymal condensation to direct skeleton development and prohibits the mesenchyme over-proliferation [3,9]. Simultaneously, most of the primary vessels disappear, and only those stimulated by vascular endothelial growth factor (VEGF) persist [5]. Secondary to transcriptional factor Runx2, mesenchymal cells commences synthesis of collagen type I, osteocalcin, osteonectin and osteopontin. Such primary selected cells are characterized by high expression of gene coded bone morphogenetic protein 6 (*BMP6*). In contrast, cells predominately directed to cartilage development have high expression of Sox family genes that regulate synthesis of collagen type II and its isoform IIa1, as well as collagen IXa1 and XIa2 [3,10,11]. *Sox9* plays a crucial role in the chondrogenesis initiation [12]. However, it was already proven that due to unknown molecular mechanism, some cells with *Sox* expression may develop to osteoprogenitor cells [13]. Differentiation of prechondrocytes leads to active chondrogenic cells called chondroblasts, which rapidly proliferate and build new bone tissue. They expressed *Sox9* which is required for further differentiation [14]. *Sox9* is highly expressed in prechondrocytes and chondroblasts, however, once the cells undergo prehypertrophy or hypertrophy, the gene turns off [15]. In the study of Akiyama *et al.* [12] inactivation of *Sox9* using Prx1Cre transgene, a gene expressed in early limb bud mesenchymal cells, resulted in lack of appendicular cartilage elements in embryo. Such observations have suggested that transcription factors have been identified to control mesenchymal cell migration, proliferation, survival, and condensation in one or a subset of cartilage elements. These elements control the skeletal development in multiple species and are also referred to as patterning factors since they determine the shapes and sizes of the skeletal elements [1,14].

Prechondrocyte differentiation leads to the next stage, known as early chondroblasts. Early chondroblasts develop cartilage growth plates by assuming a flattened shape and organizing into longitudinal columns. They proliferate at a high rate until, one layer at a time; they exit the cell cycle and start to increase in size, undergoing prehypertrophy followed by full hypertrophy. They undergo apoptosis to allow primary ossification centers to expand. Cells located in the middle of the epiphysis of future long bones undergo a similar maturation process that leads to the formation of secondary ossification centers [1,2,16].

Chondrocytes are metabolically active cells that synthesize various elements of the extracellular matrix (ECM) [17] They are also a source of so called matrix vesicles, 100 nm in diameter-follicles, formed by separation of cellular membrane [4,18,19]. The calcium accumulation in matrix vesicles seems to be directly related to calcium channel molecules – annexin II, V and VI that are found in the external lipid bilayer of the vesicles. Collagens type II and X bind to matrix vesicles and, in turn, interact with the annexin V [20]. The role of type X collagen, primarily found in the hypertrophic zone (see below), is to facilitate the deposition of calcium within the matrix [21]. Some other matrix elements are also involved in this process mostly proteoglycans, *e.g.*, aggrecan which aids in the osmotic properties that are essential for resisting compressive loads, as well as decorin that regulates collagen fibrillogenesis. On the contrary, cartilage oligomeric protein is a non-collagenous calcium binding glycoprotein found in the ECM [22].

Growth plate morphology and physiology

Each growth plate is a sandwich-like, multilayer structure divided into four well defined zones: reserve, proliferative, transformation and degeneration (Fig. 1). The last zone is adjusted to the primary spongiosa zone that finally gives origin for the secondary spongiosa zone.

Resting cartilage cells lying within the reserve zone (also known as resting or germinal zone) are formed by small, uniform, compactly located chondrocytes that occur singly or in pairs and are rich in lipid and cytoplasmic vacuoles (Fig. 2A). Additionally, in the zone ECM take more place than cells [1]. This zone is characterized also by low rates of proliferation and proteoglycan and collagen type IIB synthesis [1,13]. It is surrounded the groove of Ranvier, that hosts chondrocyte progenitor cells, which flow into the cartilage of this germinal layer [1,22].

The direct continuation of the reserve zone is the second layer known as proliferative zone. Its chondrocytes are flat and well divided into longitudinal columns (Fig. 2B). The mitotic activity is found only

