Review

Genetic mouse models for the functional analysis of the perifibrillar components collagen IX, COMP and matrilin-3: Implications for growth cartilage differentiation and endochondral ossification

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Summary. The mutual interaction of the two supramolecular compartments, the fibrillar and extrafibrillar matrix is a prerequisite for stability and integrity of the cartilage extracellular matrix. The fibrillar periphery, composed of collagen IX, matrilins and cartilage oligomeric matrix protein (COMP) among other components, constitutes the interface which mediates interactions between the two compartments. Mutations in these peripheral macromolecules cause a broad spectrum of skeletal conditions such as pseudo-achondroplasia (PSACH) and multiple epiphyseal dysplasia (MED), which severely affect the organization and integrity of the cartilage growth matrix in humans. Transgenic and knockout mouse models for collagen IX, matrilin-3 and COMP and combinations thereof display cartilage abnormalities and pathologies of varying severity. Absence of collagen IX appears to cause the most severe growth plate phenotype with a profoundly disturbed morphological organization affecting size and shape of the long bones. Notably, similar growth plate phenotypes, including irregularities in the proteoglycan content, hypocellular central regions, disorganized proliferation columns with atypically shaped and oriented chondrocytes and alterations in the hypertrophic zone are observed in transgenic mice lacking other macromolecules or carrying mutations therein. These include collagens II and XI, integrin subunits, integrin linked kinase (ILK), HIF-1α, VEGFα and the tumor suppressor PTEN. Notably, mutations in ciliar proteins such as Kif3α, polaris or Smo/Gli severely affect the ability of chondrocytes to move and to become arranged in columns. Absence or mutational changes of a variety of different, non-related cartilage macromolecules apparently cause similar pathologies and abnormalities of the growth cartilage, suggesting a limited number of underlying molecular mechanisms.

Key words: Collagen IX, COMP, Matrilin-3, Endochondral ossification, Growth plate

Introduction

Interactions between the fibrillar and extrafibrillar cartilage matrix

In cartilage, the extracellular matrix occupies the major volume fraction of the tissue and is responsible for its main functions, i.e. load bearing and, in the case of joint cartilage, allowing smooth articulation of long bones. These functions are engendered by two supramolecular systems, the collagen-containing fibrils and the extrafibrillar matrix, which comprises mainly the large, cartilage-specific proteoglycan aggrecan. Cartilage fibrils are complex structural aggregates containing at least collagens II and XI and, optionally, collagen IX (Mendler et al., 1989; Hagg et al., 1998) and collagen XVI (Kassner et al., 2003). In addition, some fibril populations are associated with small leucine-rich proteins or proteoglycans, such as decorin, biglycan and fibromodulin (Hedbom and Heinegard 1993; Hagg et al., 1998). In order to warrant tissue stability, a mutual interaction of the two supramolecular compartments is

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required. The fibril surface is densely populated with several types of macromolecules, including collagen IX, which all are potential adapters between the fibrillar and the extrafibrillar matrix. Thus, the molecular nature of the fibrillar periphery is of substantial interest and, in this respect, further cartilage matrix components are likely to be of particular biological importance. These further components include matrilins and COMP (also referred to as thrombospondin-5), which are neither collagens nor proteoglycans.

Unlike the collagenous fibrillar structures and the aggrecan matrix themselves, their interface therefore, has a highly complex biochemical composition. Sometimes such perifibrillar molecules occur in a complementary fashion in different regions of the tissues. Collagen IX stabilizes the thin, prototypic cartilage fibrils preferentially found in the territorial matrix as baskets around chondrocytes (Bruckner et al., 1988). The protein forms a macromolecular alloy together with collagens II and XI, but also projects its amino terminal domains COL3 and NC4 away from the surface into the fibril-proximal environment ready to undergo interactions with other adapter proteins or the aggrecan matrix (Vaughan et al., 1988). Matrilins, COMP, decorin, fibromodulin, and biglycan bind directly or indirectly to the collagenous bodies of the fibrils and, therefore, are interface components potentially mediating interactions between cartilage fibrils and the extrafibrillar matrix (Budde et al., 2005) (Fig. 1). Deficiencies in such components generally have much less severe consequences than the absence of the core components of the suprastructural compartments.

However, mutations that lead to structural abnormalities rather than the absence of these perifibrillar components can cause human or animal disease phenotypes.

Human diseases

Mutations in cartilage extracellular matrix proteins, including COMP, collagen IX and matrilin-3 cause a broad spectrum of skeletal conditions (Superti-Furga and Unger, 2007). Two of them, pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED) are autosomal dominant inherited forms characterized by severe to moderate disproportionate dwarfism and pronounced joint laxity. In these patients premature osteoarthritis often requires early joint replacement. The more severe form PSACH is caused exclusively by mutations in COMP (Briggs et al., 1995; Hecht et al., 1995). Electron microscopy of PSACH patient biopsies showed that mutated COMP is retained in typical granular or lamellar inclusions in the endoplasmic reticulum (ER) of chondrocytes (Stanescu et al., 1993). The accumulation of COMP in turn leads to co-retention of other matrix components. Interestingly, collagen IX and matrilin-3 are also retained but the trafficking and secretion of collagen II remains mostly unaffected (Maddox et al., 1997; Dinser et al., 2002; Hecht et al., 2005). Impaired protein trafficking compromises normal ER function and eventually leads to ER stress, which in turn results in an increased rate of apoptosis. Indeed, in the growth plate of affected individuals many dead cells were detected and a general disorganization with chondrocytes being arranged in clusters rather than in
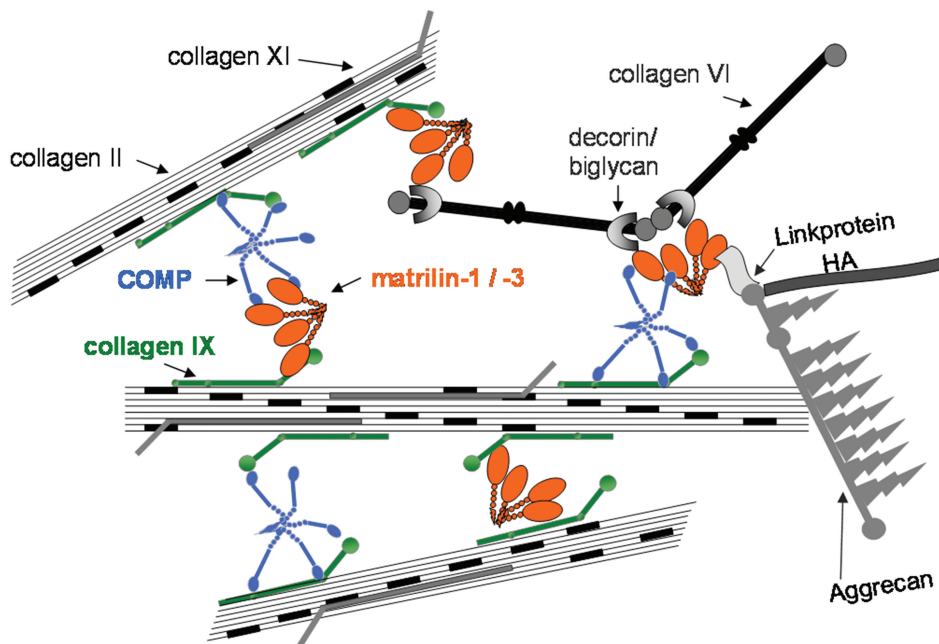

