Susanne Grässel · Attila Aszódi *Editors*

Cartilage

Volume 1: Physiology and Development

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Editors

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Preface

Volume one of a book series comprised of three volumes is dedicated to provide an overview about physiology and biology of permanent cartilage tissue and its role as a template in development and skeletal growth.

The text is designed to be of use to multiple medical and basic science disciplines as orthopedics, rheumatology, and trauma surgery and all basic investigators working in the field of cartilage and joint physiology and development.

Three types of cartilage (hyaline, elastic, and fibrous) have been characterized on the basis of histological criteria and mechanical properties. The most prevalent type is hyaline cartilage which is a visually uniform, translucent tissue found in the skeleton of all vertebrates. Articular cartilage, the most familiar hyaline permanent cartilage, forms the smooth gliding surface of joints, such as the knee and hip that permits locomotion in humans and animals. Injuries to this tissue and degradative diseases as osteoarthritis impair joint mobility and are a great challenge of modern regenerative medicine. Hyaline cartilage also comprises the growth plate, the transient and temporary template required for endochondral bone formation in fetal development, skeletal growth, and repair processes, i.e., after fracture. In addition, hyaline cartilage occurs as a permanent structural tissue in costal cartilage and tracheal reinforcing rings.

Cartilage is a matrix-dominated tissue, and with regard to its abundance, the matrix is mainly composed of collagens and proteoglycans. These two main components form suprastructures interconnected by plenty of proteins that way forming a kind of alloy. Cartilage fibrils vary in their molecular organization, their width, and their orientation in the tissue in order to resist forces generated by external load. Proteoglycans, especially the lectican family, provide the required tissue elasticity and resilience by dissipating load. The interconnecting molecules, sometimes also referred to as adaptor proteins, are from a biochemical point of view mainly non-collagenous glycoproteins and small leucine-rich repeat proteoglycans which closely regulate the assembly and connection of the fibrillar and extrafibrillar matrices. Chapters 1, 2, and 3 of this volume summarize information about the impact of proteoglycans, forming the extrafibrillar matrix, on cartilage physiology and integrity; the role of the different collagens in cartilage matrix homeostasis and formation of fibrillar suprastructures; and the role of non-collagenous matrix adaptor proteins in growth factor binding, mediation of inflammatory and immune responses, and their use as biomarkers in cartilage-associated diseases.

In long bones, a specialized structure called the growth plate is responsible for the linear growth and forms just below the epiphysis at both ends of the cartilaginous mold. The growth plate is organized into zones which reflect the sequential differentiation stages of chondrocyte proliferation, maturation, and hypertrophy. The differentiation process is accompanied by the establishment of cellular anisotropy and planar polarity that generates the unique spatial structure of the tissue. Chondrocyte differentiation and polarity are essential and mutually interacting foundations of the normal growth plate function, and their disturbance results in chondrodysplasias with impaired longitudinal growth. Chapter 4 will focus on the mechanisms responsible for the establishment and maintenance of the structural polarity of the cartilaginous growth plate.

The cell fate of hypertrophic growth plate chondrocytes at the chondro-osseous junction has been a subject of discussion for several decades: on the one hand, there is ample evidence for programmed cell death by apoptosis or other mechanisms in the lower hypertrophic zone; on the other hand, several studies have indicated that some hypertrophic chondrocytes may not be "terminally differentiated" but are able to further differentiate into osteoblasts. Comprehensive insight into this novel concept of the fate of hypertrophic chondrocytes is provided by Chap. 5. Hypoxia-driven pathways, governed by the hypoxia-inducible factors (HIFs), are absolutely essential for the survival and functioning of chondrocytes in these challenging conditions. HIF-mediated signaling has also been implicated in joint formation and the integrity of the adult articular cartilage. Thus, the oxygen-regulated genetic program mediated by HIFs is key to the controlled development, growth, health, and disease of endochondral bone summarized in Chap. 6.