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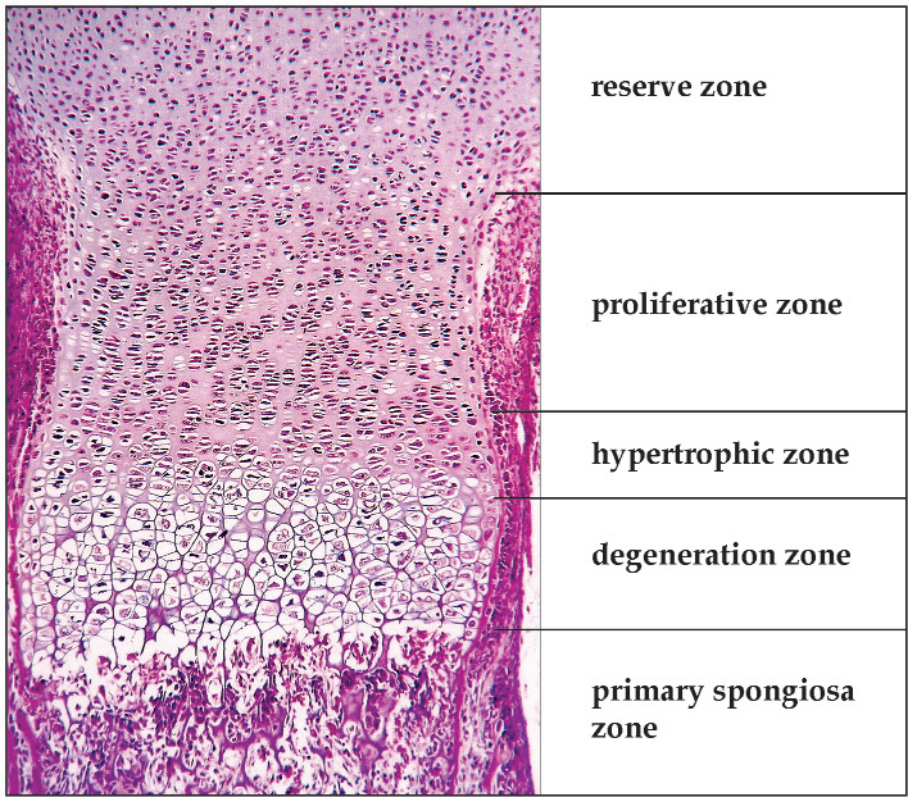

Fig. 1. Longitudinal section of the proximal femoral epiphysis from 21-day-old rat pup (H+E, original magnification ×100).

in the base of the columns. This true germinal layer has increased type II and type XI collagen synthesis [22].

Below the proliferative zone is another layer known as transformation zone, divided into upper and lower hypertrophic zone and a farther located degeneration zone. The most characteristic feature of chondrocytes of this level is lack of cellular division and decreased DNA synthesis. Instead of proliferation the cells synthesize high amount of various elements of ECM. It is worth to mention that this is the only zone that contains short-chain collagen and the first to produce alkaline phosphatase, responsible for the widening of the growth plate by increasing phosphate ions that are required for calcification [1,2,22]. Compared to the other zones chondrocytes of transformation one are relatively larger. They begin to terminally differentiate and become swollen (Fig. 2C and 2D). Closer to the primary spongiosa zone the amount of cells with features of degeneration increases. Chondrocytes lose intercellular junctions and are located in special vesicles formed by ECM [1].

Major phenotypic changes occur when chondroblasts divide into prehypertrophic and hypertrophic chondrocytes. Cytoplasmic volume increases ten times and levels of mRNA increase for most early cartilage matrix genes [14]. Once they reach the hypertrophic stage, they no longer express early cartilage matrix genes. Cells located centrally to the cartilage anlagen differentiate directly to prehypertrophy from prechondrytic or early chondroblastic stage [1,14,17]. This process is regulated by genes *Sox*5/*Sox6*. These genes function is to hinder the prehypertrophy rate and to promote hypertrophy [23].

Chondroblast columnar proliferation is also assisted by a transcriptional activator of the Runt domain. This domain holds the activator Runx2 which directs organization of columns by influencing chondroblast proliferation. *Runx2* is expressed in chondrogenic mesenchymal cells and hypertrophic and terminally differentiated chondrocytes but is inactivated in chondroblasts [24]. *Runx2* and *Runx3* both induce chondrocyte prehypertrophy and hypertrophy. When *Runx2* was expressed in chondroblasts of transgenic mice, the cells showed ectopic maturation, however, when the negative form was expressed in the same cells, events of prehypertrophy followed [25]. Therefore, it seems that Runx2 is a direct transcriptional activator of chondrocyte maturation markers.

The maturation of chondrocytes is due to physical and biochemical changes that occur in a spatial and temporal pattern. In differentiation, there is a 5-10-fold increase in intracellular volume, which is not a passive swelling. Instead, it is an active process by which there is an increase in organelles such as mitochondria and the endoplasmic reticulum [5,26]. The chondrocytes located in bones with rapid growth such as the femur, increase at a faster pace than chondrocytes in bones, like the radius, primarily due to the proportions of size [27].