Fig. 1. Model for supramolecular assembly of cartilage fibrils and filaments into fibrillar networks, Matrilin-3 and COMP act as adaptor molecules to interconnect D-periodically banded fibrils with each other and/or with collagen VI beaded filaments to generate a heterotypic fibrillar network. The interaction may be mediated either by matrilin-1 / -3 binding directly to collagen IX or via COMP, which associates with the NC domains of collagen IX. Modified from Budde et al. (2005).

columns was described (Hashimoto et al., 2003; Hecht et al., 2004). It is therefore believed that chondrocyte depletion in the PSACH growth plate is the main reason for the diminished linear growth leading to disproportionate short stature.

The milder form MED has, in addition to COMP, been linked to at least four other matrix genes coding for each of the three chains of collagen IX (Muragaki et al., 1996; Czarny-Ratajczak et al., 2001) and for matrilin-3 (Chapman et al., 2001). Interestingly, mutations in matrilin-3 and the α3 chain of collagen IX lead to very similar intracellular inclusions as observed in PSACH patients (Bonnemann et al., 2000; Cotterill et al., 2005). Based on the intracellular retention of mutated proteins several studies have suggested that PSACH and MED are mainly storage diseases of the ER. However, it was demonstrated in cell culture models that not all disease causing mutant proteins are retained. Instead, secreted mutant proteins cause a disruption of extracellular matrix structures, indicating that both intra- and extracellular pathogenic pathways can contribute to the disease mechanism (Dinser et al., 2002; Schmitz et al., 2006). Nevertheless, it is puzzling how mutations in genes coding for structurally unrelated proteins cause a more or less identical phenotype. Like COMP, matrilin-3 and collagen IX have been shown to interact with each other to fulfill their structural role in the ECM. Due to mutations in each of these proteins these interactions might take place already intracellularly, leading to premature formation of highly ordered but insoluble aggregates (Merritt et al., 2007).

Endochondral ossification

Endochondral ossification is particularly important for bone development and repair, as well as for longitudinal growth of long bones. Within the highly organized structures of growth plates, located between the epiphyseal and the metaphyseal part of long bones, differentiated cartilage cells transit through a temporo-spatial cascade of late differentiation events that sequentially include proliferation and several steps of maturation, culminating in chondrocyte hypertrophy. Longitudinal growth is collectively achieved by proliferation and hypertrophic differentiation of chondrocytes, along with appropriate matrix synthesis. After invasion of blood vessels from the subchondral bone, the majority of hypertrophic cells undergo apoptosis and the cartilage template is remodelled into trabecular bone. The overall process is regulated by systemic hormones, as well as by locally acting autocrine signals derived from chondrocytes themselves or by paracrine signals originating from cells of surrounding tissues, e.g. the perichondrium or subchondral blood vessels. The interaction of chondrocytes with their surrounding matrix via cell surface receptors is thought to play a key role in the regulation of survival, proliferation and maturation of cartilage cells (Olsen, 1996; Kronenberg, 2003; Lefebvre and Smits, 2005; Goldring et al., 2006).

Transgenic and knockout mouse models of perifibrillar cartilage macromolecules

Collagen IX knockout and transgenic mice

Collagen IX, a heterotypic collagen belonging to the FACIT family, is assembled from α1(IX), α2(IX), and α3(IX) polypeptide chains and is a flexible molecule containing rod-like, triple helical collagenous (COL) domains interrupted by more supple, hinge-like non-collagenous (NC)-domains (Shaw and Olsen, 1991). It is a component of D-periodically banded cartilage fibrils and is assembled with collagens II and XI in an anti-parallel fashion (Eyre et al., 2004). Its N-terminal triple helical domain 3 (COL3) and non-collagenous domain 4 (NC4) point away from the body towards the periphery of the fibril (Wu et al., 1992). The interaction sites of collagen IX within the fibril body include the triple helical domain 1 (COL1), the most conserved sequence occurring in all FACITs (Eyre et al., 2004). In adult tissues, collagen IX is present in a subpopulation of thin fibrils preferentially located in the territorial matrix and mostly lacking decorin. In embryonic cartilage, collagen IX is distributed more ubiquitously and occurs in fibrils with a uniform width of about 20 nm (Fig. 2) (Muller-Glauser et al., 1986; Hagg et al., 1998). Collagen IX stabilizes the individual fibrils (Eikenberry and Bruckner, 1999) and directs their organization into a fibrillar network. Deletion of the α1 (IX) chain of the heterotrimeric molecule leads to a functional knockout of the entire collagen IX protein (Fassler et al., 1994; Hagg et al., 1997). Lack of collagen IX impairs cartilage matrix integrity by subsequent loss of COMP and matrilin-3. The association of matrilin-3 with collagen fibrils is critically dependent on the presence of collagen IX and to some extent of COMP, as fibrils isolated from α1 (IX) collagen deficient mice are almost devoid of matrilin-3, and COMP deficient collagen fibrils exhibit a reduced matrilin-3 decoration (Budde et al., 2005). α1 collagen IX deficient mice develop an OA-like joint phenotype at an age of 6 months with proteoglycan depletion and loss of intact collagen II. These degradative changes are possibly mediated by a concurrent increase of MMP-13 expression stimulated by induced activation of the DDR-2 receptor through enhanced exposure of chondrocytes to collagen II fibrils, which normally are not located close to the cell surface (Hu et al., 2006; Lam et al., 2007). Increased MMP activity produces fragments of collagen II and fibronectin which then induce more proteinases through binding integrins α2ß1 and α5ß1, respectively (Li et al., 2007). In addition, the structural changes affect the tectorial membrane of the α1 (IX) collagen deficient mice which results in progressive hearing loss (Asamura et al., 2005; Suzuki et al., 2005).