Chapter 7 focuses on our current understanding at the cellular and molecular levels, from creation to maturation of a synovial joint. Morphologically, we know there is the formation of interzone regions at the presumptive sites of the future joint. Molecularly, we have some insights into signals that direct the initiation and progression of interzone regions toward a joint. And through innovative technologies in mouse genetics and genomics, we are beginning to understand the developmental processes, with the identification of progenitor cell pools, and to trace origin of cells and track the fate of descendent cells from initiation to formation of the complete joint.

Chapter 8 provides an overview about signaling factors which control cartilage formation, development, and the differentiation and maturation of chondrocytes during embryonic skeletal development. The orchestrated formation, differentiation, and degradation of cartilage and bone are regulated by a multitude of signaling systems and transcription factors. The identified signaling molecules include Ihh, PTHrP, FGF, BMP, Wnt, IGF, CNP, and CCN proteins. One essential group of regulators of chondrogenesis comprises members of the Hedgehog (Hh) morphogen family. Hedgehogs act as long-range morphogens during chondrocyte development and endochondral ossification. Mutations in Hh effectors, receptors, and co-receptors, as well as in ciliary proteins that act as modulators of Hh reception, result in skeletal and craniofacial deformities. Chapter 9 summarizes the current understanding of Hh production and signaling in chondrocytes in development and disease. Wnt signals play important regulatory roles in those processes. In the vertebrate genome,

a total of 19 different Wnt ligands are encoded which can utilize diverse signaling pathways acting either positively or negatively on chondrogenesis and during cartilage development, forming a highly interactive system addressed by Chap. 10.

Chondrogenesis, e.g., the formation of cartilage from precursor cells, is characterized by drastic changes in cell shape and size. This involves major reorganization of the cytoskeleton, in particular, the actin network. Recent years have provided new insights into both the regulation of actin organization during chondrogenesis and into the downstream mechanisms connecting actin dynamics to chondrocyte gene expression which is addressed by Chap. 11.

Bringing together international experts from diverse fields of musculoskeletal research was a demanding task requiring patience and persistence. For that we are very grateful to our authors of this volume who managed to complete their chapters on time and who dedicated their spare free time to writing their reviews.

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1 Cartilage Proteoglycans

Anders Aspberg

Abstract

Proteoglycans are key components of the cartilage extracellular matrix and essential for normal tissue function. The core protein and the glycosaminoglycan chains both contribute to function and provide different properties of the individual proteoglycans. This review is focused on the two main families of cartilage proteoglycans.

The first of these is the lectican family, including aggrecan, versican, and the cartilage link protein. The aggregating proteoglycan network formed by aggrecan, link protein, and hyaluronan provides biomechanical properties that give the tissue its ability to withstand and distribute load.

The second group discussed is the small leucine-rich repeat proteoglycan family, which includes decorin, biglycan, asporin, fibromodulin, lumican, keratocan, osteoadherin, proline-/arginine-rich end leucine-rich repeat protein, epiphycan, mimecan, opticin, chondroadherin, and chondroadherin-like. These proteoglycans bind collagens and are important regulators of cartilage extracellular matrix assembly. In addition, some of these proteoglycans bind and regulate growth factors and their receptors and regulate innate immunity through interactions with Toll-like receptors or the complement system.

This review will give an overview of the structure and function of the different aggregating proteoglycans and small leucine-rich repeat proteoglycans in normal cartilage extracellular matrix.

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1.1 Introduction

Articular cartilage function depends on the molecular composition and organization of its extracellular matrix (ECM). This complex protein network fills the space between the cells and provides a structural scaffold, giving the tissue its unique biomechanical properties.

Disturbed cartilage ECM composition or organization, either by failure to produce and assemble the ECM building blocks or dysregulated ECM degradation, is a key factor in the development of joint disease such as osteoarthritis.

The components of cartilage ECM are usually grouped into proteoglycans, collagens, and non-collagenous proteins, each providing specific functionalities to the composite ECM material. This chapter will give an overview of cartilage proteoglycans, while the latter molecular classes will be discussed in Chaps. 2 and 3, respectively.