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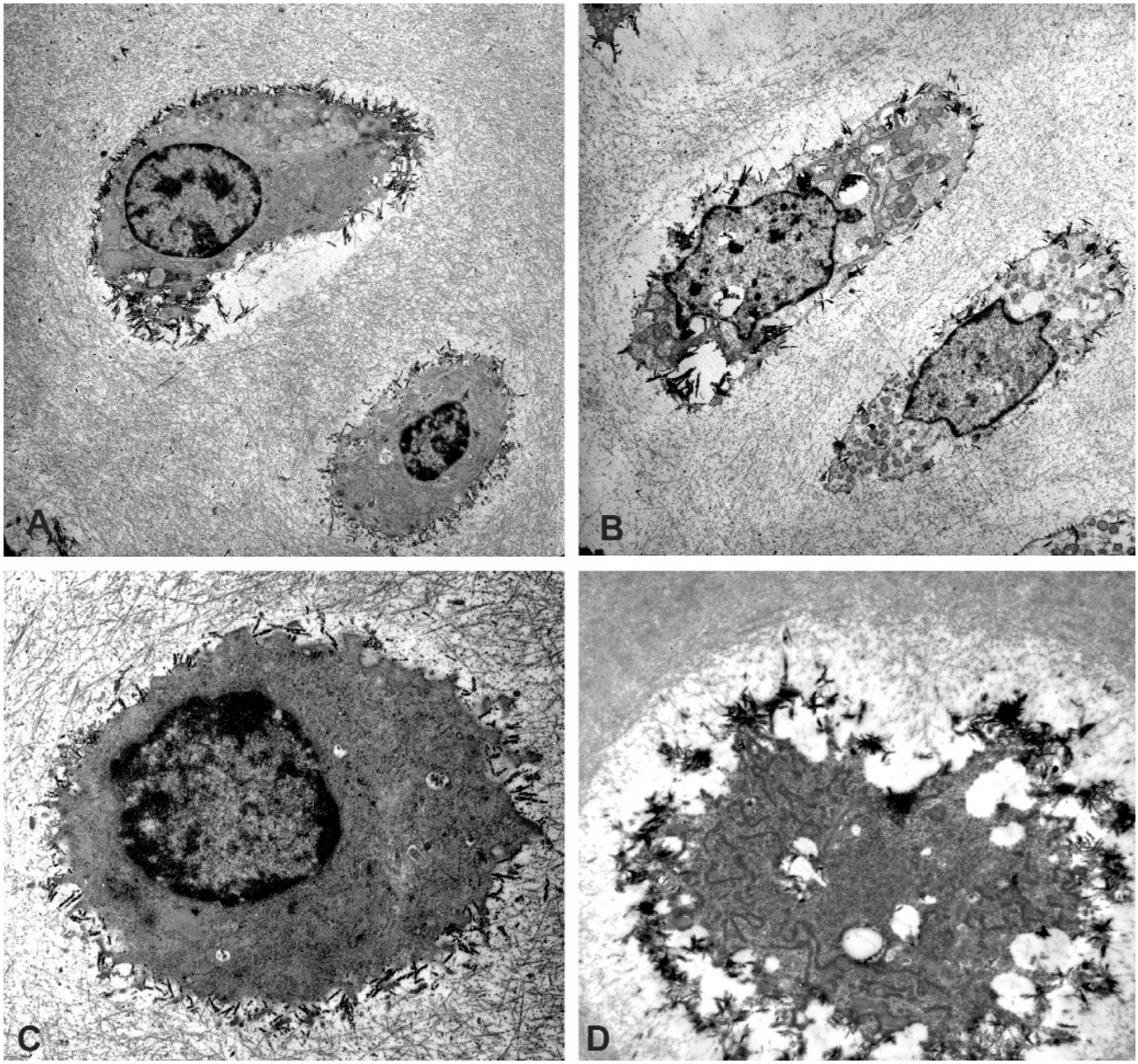

Fig. 2. Chondrocytes of the reserve (**A**), proliferative (**B**), hypertrophic (**C**), and degenerative (**D**) zones of the proximal femoral epiphysis from the 7-day-old rat pup (TEM, original magnification ×3000).

Despite of above mentioned alkaline phosphate, one of the most important factors for chondrocyte hypertrophy and terminal differentiation is the transcription core binding factor 1 (CBFA1) [25]. However in mice lacking CBFA1 in their growth plate, normal hypertrophy is observed, which indicates that other transcription factors like Smad-1, -5 and -8 also play an important role in this process [25,28,29].

The morphology of the lowest zone – primary spongiosa – is similar to the lower level of degeneration zone. However, it is characterized by presence of osteoprogenitor cells. Due to initiation of mineralization process, primary ossified bone lamella and small blood vessels are also visible [1,2,22].

As hypertrophy shifts to terminal differentiation, the gene *Col10a1* ceases to be expressed in chondrocytes leading to the activation of a new group of genes [14]. They induce extracellular matrix mineralization and as current view states, they result in cell death, or apoptosis. The importance of a terminally differentiated chondrocyte seems to be the preparation of calcification by the matrix; since once the cartilage calcifies, apoptosis is required to remove the terminally differentiated chondrocytes. After the cartilage has undergone calcification, the removal of differentiated chondrocytes allows for access by the vascular channels and bone-marrow stromal cells [30-35]. Chondrocytes in the hypertrophic zone have similar morphological

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Table 1. Basic morphological features in various types of programmed cell death of chondrocytes (based on Roach and Clarke [44] with own modification).