Ablation of collagen IX not only affects integrity and organization of costal or permanent articular cartilage in joints and intervertebrae, but also growth cartilage, the bone template in endochondral ossification. In adults, collagen IX deficiency delays callus maturation in tibial fracture healing. In particular, terminal hypertrophic chondrocyte differentiation and bone formation are delayed (Opolka et al., 2007). This agrees with the observation that young adult α1 (IX) collagen deficient mice exhibit loss of trabecular bone and progressive bone deterioration with age. Possibly, the short form of collagen IX, expressed by osteoblasts, is involved in the pathogenesis of osteoporosis (Wang et al., 2008). In newborn and growing mice, the prenatal and perinatal organization of the growth plate is profoundly disturbed in collagen IX deficient mice,
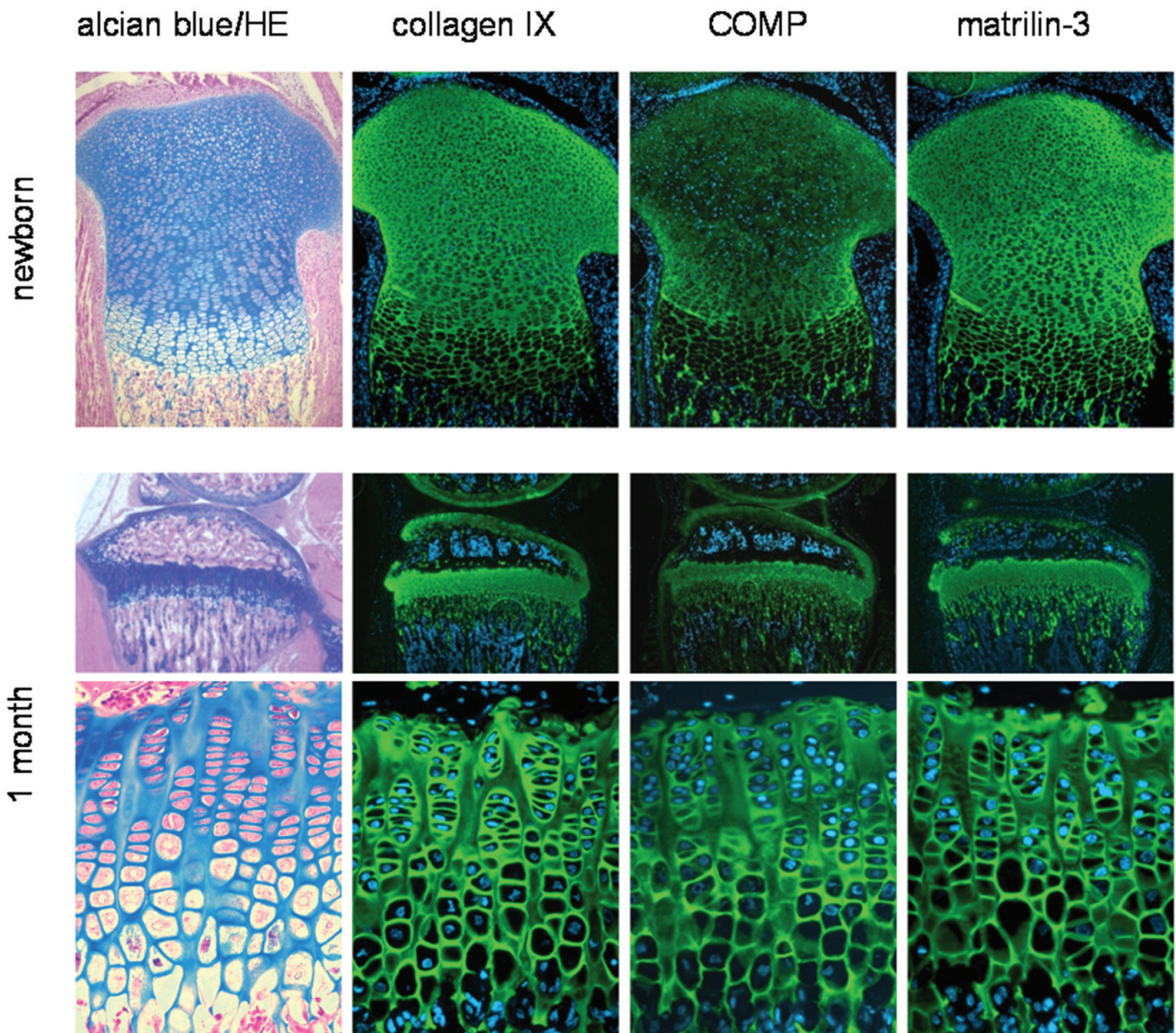