1.2 Proteoglycans

A proteoglycan is a protein posttranslationally modified with one or several glycosaminoglycan (GAG) chains, a type of linear carbohydrate polymers. The GAG chains are composed of repeating disaccharide units, with different specific disaccharides used in the different types of GAGs: hyaluronan, chondroitin/dermatan sulfate (CS/DS), heparan sulfate (HS)/heparin, and keratan sulfate (KS). The proteoglycan-forming GAGs CS/DS and HS are attached to the core protein by linkage to serine residues in Ser-Gly sequence motifs through a specific tetrasaccharide linker. Keratan sulfate is either O-linked (KS type II) to serine or threonine residues or N-linked (KS type I). Unlike other GAGs, hyaluronan is not attached to a protein core but is extruded into the extracellular environment by transmembrane hyaluronan synthases. Further variation and specificity in GAG structure are achieved through sulfation at different positions of the individual disaccharide units and through epimerization of uronic acid residues in DS and HS. The cellular synthesis of GAGs is complex and not yet entirely understood, with a large number of different enzymes involved in producing and modifying the GAG chains. The details of structural variation and synthesis of GAGs are beyond the scope of this chapter and have been the subject of many excellent recent reviews; see, for example, (Mikami and Kitagawa 2013).

In cartilage, a key function of proteoglycan is to provide swelling pressure, allowing the tissue to take up and distribute mechanical load. This is achieved by the aggrecan-hyaluronan matrix (see below). Other cartilage proteoglycans play vital roles in guiding the ECM assembly, functioning as tissue reservoirs for soluble factors or as cell surface receptors. Additional functions of proteoglycans include regulating the innate immune system through interaction with complement components and Toll-like receptors (TLRs), which may lead to an inflammatory response and contribute to osteoarthritis pathogenesis (Orlowsky and Kraus 2015).

All proteoglycans found in cartilage are present in other tissues as well. Even aggrecan, the quintessential cartilage proteoglycan is prominent in the central nervous system ECM. This chapter will give an overview of the structure and function of the main proteoglycans of cartilage, including some functional information gained from studies in other tissues. Other proteoglycans with important functions are present in cartilage, including the basement membrane proteoglycan perlecan and cell surface proteoglycans such as syndecans. In addition, several part-time proteoglycans such as lubricin (proteoglycan 4) are important for cartilage and joint function. For space reasons, these are not discussed, and this chapter is focused on the two main extracellular matrix proteoglycan groups – the aggregating proteoglycan network and small leucine-rich repeat proteoglycans (SLRPs).

1.3 The Aggregating Proteoglycan Network

The large aggregating cartilage proteoglycan aggrecan is perhaps the most studied of all proteoglycans. Aggrecan, together with versican, neurocan, and brevican, forms the lectican family (Ruoslahti 1996), also known as hyalectins. These proteoglycans have an elongated core protein carrying CS and KS chains, with globular interaction domains at the N- and C-termini (Fig. 1.1). The N-terminal G1 domain interacts with hyaluronan and the hyaluronan-proteoglycan link proteins. The C-terminal G3 domain binds multimeric ECM molecules such as tenascins and fibulins. The result of these interactions is that the PG is organized into an enormous molecular network, by G1 hyaluronan anchorage and G3 cross-linking.

Aggrecan and cartilage link protein are fundamental for cartilage function. Versican is also found in cartilage and may play a role during development, whereas neurocan and brevican are only found in the nervous system.

1.3.1 Aggrecan

The aggrecan domain structure was first revealed by molecular electron microscopy studies, showing an N-terminal G1 domain separated from a second globular domain (G2) by a short interglobular domain, an elongated domain carrying KS and CS chains, and a C-terminal globular G3 domain (Fig. 1.1). Subsequent sequencing of the aggrecan cDNA identified the structural protein repeats forming the globular domains and the homologies between the G1 and G2 domains with proteoglycan hyaluronan link protein (LP) and between the G3 domain and selectins; see (Aspberg 2012) for references.