	Apoptosis	Dark chondrocyte	Paralysis chondrocyte
Nucleus	rounded nucleus; chromatin condensation into rounded bodies, crescent or caps marginated to the nuclear envelope; fragmentation of nucleus and formation of apoptotic bodies; digestion during phagocytosis of apoptotic bodies by nearby cells; chromatin condensation before or simultaneously with cell shrinkage	dark nucleus with convoluted outlines; chromatin condensation into small "patches" located throughout the nucleus; prominent nucleolus; chromatin condensation after or simultaneously with secretion and vacuolization	dark nucleus with convoluted outlines; chromatin condensation into small "patches" located throughout the nucleus; chromatin condensation after cytoplasmic digestion
Cytoplasm	condensed; formation of apoptotic bodies; digestion during phagocytosis of apoptotic bodies by nearby cells	partly digested within cell	enclosed and replaced by expanded rough endoplasmic reticulum; digestion of cytoplasmic "islands" with formation of "worm-like" structures
Lysosoms	intact within apoptotic bodies	probably intact	digested within intracellular "islands"
Other organelles	apparently intact but condensed; formation of apoptotic bodies; digestion during phagocytosis of apoptotic bodies by nearby cells	well developed Golgi complex and endoplasmic reticulum, many secretory vesicles and large mitochondria	distension of rough endoplasmic reticulum with formation of cytoplasmic "islands" containing organelles and their digestion; small dark inclusions, secretory vesicles, Golgi complex and swollen mitochondria
Cell membrane	budding – formation of large cytoplasmic protuberances to produce apoptotic bodies (late event)	extensive blebbing – formation of small protuberances to produce vesicle-like structures (all stages)	blebbing (late event)

characteristic to other cells undergoing apoptosis such as the condensation of the nuclear chromatin, cell shrinkage, and plasma membrane blebbing [1,4,9,22].

It should be noted, that early growth plate – visible before the secondary ossification centers are formed – is an avascular structure which relies on receiving nutrients and oxygen via diffusion from the vascular arcades presented in the metaphyseal side of the epiphysis [31]. The anatomical location of the vascular channels is between the calcified cartilages under the last row of hypertrophic chondrocytes [30]. VEGF is the key factor responsible for vascular ingrowths, which is physiologically expressed solely by the hypertrophic chondrocytes [5,22].

Physiological chondrocytes elimination

Chondrocytes similar to most other mammalian cells are physiologically eliminated via programmed cell death. However, unlike in other greatly vascular tissues, removal of apoptotic bodies is difficult due to large amounts of ECM, and is only possible in the primary spongiosa zone [4,9,33,34].

Typically apoptotic cells are characterized by nuclear chromatin condensed into dark crescents, caps, spheres, and smaller so called shrinks size. Such changes are secondary to DNA fragmentation and intracellular disintegration. The cells and apoptotic bodies are typical for adult cartilage; however, rapidly undergo phagocytosis [36]. In fetal and early formed growth plates, some other types like authophagal bodies, paralysis and dark chondrocytes are also observed (Table 1) [4,18,32].

Authophagal bodies

It was found that morphology of some hypertrophic chondrocytes was diverse that of apoptic cells with irregular nuclei and an exaggerated endoplasmic reticulum [37]. These cells were unable to live or die analogous to a paralytic state, a form of cell death similar to dark chondrocytes (see below). These cells underwent autophagy [38,39] that is a process that involves the cell using its own components to degrade itself by lysosomes. These cells contain double-membrane autophagic vacuoles, termed autophagosomes that are used for self degradation. Autophagy protects the cells by consuming its own lipids and nutrients to generate energy when nutrients are deficient [40]. Protein Tor is the main component involved in detection of nutrients

concentration. It blocks autophagy when nutrients are available [41,42]. A negative regulator of Tor protein is AMP-dependent protein kinase associated with the cell redox status, which blocks Tor complex. Autophagy is also regulated by low oxygen and/or low protein levels. Additionally, low glucose level signals glycolysis which lowers ATP and use of nutrients, promoting autophagy [42].

Paralysis chondrocytes

The paralysis chondrocytes develop secondarily to the focal digestion of cytoplasm and organelles that occurs within an internal "island" formed by an enlarged and highly hydrated lumen of the endoplasmic reticulum [43]. The next step in cellular disintegration is always chromatin condensation with formation of typical small patches throughout the nucleus that usually has a convoluted outline. Similar to other types of programmed cell death, and unlike necrosis, release of proteolytic enzymes has not been observed. In the late stages, formation of small, blister-like, vacuolated structures from pseudopodias, which is presently known as blebbing is observed. Elimination of paralysis and dark chondrocytes are probably similar and will be explained below [33,43,44].

Dark chondrocytes

In the last few decades numerous authors have reported a presence of chondrocytes which have a different morphology from so-called normal (light) cells in the same growth plate zone. Due to their characteristic images in transmission electron microscope (TEM) they are known as dark chondrocytes (Fig. 3) [4,18].

Dark chondrocytes have different signs of degeneration, like the separation of a nucleolus "cap", single blebs of plasmalemma, and are also seem to be more viable. The typical nucleus of a dark cell includes the tandemic arrangement of chromatin condensations, large, compact, nucleolus, and undeveloped accessory nucleoli. Contrary to them, light chondrocytes possess evenly shaped nuclei with dispersed chromatin. As dark cells become more advanced, the density of their karyoplasms increases and condenses into chromonema-like strands or irregular clumps (less often). Both light and dark cells usually contain one or two centrally located nucleoli of differing types, the "compact nucleolus". However, in more advanced dark cells, the nucleoli appeared to be hyper-compacted and smaller, suggesting degeneration. It is worth mentioning that their nuclear membrane often contains defects. The segments ravel into small inverted buds and dissolve together with adjacent chromatic clumps to release pore complexes [4,18,33,38].