Fig. 2. Histochemical and immunofluorescence staining of tibiae from newborn and 1 month old wild type mice. Paraffin sections were either stained with haematoxylin eosin / alcian blue (left column) or processed for immunofluorescence staining using specific antibodies directed against collagen IX, COMP and matrilin-3. The epiphyses of newborn animals are completely cartilaginous, as indicated by the blue proteoglycan staining. Collagen IX, COMP and matrilin-3 are expressed throughout the matrix. At 1 month of age, the secondary center of ossification and the characteristic columnar arrangement of chondrocytes within the growth plate is fully developed. The higher magnification shows the central region of the growth plate. The localization of collagen IX, COMP and matrilin-3 is still similar, with all three proteins being expressed in both articular and growth plate cartilage.

however, the anomalies become attenuated in adult mice. In contrast to the wild type mice, newborn α1 (IX) collagen deficient animals have broadened tibial condyles with large hypocellular central regions almost devoid of chondrocytes. In addition, the central hypocellular regions are often poorly stained with alcian blue, indicating profound irregularities in proteoglycan content. Furthermore, the formation of the typical proliferation columns in the growth plates is grossly disturbed. Cells often form proliferation patterns in a direction perpendicular rather than parallel to the long bone axis and proliferative cells are less flattened. Remarkably, cells in the proliferative and the hypertrophic zones seem to orient themselves horizontally rather than vertically along the long axis and the columnar arrangement of cells is mostly absent (Fig. 3). Within the hypertrophic zone the cells are enlarged, but less densely packed and surrounded by more extracellular matrix. Also, the cell layers are unusually irregular; some regions contain only 1-2 chondrocytes, other areas are composed of 5-6 cells in height. Presumably for this reason, the border between proliferative and collagen X producing hypertrophic cells is poorly defined in older Col9a1 - deficient animals. In these mice, the arrangement of cells is altered and the total number of cells is decreased in the hypertrophic zone, which leaves them narrower due to a decreased number of proliferating cells. These abnormalities cause a markedly reduced length of long bones at early postnatal age, while the width is significantly increased (Dreier et al., 2008).

In addition to the model, where the entire α1 (IX) chain is deleted, a transgenic model harbouring a truncated α1 (IX) chain was generated. A mutation was introduced into the *Col9a1* gene by deleting parts of the COL2 and COL3 domains and the entire NC3 domain; these deletions then act in a trans-dominant way. The transgene is expressed preferentially in cartilaginous
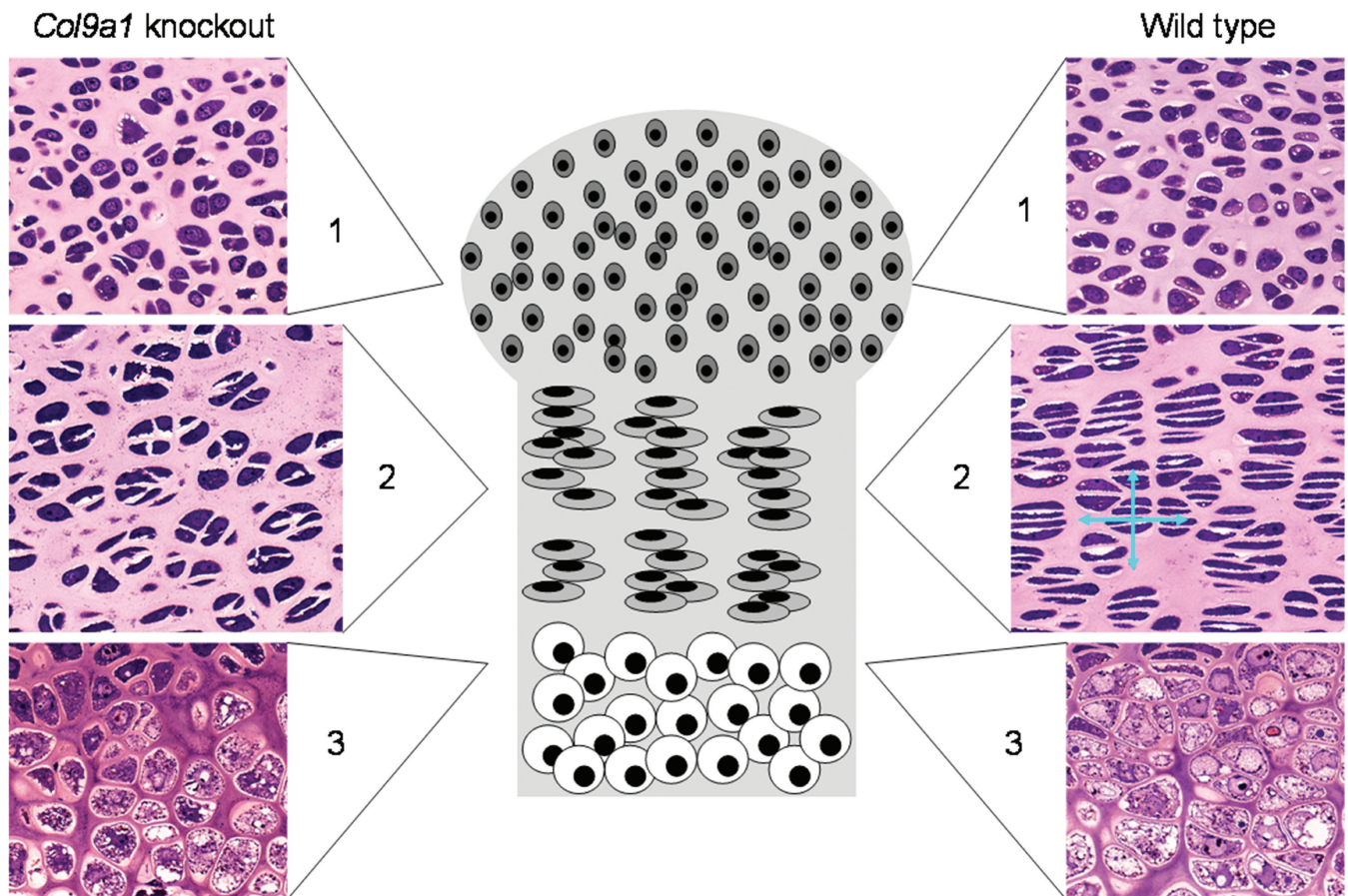