The 250 kDa human aggrecan core protein carries approximately 100 CS chains and multiple additional KS chains, resulting in a molecular weight of roughly 2.5 MDa for the proteoglycan. The CS chains are attached to serine residues in Ser-Gly motif containing repeats in the CS-attachment domain. Keratan sulfate chains are O-linked to serine residues in the region between the G2 domain and the CS-attachment region. These KS chains have been shown to interact with fibrillar

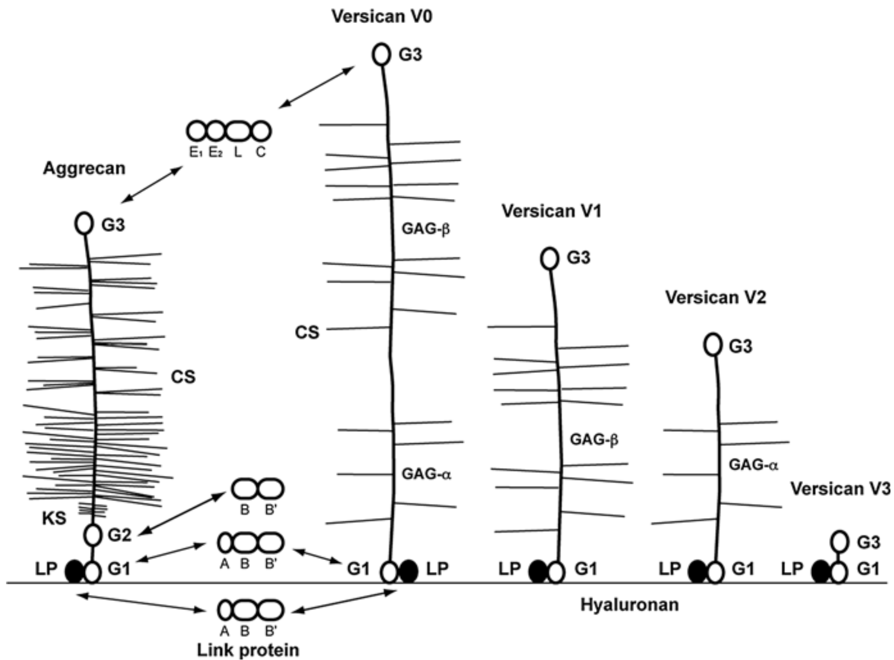

Fig. 1.1 The aggregating proteoglycans of cartilage. The figure shows aggrecan and the four splice forms of versican (*V0 to V3*) bound to hyaluronan in ternary complexes with cartilage link protein (*LP*). The subunit structure of the core protein globular domains is indicated. The G1 domain and link protein contain Ig-like repeat (*A*) and proteoglycan tandem repeats (*B, B′*); the latter also found in the G2 domain. The G3 domain consists of two EGF-like repeats (*E1, E2*), a C-type lectin repeat (*C*), and a complement regulatory protein-like repeat (*C*). Chondroitin sulfate (*CS*) and keratan sulfate (*KS*) glycosaminoglycans are indicated, and the alternatively spliced CS substituted domains of versican are labeled GAG-α and GAG-β

collagen (Hedlund et al. 1999). Interestingly, this region is polymorphic in human, with varying number of KS attachment repeats. Other O- and N-linked KS glycans are present in the interglobular domain (Barry et al. 1995). Taken together, each aggrecan molecule carries about 10,000 fixed negative charges in the form of carboxyl and sulfate groups of the CS chains.

The name aggrecan is derived from this proteoglycan's ability to form large aggregates with hyaluronan in the extracellular matrix. Seminal work by Sajdera, Hascall, Heinegård, and Hardingham clarified the mechanism of aggregation and formation of the supramolecular complexes with hyaluronan and link protein, as reviewed in (Heinegård 2009). In addition, interactions of the G3 domain with multimeric ECM proteins such as tenascins and fibulins allow complex formation through the C-terminal end of the proteoglycan (Aspberg 2012).