It should be stressed that such cells have a particular path of division. They are not present in normal mitosis, but only sandwich-like amitotic nuclear segregation. Their nuclei commonly lose nucleoli and hyper-compact, after which they dissolve. Additionally, such cells have a chromatic arrangement similar to that of condensed pro-metaphase or metaphase. These lack nucleoli, but are surrounded to some extent by a nuclear membrane. Sometimes, microtubules are located between lobes of chromatin clumps that resemble chromosomes. This pattern has been identified in some tumor and irradiated cells and is known as a "clover leaf" [38,45]. Similarly, the metaphase-like chromatin figures in interphasic cells are also found on cell imprints. All forms that look like division have characteristics of aberrancy, *i.a.*, minimal, to a lack of spindle elements, maintain nuclear membrane, hyper-compaction and chromatin dissolution [38,46].

Unlike light chondrocytes that have small mitochondria and sparse rosettes of glycogen, dark cells are characterized by plenty of glycogen and large swollen mitochondria with well developed cristae, which occasionally possess fenestrae and dark matrix. The Golgi complex is the dominant organelle. At times, even the nucleus appears as an enlarged "belly" of the Golgi. In some locations, it appears as if the entire cisternae are destined to rupture or protrude to the exterior of the cell. Besides, multiple invaginations of the ground cytoplasm in the dilated cisternae are found. Such cells contain also ribosomes and form "inverted channels". The cell membrane blebbing (zeiosis) is also typical of dark cells and less often the cytoplasm is disintegrated. Such membrane fragmentation gives origin to the above mentioned matrix vesicles [18,38,46].

It should be noted that there are also nuances in dark cells during their transitional phase. Cells which appear to be transitory are observed only in early embryos. Arguably these cells could be transitioning, just as likely, from dark to light chondrocytes. These cells have two distinct characteristics of dark cells-pattern of chromatic compactions and "inverted" membrane, but their karyoplasm and ground cytoplasm are not condensed [4,18,38].

Dark cells also exhibit atypical characteristics during cell death. At times, these small dark cells resemble apoptotic ones, however, they do not have typical condensed chromatin. There were two different degeneration patterns: one where the karyolitic dark cell forms a pair with a normal chondrocyte in the proliferating zone, and in the resting zone, the karyopycnotic dark cell is engulfed by a light chondrocyte. The generally accepted hypothesis states that elimination of dark cells or their remnants occurs in the lower primary spongiosa zone due to phagocytosis. However, in the upper part of the growth plate, the cell size reduces and their matrix lacuna becomes smaller secondary to a higher secretion of ECM elements and the release of matrix vesicles. This explains the high number of empty lacunas observed in

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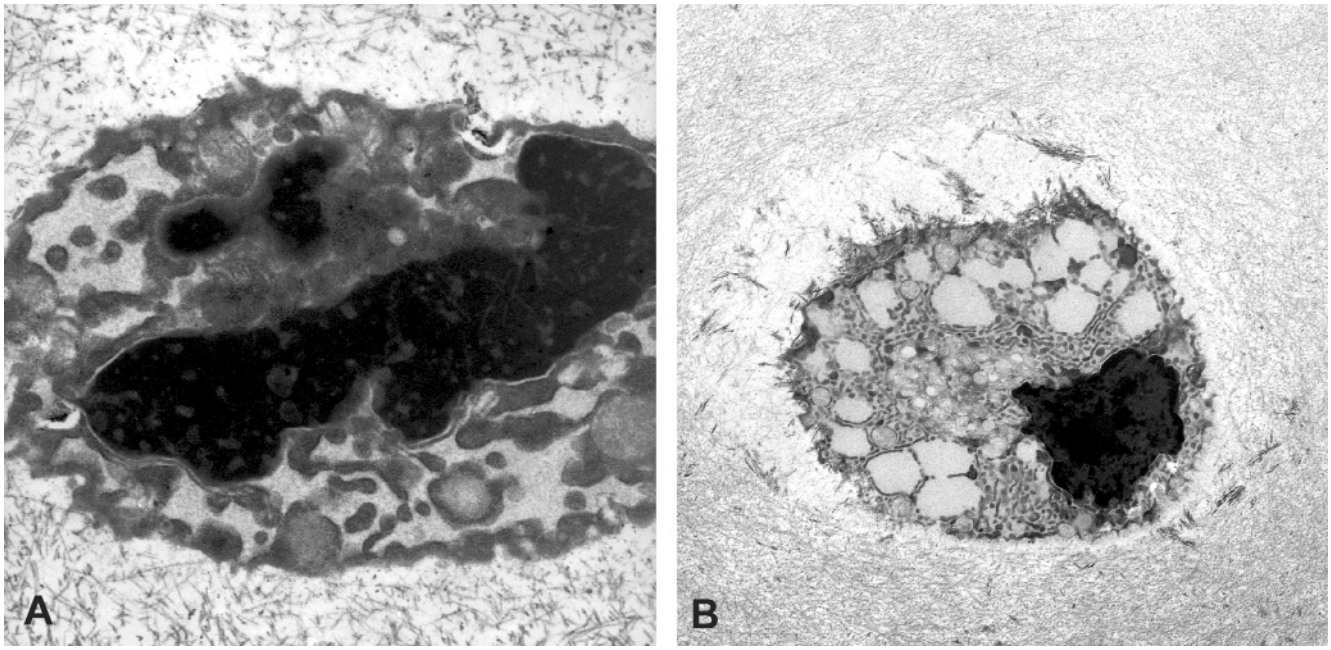