Fig. 3. Spatial organization of chondrocytes in different growth plate zones. Toluidine blue stained semi-thin sections of tibiae from newborn wild type and Col9a1 knockout mice demonstrate profound changes in the organization of chondrocytes in all growth plate zones of Col9a1- deficient animals. Marginally smaller cells are observed in the resting zone (1). In the proliferative zone (2) the chondrocytes occur in isogenous groups, indicating proliferation, but flattening of cells and arrangement to columns failed. Within the hypertrophic zone (3) these changes are maintained. Modified from Dreier et al. (2008).

tissues and in the eye, and the shortened chains assemble with the endogenous α2 and α3 chains to form abnormal collagen IX heterotrimers. The mice develop a mild chondrodysplasia phenotype, including dwarfism and spine and cornea abnormalities which predispose for early onset OA (Nakata et al., 1993). Notably, the transgene causes progressive intervertebral disc degeneration, induced by shrinkage of the nucleus pulposus and fissures in the annulus fibrosus (Kimura et al., 1996).

Recently, similar degenerative alterations in the intervertebral discs and endplates of the spine in mice lacking the entire collagen IX protein were reported. Boyd et al. detected cellular changes such as mucous degeneration, cell death, fibrochondrocyte degeneration and cell clustering in 3-12 months old *Col9a1* deficient mice. The vertebral endplates of 6 month old mice showed a more severe degeneration than in wild type mice (Boyd et al., 2008). Notably, growth cartilage abnormalities have only been assigned to mouse strains lacking the entire collagen IX protein and comparable irregularities have not been described for the mice harbouring a mutated collagen IX.

Matrilin-3 knockout mice

All four mouse matrilins contain up to two VWA-like domains, up to ten EGF-like domains and one C-terminal coiled-coil α-helix mediating the oligo-merization of single matrilin subunits. All matrilins are expressed during mouse limb development (Deak et al., 1999; Wagener et al., 2005). Native matrilin-1 and -3 are able to form homo- and hetero-oligomers with a varying stoichiometry (Wu and Eyre, 1998; Klatt et al., 2000). In mouse, matrilin-1 and -3 are often co-localized, e.g. in the cartilage primordium of the vertebral bodies, costal cartilage, sternum, ilium, the cranial bones and the joints of developing bones (Fig. 2) (Klatt et al., 2002). In costal and tracheal cartilage, matrilin-3 containing filamentous structures are situated perpendicular to the perichondrium and they possibly represent the in vivo counterpart of the fibrils observed in cell culture (Klatt et al., 2000). *In vitro* binding assays have demonstrated that matrilin-3 can interact directly with COMP (Mann et al., 2004) and *in vivo* it is associated with collagen IX containing thin cartilage fibrils (Budde et al., 2005).

Two different mouse strains with a deleted *Matn-3* gene have been generated which display different skeletal phenotypes. The *Matn-3* deficient mice generated by Ko et al. produce viable hetero- and homozygous offspring without detectable abnormalities during skeletal growth and development or concerning the growth plate morphology. The morphology of the skeleton appears normal and endochondral bone formation and intervertebral disc development proceed without obvious alteration. Expression analysis of other members of the matrilin family reveals that the lack of an apparent skeletal phenotype is not due to compensation by structurally and functionally related

proteins. Cartilage specific differentiation markers *Col2a1, aggrecan, Col10a1, Ihh and PPR* are similarly expressed, mineral deposition is unchanged and the collagen fibrillar network organization identical to the newborn wild type. Additionally, differentiation, number and activity of osteoblasts and osteoclasts are similar, and no differences could be detected in the vascular invasion front implicating normal progression of endochondral ossification without martilin-3. These results suggest that human diseases due to matrilin-3 mutations are not caused by a loss of matrilin-3 but by a mutated protein (Ko et al., 2004). Similarly, Nicolae et al. did not observe alterations in organization of resting, proliferative and hypertrophic zones of matn-1 / matn-3 double deficient mice. The length of total growth plates is unchanged, as is expression of the above listed cartilage differentiation markers at various newborn and postnatal stages. Double deficient mice at early postnatal stages (day 3) have normal collagen fibrils without alterations in diameter, and chondrocyte morphology appears inconspicuous in epiphyseal growth plates. However, from day 7 postnatal the distribution profile of collagen fibrils from the interterritorial matrix shifts towards larger diameters. Additionally, the fibrils were more densely packed with a higher proportion of thicker fibrils and an increase in collagen volume density (Nicolae et al., 2007). This observation implicates involvement of matrilin-1 and -3 in the fine control of lateral fibril growth, as was proposed earlier for collagen IX (Hagg et al., 1998) and decorin (Danielson et al., 1997; Ruhland et al., 2007).

On the other hand, van der Weyden et al. independently generated a *Matn-3* knockout strain which shows prenatal growth plate abnormalities with a temporally increased height of the hypertrophic zone. This transient expansion of the hypertrophic zone at prenatal stages results from premature chondrocyte differentiation. Additionally, the proliferation rate of columnar chondrocytes was decreased, indicating a precocious exit of the cell cycle. However, this malformation is attenuated during the perinatal period and disappears in postnatal animals. There was no skeletal malformation and no evidence for compensatory up-regulation of other matrilins after birth. However, adult matrilin-3 deficient mice show a higher bone mass density and develop OA with higher incidence and severity (van der Weyden et al., 2006).

COMP knockout mice

COMP is a pentameric member of the thrombospondin family (Hedbom et al., 1992; Morgelin et al., 1992) which is expressed in all types of cartilage, with its localization being developmental regulated (Fig. 2). In the mouse growth plate and immature joint cartilage, COMP is mainly localized in the territorial matrix. With increasing age, COMP expression is upregulated in developing articular cartilage and mainly found in the interterritorial matrix (Shen et al., 1995;

Ekman et al., 1997). COMP binds in a zinc dependent manner to collagen II (Rosenberg et al., 1998) and collagen IX (Holden et al., 2001; Thur et al., 2001). Recently, *in vitro* fibrillogenesis assays have suggested a role for COMP in catalyzing the assembly of collagen I and II. The presence of COMP promoted the early association of collagen molecules and accelerated the formation of well-defined fibrils (Halasz et al., 2007).