Fig. 3. Apoptotic (**A**) and dark (**B**) chondrocytes of the hypertrophic zone of the proximal femoral epiphysis from the 21-day-old rat fetus and 7-day-old rat pup, respectively (TEM, original magnification ×3000).

various developmental studies. The second mechanism seems to be prominent in the proliferative zone, where the cell space is taken up by the expansion of the ECM around neighboring chondrocytes [4,43,46].

Until now, dark chondrocytes were reported only in human fetal cartilage [46], mice mandibular condyle [48], physes of piglets [49], proximal epiphyses of the femur and humerus in young (0-9 day, and 5- and 20 weeks old) New Zealand rabbits [33], proximal femoral growth plate of 14-15 day chick embryos [4, 38], as well as 21-day old fetuses [18] and 7-day old pups of Wistar rats [50]. Dark chondrocytes were present in about 10-35% of avian embryos growth plates and were found in all stages of chondrocyte differentiation and in all ages of studied femurs. In every zone, such cells were revealed to be juxtaposed to light ones. They were even observed in mineralized areas with signs of degeneration, and found to be abundant in proliferating cells. Sporadically, they were even seen sharing a common lacuna [4,38]. Unlike those findings, such dark cells were present only occasionally in rat offsprings [18]. Among the six proximal epiphyses of the fetal femur, they were found only in four separate specimens. The prenatal fetuses exposed to various cyclooxygenase inhibitors, possessed dark chondrocytes in 23 out of the 32 examined samples, without any association with administered drugs. Similar frequency and the same localization of dark cells, limited to the germinal and proliferative zones, were later reported by Solecki [50]. The last two cited reports are the only fully available ones that presents such chondrocytes in animals exposed to xenobiotics.

Regulation of differentiation

There are two main mechanisms that eliminate hypertrophic cells. One mechanism is apoptosis, where terminally differentiated cells die. The second mechanism is transdifferentation, where mature chondrocytes are removed by converting them into bone forming cells or osteoblasts [22]. The latter mechanism allows the cells to undergo a drastic change in phenotype and new genes to be expressed [33,34].

On the other hand, there are several molecular, biochemical, as well as morphological changes that take place during differentiation. The growth of bones depends solely on the proliferation and hypertrophy of the chondrocytes located within the growth plate. Apoptosis is also crucial for the terminal chondrocyte maturation, which relies on the secretion of matrix. This process assists in the matrix calcification and is regulated by interactions with various hormones, peptides, and growth factors, which are synthesized locally [33-35].

The key enzymes in apoptosis initiation are caspases, which belong to the cysteine protease family. Caspases are found in the cytoplasm of all cells in the inactive form awaiting a primer to activate apoptosis [22,35]. *In vitro* experiments have shown that chondrocytes with high phosphate concentrations exhibit an increased apoptotic activity [51]. This phenomenon is more common in differentiated than in less differentiated chondrocytes. This increase in phosphate is associated with abnormal mitochondrial function of chondrocytes, which causes a greater reliance on glycolysis with progression towards hypertrophy [52].

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On the other hand, parathyroid hormone related peptide (PTHrP) is a potent inhibitor of apoptosis, through the mechanism of up-regulation of the apoptosis inhibitor Bcl-2. In addition to phosphate, adverse effects have been seen by glucocorticoids and radiation, which yields and increase in apoptosis [52-55]. In animals treated with a ten-day course of glucocorticoids, an increase of apoptosis in the hypertrophic chondrocytes yields a growth plate with a reduced width was revealed [53]. Both, PTHrH and parathyroid hormone are the main stimulators of the perichondrium. The physiological balance between both factors regulates chondrocytes proliferation and differentiation. Maturation and terminal differentiation also depends on the Indian Hedgehog (Ihh) peptide, which regulates expression of genes coded on the hedgehog receptor and transcriptional factor Gli1 [56,57]. Ihh synthesis begins throughout mesenchymal condensation, increases during cartilage formation and later decreases. Without the factor, the perichondrium transforms into periosteum [59]. Its overexpression up-regulates the expression of perichondrial PTHrP, which keeps chondrocytes in the proliferating state, and slows or even inhibits hypertrophy. Ihh regulates the proportion of chondrocytes dividing and withdrawing from the cell cycle through negative feedback [60,61].