COMP deficient mice have a completely normal skeletal development (Svensson et al., 2002). A detailed anatomical, histological and ultrastructural investigation did not reveal any differences compared to wildtype littermates at any stage of development. The long bones were similar in size, the structure of the growth plate was unchanged and endochondral ossification appeared normal. Up to 14 months of age no signs of osteoarthritis were detected. Even though *in vitro* data point to a function of COMP in fibrillogenesis (Halasz et al., 2007), ultrastructural analysis of growth plate cartilage, articular cartilage, and Achilles tendons showed normal cell morphology and a normal collagen fibrillar network. Several combined knockout strains have been generated to gain more insight into the role of COMP in skeletal development. However, the finding that mutant mice did not display any clinical manifestations of PSACH or MED indicates that the development of these chondrodysplasias does not result from COMP being absent or present in decreased amounts.

Transgenic COMP and matrilin-3 mouse models

Transgenic and knock-in mouse models were generated to study the mechanisms underlying the development of chondrodysplasias. Three genetically different transgenic lines expressing the same mutant COMP (deletion of a single aspartate at amino acid position 469) were established. Several features of the human disease were recapitulated, including disorganization of the growth plate, intracellular retention of COMP, improperly formed collagen fibrils and a variable degree of short stature (Posey et al., 2008; Schmitz et al., 2008). These findings clearly demonstrate that the disease is caused by the presence of a mutant protein rather than by its absence. Another murine model was established by a knock-in approach (Pirog-Garcia et al., 2007). Here, a mutation in the C-terminal domain of COMP (exchange of threonine at amino acid position 583 to methionine) was introduced. Surprisingly, mutant COMP was secreted into the extracellular matrix, further corroborating the hypothesis that not only intracellular mechanisms apply. Even though mutant COMP was not significantly retained within the ER, signs of an unfolded protein response were detected and chondrocyte proliferation was significantly reduced, whilst apoptosis was both increased and spatially dysregulated. By 9 weeks of age tibias of homozygous mutant mice were shorter and hip dysplasia was detected. Finally, by 16 months of age mutant animals exhibited severe degeneration of the articular cartilage, which is consistent with the early onset of osteoarthritis seen in patients.

The pathophysiology of MED caused by mutations in matrilin-3 was further studied in a second knock-in mouse model (Leighton et al., 2007). Mice that expressed a disease-associated mutation in matrilin-3 (exchange of valine at amino acid position 194 to aspartate) were normal at birth but later developed short-limbed dwarfism. Again the cartilage growth plate was disorganized and the mutated protein was retained in the ER of chondrocytes leading to upregulation of chaperones. Intracellular accumulation of mutant protein was also reported in patient biopsies and cell culture models expressing mutant matrilin-3 (Cotterill et al., 2005; Otten et al., 2005). Within the growth plate a reduced proliferation and a spatially dysregulated apoptosis of chondrocytes was observed. These mouse models display important features of the human diseases and allow not only to dissect the contribution of intra- and extracellular pathways, but also to analyse the physiological role of individual proteins in skeletal development. However, it is noteworthy that the phenotype is generally weaker in mice and in some cases even both copies of the mutant allele were required to develop a detectable chondrodysplasia. This phenomenon could be explained by differences between mice and men with respect to the development of the skeletal system and the different biomechanical loading on individual skeletal elements.

Combined knockouts

The lack of a skeletal phenotype in mice deficient in various cartilage matrix proteins was often explained by compensatory upregulation of a structurally related protein. However, in COMP deficient mice the expression of all other members of the thrombospondin family was unchanged (Svensson et al., 2002). A similar observation was made in matrilin-3 deficient animals where the expression of other matrilins was unaltered (Ko et al., 2004). Recently, several mouse lines with a combined deletion of structurally different extracellular matrix proteins which share similar functions have been generated. Mice deficient in both COMP and collagen IX have shortened and widened long bones, as well as an altered bone structure (Blumbach et al., 2008). The most striking phenotype is a large uncalcified hypocellular area in the central region of the tibia. Cells in the proliferative and hypertrophic zone failed to arrange in columns. These malformations were also reported in mice lacking only collagen IX in two independent studies (Blumbach et al., 2008; Dreier et al., 2008). Even though the contribution of COMP to the overall phenotype appears to be minor, some discrete changes in the deposition of extracellular matrix proteins like matrilin-3 are only detected in collagen IX/COMP deficient animals. Another group demonstrated that both collagen IX and COMP play a role in maintaining homeostasis of articular cartilage (Posey et al., 2008).

When subjected to running exercise, flattening of the articular surface was seen in both single and double deficient animals. However, only in mice lacking both collagen IX and COMP, fibrillation of the surface was detected. Also, mice lacking either thrombospondin-1, -3, COMP and collagen IX and several combinations thereof were generated. From the study of these animals it appears that collagen IX plays a more significant role in the growth plate architecture than any member of the thrombospondin family. However, all of the individual combined knockout strains show growth plate alterations with more chondrocytes arranged in clusters than in columns. The highest degree of disorganization was observed in the thrombospondin-3/COMP/collagen IX triple knockout. The absence of thrombospondin-1 leads to alterations in the timing of the growth plate closure, especially when other proteins are lacking (Posey et al 2008).

Mechanistic explanations of growth cartilage alterations

Other macromolecules responsible for similar growth plate phenotypes

In order to determine the mechanisms which underlie these growth plate abnormalities it might be helpful to evaluate phenotypes of transgenic mice lacking fibrillar collagens, such as collagen II and XI, or those carrying mutated collagens. Collagen II which, together with collagen XI, forms the body of D-banded cartilage fibrils is highly sensitive to small mutational changes of even single amino acids. Such substitutions can change the physicochemical and structural characteristics of the collagen chain and thereby affect interactions with other molecules. Transgenic mice homozygous for the Del1 mutation within the triple helical domain (deletion of whole exon 7 and intron 7) in the *Col2a1* gene even die at birth. The architecture of the growth plate of these mice is grossly disturbed, with a marked reduction of proliferating chondrocytes, an absence of the typical columnar arrangement and an increase in the height of the zone of hypertrophic chondrocytes. In addition, these mice have a markedly reduced amount of collagen fibrils and, thus, a total reduction in cartilage matrix (Metsaranta et al., 1992). Here, the abnormal growth plate morphology is clearly connected to a lack of cartilage fibrils, which are necessary to ensure a proper separation of the clonally derived chondrocytes in order to form typical columnar structures. Interestingly, mice harbouring single amino acid mutations, such as exchange of glycine to cysteine (G85C and G904C which affects one of the cross-linking lysine residues) and arginine to cysteine (R789C which affects the amino acid in the Y-position of the G-X-Y sequence), develop similar morphological growth plate anomalies (Garofalo et al., 1991; So et al., 2001; Gaiser et al., 2002). The G85C and G904C mice have a reduced number of thin collagen fibrils in growth cartilage

which, in the case of the G85C mutation, are replaced by large fibrils frequently arranged in thick bundles. G904C mice have no such abnormal collagen fibrils. Possibly, conformational changes induced by the G904C mutation have no impact on cross-linking due to the position of amino acid 904 within the collagen chain. Obviously, alterations in pro-collagen assembly, increased intracellular degradation and a more rapid turnover of the mutated collagen contribute to a decreased number of typical cartilage collagen fibrils. A reduction of these collagen fibrils seems to prevent post-mitotic chondrocytes from separating in order to form the typical columns. In mice lacking the α2 chain of collagen XI, chondrocytes in growth plates of all long bones were disorganized and failed to align in columns. Also here, the collagen fibrils of homozygous mutants appear to be less organized (Li et al., 2001).

The lack of the major cartilage proteoglycan aggrecan leads to severe dwarfism, a short snout, and cleft palate in so-called *cmd* mice. A detailed analysis of the growth plate revealed that the expression pattern of other cartilage matrix proteins, including collagens II, IX, X, and XI is changed. In addition, abnormal collagen fibrils with increased diameter and bundling formations were detected with electron microscopy. These effects are believed to contribute to the disrupted cellular architecture observed in the *cmd* growth plate (Watanabe and Yamada, 2002). However, in collagen IX deficient mice fibrillogenesis and the number of typical thin cartilage fibrils appears normal, except for a slight increase in fibrillar diameter (Budde et al., 2005). Thus, other mechanisms must contribute to columnar disorganization and alterations in the growth plate architecture.

Several mouse models lacking integrin subunits demonstrate that not only the ECM itself, but also cellular matrix receptors and their outside-in signalling pathways influence the arrangement of chondrocytes into columns. Here, the disturbance within the growth plate results finally in growth retardation and/or chondrodysplasia-like phenotypes.

Integrins α1ß1, α2ß1, and α10ß1 are the major receptors for fibrillar collagens in cartilage and their role in chondrocytes spatial arrangement has been intensively studied. Cartilage specific deletion of the common ß1 integrin subunit results in severe growth plate disorganization, leading to chondrodysplasia. Chondrocytes in the proliferative zone remain roundish, display an abnormal rotational movement and an abnormal organization of the cytoskeleton (Aszodi et al., 2003). The growth plates of mice lacking the α10 subunit were also characterized by a disturbed columnar arrangement, abnormal shape and reduced proliferation of chondrocytes (Bengtsson et al., 2005). Notably, integrins α1ß1, α2ß1, and α10ß1 were reported to be involved in chondrocyte adhesion and spreading to collagen IX, matrilin-1 and COMP (Makihira et al., 1999; Kapyla et al., 2004; Chen et al., 2005). Integrin-linked kinase (ILK) is a protein serine/threonine kinase

that has been implicated in integrin-dependent signaling pathways and also in the regulation of chondrocyte shape and proliferation. Interestingly, chondrocyte specific deletion of the ILK gene leads to rounded proliferative chondrocytes, a disorganized columnar arrangement and irregular actin distribution. As a result, these mice remain too short (Grashoff et al., 2003). A similar phenotype has been described in mice lacking the Rho GTPase Rac1 in cartilage. Disorganized growth plates with hypocellular areas and chondrocytes that are abnormal in shape and size were reported, demonstrating that Rac1 is required for normal cell matrix interaction in cartilage (Wang et al., 2007). Thus, integrins, ILK and Rac1 seem to act in a common pathway regulating cartilage development, and disturbances in outside–in signalling from the ECM to the cytoskeleton might contribute to a severe skeletal phenotype. Whether the absence of collagen IX results in or contributes to disturbances of this signalling pathway remains unresolved at this time.

Growth plate cartilage is a rather hypoxic structure in which the transcription factor HIF1α supports chondrocyte survival. Differences in ECM composition and supramolecular structure might influence diffusion processes, resulting in alterations in oxygen tension. Interestingly, some aspects of the morphological phenotype of collagen IX deficient mice closely resembles that of animals lacking HIF1α (Schipani et al., 2001) or cartilage specific knock outs of VEGFα (Zelzer et al., 2004) and the tumour suppressor PTEN (Yang et al., 2008). In all these mouse lines, central hypocellular areas in the epiphyses, as well as a disruption of growth plate organization and architecture were detected. However, it remains to be determined whether excessive hypoxia, HIF1α- dependent pathways and VEGF signalling are causative for hypocellular regions in the tibial growth plates in collagen IX deficient animals or if other and/or additional mechanisms apply.