It should be noted, that the periarticular chondrocytes produce PTHrP, but its receptor is found particularly in the prehypertrophic cells and the lower proliferating zone. The mechanism of PTHrP activity is achieved by delaying the hypertrophic differentiation in the lower proliferating zone by maintaining cells in the prehypertrophic phenotype. Secondary to activation of PTH/PTHrP receptors, PTHrP stimulates cell proliferation by G protein activation and delays their transformation into prehypertrophic and hypertrophic chondrocytes. When proliferation is completed, chondrocytes release Ihh, which stimulates PTHrP synthesis via a feedback loop [56,57].

Transgenic mice lacking PTHrP or its receptor have shown signs of dwarfism caused by the accelerated differentiation and premature hypertrophy. In contrast overexpression of PTHrP causes slower cellular differentiation and a secondary reduction of bone length [61]. In humans, a mutated PTHrP and receptor results in Jansen metaphyseal chondrodysplasia, a dwarfing condition due to a postponement in growth plate mineralization and hypocalcaemia [62].

The next important biological system is related to the thyroid hormone working with major regulatory pathways that govern the proliferation and differentiation of chondrocytes and changes in the ECM [63,64]. Thyroxin stimulates type X collagen synthesis along with other various maturational features through the introduction of BMP-2 [65]. The expression of genes that code these hormones is dependent upon the particular tissue and is regulated at different stages of development. One of the thyroid receptors, TRα1, is predominant in bone, heart, and central nervous system [66]. Deletion of TRα isoform results in stunted linear bone growth, disorganized columnar scaffold of growth plates, lagging cartilage mineralization, and retarded chondrocyte hypertrophy. On the other hand, the deletion of TRβ did not produce any of these abnormalities [63].

When thyroid hormone is absent or inhibited, the resulting clinical manifestation is hypothyroidism. Relevant symptoms of hypothyroidism in skeletal system include: decreased rate of bone formation, decreased thickness of growth plates of long bones, disorganization of the normal cartilage columns of the growth plates, and hindered differentiation of growth plate chondrocytes into hypertrophic cells [67]. Additionally, it has been noted that the receptor proteins are expressed in reserve and proliferating zone chondrocytes, but not in hypertrophic one in rat growth plate [67]. However, in humans, TRα1 is present in undifferentiated, proliferating, mature and hypertrophic chondrocytes [68].

According to *in vitro* observations, triiodthyronine (T3) stimulates growth and maturation of porcine scapular and pelvic growth plate cartilage [70]. Its enhancement also causes an increase in mRNA and protein levels of type X collagen, cellular hypertrophy, and alkaline phosphatase activity, which are all indicators of terminally differentiated growth plate chondrocytes [33,34,64,69].

Thyroid hormone is also a modulator of other biological factors. Insulin-like growth facor-1 (IGF-1), for example, is a downstream effector for growth hormone that stimulates the local synthesis of IGF-1 and causes an increase in the rate of cell division [70,71]. In addition, thyroid hormone may be able to act directly on growth plate chondrocytes through growth hormone-independent mechanisms [34,64]. Further, transgenic mice deficient of the IGF-1 have growth retardation with intense defects in skeleton and various internal organs. This causes death within twenty-four hours after birth [72]. In humans, however, the mutated IGF-1 causes Laron syndrome, characterized by hereditary dwarfism resulting from low serum factor levels [73]. There is also an implication that thyroid hormone could potentially be involved in regulating the set point of the PTHrP-Ihh feedback loop [74]. The hormone enhances FGF signaling that could regulate the inhibitory effects of thyroid hormone on growth plate cell proliferation [63,75].

The effects of thyroid hormone inhibitors have also been studied. Peroxisome proliferator activated receptors (PPARs) are negative regulators of thyroid hormone signaling [76]. PPARγ and ciglitazone – one of its ligands – inhibits thyroid hormone-induced increases in alkaline phosphatase activity by increasing apoptosis. It is believed, that the receptor is one of the few

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negative regulators of thyroid hormone-mediated skeletal growth and gene transcription [63].

It was also observed that thyroid hormone activates Wnt/β-catenin signaling in chondrocytes of growth plate. Wnt-4 activates β-catenin signaling and encourages terminal differentiation. The main role of β-catenin is to stimulate differentiation of growth plate chondrocytes and increase alkaline phosphatase activity [64]. When Wnt is inhibited, T3-induced terminal differentiation is blocked and alkaline phosphatase activity is decreased. Based on the above observations, it seems that T3 stimulates growth plate chondrocyte terminal differentiation by amplifying Wnt-4 expression, and increasing β-catenin, TCF/LEF transcriptional activity, and expression of the Wnt/β-catenin target gene *Runx2/cbfal* [64,77].

Iodothyronine deiodinase is another active hormone in the growth plate. An insufficient expression of iodothyronine deiodinase type 2 (DIO2) results in tibial dyschondroplasia, the inability of chondrocytes to undergo terminal differentiation [78]. When *DIO2* is expressed via WSB-1 complex, PTHrP is also presented and chondrocyte differentiation is modulated [63].

Furthermore, just as thyroid hormone is regulated, apoptosis is monitored as well. There are many ways in which apoptosis is monitored or regulated. One of the ways, apoptosis in the growth plate is regulated by balancing the *Bcl-2* protein and an apoptosis inhibitor Bax [79]. If this balance is interrupted, growth plate function is affected and the length of long bones is distorted. The deletion of the *Bcl-2* gene accelerates apoptosis of chondrocytes, and causes a decrease in thickness of the growth plate and shortened long bones [80].

One of the most important components in activating apoptosis is the calcium and phosphate (Pi) ion pair [81] that decreases the mitochondrial membrane potential and initiates the process of apoptosis [52]. Pi causes an increase of nitric oxide (NO). Inhibitors of NO synthase and Pi transport cause a decrease in NO concentration, thus preventing cell death [82]. In addition, NO production causes sheer stress, which reduces the expression of type II collagen mRNA and aggrecan [83]. Elevated levels of NO, its synthase (iNOS), and nitrate have been linked to osteoarthritis. In addition, iNOS is expressed when chondrocytes are exposed to cytokines such as IL-1 and TNF-α. IL-1, which is activated by NO, and in turn can inhibit chondrocyte proliferation by acting in conjunction with PGE_2, a chondrocyte growth inhibitor [84].

There are two main ways that NO sensitizes chondrocytes to apoptosis: decreases the thiol reserve [86] and affects the survival of the cell under oxidative or nitrosative tension. Furthermore, NO decreases mitochondrial membrane potential, which causes a loss of tight coupling, an essential prerequisite for generating ATP by means of oxidative phosphorylation. Interference with mitochondrial activity causes a loss of ATP production leading to a low energy state, which makes chondrocytes more susceptible to enter apoptosis [86]. NO also interferes with cellular adhesion to the extracellular membrane. Chondrocytes depend on adhesion due to survival signals are provided for them by the membrane through matrix interactions and proteoglycans [87]. Similarly, β1-integrin-mediated cell matrix interaction gives signals to chondrocytes for survival. The loss of this interaction accompanied by the incapability to respond to IGF-1 can cause osteoarthritis via hypocellularity and matrix degradation [88].

Several other factors are associated with chondrogenesis, *i.a.*, TGFβ, heparan sulfate, fibroblast growth factor receptor-3 (FGFR-3), pleiotrphin/midkine [59].

TGFβ is an influential inhibitor of maturation, which includes cell hypertrophy, type-X collagen synthesis, and alkaline phosphatase activity. The actions are mediated by Smad-3, which is also a transcription factor [89,90]. Studies have illustrated that Smad-3 deficient mice have a normal skeleton at birth, but at three weeks of age, they show signs of cartilage abnormalities, which result in decreased longitudinal growth [91]. PTHrP, Ihh and TGFβ seem to be the primary regulators of cell proliferation in the growth plate, but other factors such as FGFR-3 also play an important roles [92,93]. A mutation in the gene coding the receptor protein results in achondroplasia, which is a dwarfing condition caused by the reduction of proliferation in the growth plate [94].

Heparan sulfate (HSPG) in mouse cartilage is found mainly in proliferating, differentiating, and prehypertrophic chondrocytes, but is reduced in terminally differentiated hypertrophic chondrocytes. Furthermore, *HIP/RPL29*, a heparan sulfate gene, is up-regulated in carcinomas. Fibronectins are thought to be involved with HSPGs and cartilage integrin α5β1, influencing cellular morphology [59]. At the moment, there is inadequate evidence regarding the expression of HSPG remodeling enzymes in developing and mature cartilage. However, heparan sulfate is needed for the morphogenesis of individual organs and cartilage formation, which is necessary for skeletal structure [95,96].

Conclusion

The cartilage growth plate is a highly specialized skeletal structure, essential for bone growth and development. Its formation is a final step of chondrogenesis and is regulated by various factors that modulate cellular proliferation and terminal differentiation, allowing the transformation to apoptotic and autophagal bodies, paralysis, and dark chondrocytes. There is also another highly unknown transdifferentation process in

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which chondrocytes transform into osseous cells. All these mechanisms lead to bone growth and elongation. It is estimated that 60% of the bone growth is due to chondrocyte hypertrophy, 10% to cell proliferation and the remainder to matrix synthesis.

Any disturbance of the plate physiology cause developmental abnormalities. For example, the final manifestation of mutations in genes coding specific types of collagen is: Stickler syndrome (collagen type XI), Schmid metaphyseal chondrodysplasia (collagen type X) or spondyloepiphyseal and Kniest dysplasia (collagen type II) [97,98,99]. Furthermore, abnormalities in IGF1, hedgehog pathway PTHrP and, as well as other important differentiation factors, may induce changes that manifest as dwarfism [100,101]. Various pathological factors are also known, while the etiology of other serious growth plate diseases is still unidentified. Intensifications of basic studies will provide prospects for new physiological pathways of the chondrocyte cell cycle and will be helpful in evaluating of such illnesses and their treatments in the future.

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