Cell movement and orientation

In the proliferative zone of the post-natal growth plate, chondrocytes are arranged in columns parallel to the long axis of the bone. Post-natal proliferating zone chondrocytes are discoid in shape and orient themselves into columns of 6 to 8 cells. Although the cells form columns along the longitudinal axis of the bone, the cells demonstrate specific polarity and divide along the perpendicular axis. After the cells divide they flatten and move so that one daughter cell is on top of another in the column. This process has been termed rotation, which maintains columnar organization, although very little is known about how it is regulated (Aszodi et al., 2003). Involved in this gliding movement are primary cilia, which are non-motile microtubule base appendages extending from the surface of almost all vertebrate cells (Davenport and Yoder, 2005). Growth plate defects can also be associated with chondrocyte cytoskeletal

abnormalities caused by different alterations affecting primary cilia. The process of intraflagellar transport (IFT) is responsible for building and maintaining the structure and the function of cilia (Rosenbaum and Witman, 2002). Motor proteins like kinesin and dynein are required to move components into and out of the cilium axoneme. Disruption of *Kif3α*, a component of the kinesin II motor complex, disables anterograde IFT and leads to failure and maintenance of cilia (Hirokawa, 2000). Deletion of the *Kif3α* gene in chondrocytes via Col2a-Cre mediated recombination results in reduced proliferation and loss of columnar organization of growth plate chondrocytes, suggesting that IFT/cilia mediate the process of chondrocyte rotation. Specifically, the polarity of the cells and axis of cell division are normal but the shape and orientation of the cells relative to each other and relative to the long axis of the bone are disrupted. The authors suggest a model in which the loss of cilia results in alterations of cell-ECM interactions, which in turn results in defects in chondrocyte rotation (Song et al., 2007). A very similar phenotype with regard to shape of chondrocytes and columnar orientation was observed in mice which carry a hypomorphic mutation in the *Tg373* gene, resulting in the loss of polaris, a protein essential for ciliogenesis. In these mice primary cilia were significantly shorter and were identified only on very few cells (McGlashan et al., 2007). Notably, cell shape and columnar orientation were comparably disrupted, as in the *Col9a1* deficient growth plate. These similarities point to a defect in the process of rotation and cell movement in the absence of collagen IX as well.

Reduced proliferation of chondrocytes and accelerated hypertrophic differentiation might also result from an altered signaling of the Ihh / PTHrP system. Smo and Gli as part of the intracellular hedgehog signalling pathway are either permanently localized in the cilia (Gli) or translocated to the cilia body after activation of the hedgehog signalling pathway (Smo) (Haycraft et al., 2005; Song et al., 2007). Overall, these results suggest that cilia and/or IFT are required in the mesenchyme at early stages of limb morphogenesis for Shh signaling to determine anteroposterior patterning of the digits, and at later stages for proper endochondral bone formation (Haycraft et al., 2007).

It is known that mechanical stress plays an important role in endochondral bone formation. The optimal mechanical force will promote the proliferation and differentiation of growth plate chondrocytes, while excessive force will cause cessation of growth and cell death. Not much is known about how mechanical force is received by chondrocytes. However, it was suggested that chondrocyte cilia function as mechanosensors (Jensen et al., 2004). Studies of the ultrastructure of cilia in primary cultures of sternal chondrocytes showed that the cilia interdigitate among collagen fibers and proteoglycans. Bending patterns of the cilia suggesting response to shear stress were observed, as well as patterns suggesting deflection by ECM contact. It was

suggested that cilia could transmit mechanical force through their interaction with the surrounding ECM (Song et al., 2007). Possibly, lack of collagen IX influences formation of signalling and growth factor gradients and sensation of mechanical stress in a secondary way. Collagen IX and / or matrilin-3, and not collagen II, might be the primary interaction partners for cell surface cilia of chondrocytes, since they are located at an exposed position within the fibril periphery.

Proteoglycan distribution

Loss of matrix proteins, e.g. matrilin-3 due to the absence of collagen IX, may contribute to the aberrant distribution of proteoglycans which was observed in central epiphyseal growth cartilage regions. Besides their role in generating large osmotic swelling pressure, important for the ability of cartilage to resist compressive forces, different proteoglycans are held responsible for the generation of growth factor gradients affecting chondrocyte proliferation and maturation. Moreover, heparan sulphate proteoglycans have been shown to control signalling of Indian hedgehog, a mediator produced by pre-hypertrophic chondrocytes influencing chondrocyte proliferation and differentiation. Mice with genetically ablated glucosyltransferases Ext1 and Ext2, participating in the synthesis of the proteoglycan heparan sulphate chains, were shown to have an Indian hedgehog signalling-deficient phenotype (Hilton et al., 2005; Stickens et al., 2005).

It is attractive to speculate that the stable cohesion of the fibrillar and extrafibrillar matrix compartments is profoundly disturbed in *Col9a1* deficient animals and that, as a consequence, growth factor gradients arise less accurately. Consequently, chondrocytes in collagen IX - deficient tibial growth plates proliferate at reduced levels. This may account in part for the development of hypocellular regions in different areas of the epiphyseal and growth plate cartilages.

Along this line, it has been demonstrated recently that proteoglycan desulfation is also crucial for normal skeletal development (Settembre et al., 2008). Ablation of the sulfatase-modifying factor 1 (SUMF1) which activates sulfatases, involved in proteoglycan desulfation, leads to severe skeletal abnormalities. In SUMF1 deficient animals, endochondral ossification, ECM production and turnover was affected and columnar organization of proliferative chondrocytes in the growth plate was lost.

Conclusion

We summarize that changes in the macromolecular composition of the cartilage matrix and its density may lead to an altered diffusion of oxygen, nutrients and growth factors within the growth plate. This in turn might result in irregular distribution of extrafibrillar proteoglycans and the generation of hypocellular regions. These changes can also affect mechanical properties and transduction of mechanical loading, leading to alterations in the growth plate architecture and disturbance of the endochondral ossification process. Cell-matrix interactions, proper movement and rotation of the chondrocytes within the matrix are essential for cellular orientation and shape. This process can be profoundly disturbed through interference with cell matrix receptors, collagen fibrillogenesis and impairment of primary cilia function. The lack of phenotypes in some of the knockout models might be due to compensation by structurally similar or functionally redundant proteins. Here, an appropriate challenge, e.g. mechanical loading, might be necessary to uncover possibly 'hidden' phenotypes. As has been reported, combined knock out mice models or animals carrying matrix protein mutations instead of absence of the resulting protein often show a more severe phenotype.

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