The formation of the aggrecan-hyaluronan network results in the immobilization of a vast number of negative charges in the tissue. By attracting counterions, this results in the formation of a Donnan equilibrium; thus, by osmotic processes, the PG draws water into cartilage tissue. This provides the biomechanical properties

critical for articular cartilage function, namely, the ability to absorb and distribute mechanical load over the joint surface. Recent technical advances in atomic force microscopy have allowed detailed analysis of the nanomechanical properties of cartilage microdomains. This has emphasized corresponding changes in aggrecan GAG chain; for review, see (Han et al. 2011)

The vital importance of aggrecan for cartilage development and function is emphasized by the identification of a variety of mutations in the aggrecan gene or affecting aggrecan posttranslational modification; see (Aspberg 2012) for review. Aggrecan null or functional null mutations have been described in a number of species, including humans. These dominant mutations result in skeletal dysplasia in heterozygous individuals and animals, whereas homozygosity for null mutation results in perinatal lethality in mice, cattle, and chicken. Missense mutations in the aggrecan G3 domain impair interactions with other ECM proteins, with clinical phenotypes ranging from short stature and osteochondritis dissecans with early-onset osteoarthritis to severe chondrodysplasia (Nilsson et al. 2014; Stattin et al. 2010; Tompson et al. 2009). A third group of mutations affect glycosyltransferases and transporter enzymes involved in GAG synthesis and sulfation. When homozygous, these mutations result in a spectrum of phenotypes ranging from short stature to lethal chondrodysplasia.

Aggrecan turnover in the tissue is relatively fast, with a reported half-life of 3.4 years for the full-length proteoglycan (Maroudas et al. 1998). Aggrecan degradation is achieved through matrix metalloproteinases (MMPs), aggrecanases, and other proteases (Troeberg and Nagase 2012). In mature cartilage, a mixture of truncated aggrecan fragments is found, reflecting cleavage at progressively more N-terminal sites and gradual loss of the more C-terminal fragments of the proteoglycan. Thus, G1 domain concentration is higher than G3 concentration, and G1 half-life in the tissue is around 24 years (Maroudas et al. 1998; Verzijl et al. 2001).

A key step in osteoarthritis disease progression is the loss of aggrecan by metalloproteinase and aggrecanase degradation. Aggrecan degradation occurs relatively early in the disease progression, and proteoglycan loss exposes the collagen fibrils to degradation. It is now becoming clear that the aggrecan fragments released by proteolysis have biological activities in regulating innate immune responses. Thus, the aggrecan G3 domain binds complement factors C1q and C3 and activates the classical and alternative complement pathways (Melin Fürst et al. 2013). Furthermore, the 32mer aggrecan fragment released by MMP and aggrecanase cleavage between the G1 and G2 domain activates TLR2 (Lees et al. 2015). Such fragments may thus maintain inflammatory processes and lead to further cartilage degradation.

1.3.2 Link Protein

The cartilage link protein is not a proteoglycan in itself but critical for cartilage proteoglycan function. It forms a ternary complex with hyaluronan and aggrecan G1 domain, stabilizing the proteoglycan aggregate (Heinegård and Hascall 1974;

Hardingham 1979). Interestingly, the link protein and the G1 domain are homologous, sharing a domain organization with an immunoglobulin-like domain followed by two proteoglycan tandem repeats (Deak et al. 1986; Doege et al. 1986, 1987; Neame et al. 1986). Four different members of the link protein family have been identified, but of these, only the classical cartilage link protein appears to be present in cartilage (Spicer et al. 2003). Mice lacking link protein showed perinatal lethality due to respiratory problems, disorganized growth plates, and shortened limbs, a phenotype similar to but milder than the aggrecan-deficient cmd mice (Watanabe and Yamada 1999). Interestingly, an N-terminal 16 amino acid long peptide, released from cartilage link protein by proteolysis, binds bone morphogenetic protein (BMP)-receptor type II and stimulates aggrecan and collagen production in chondrocytes (Wang et al. 2013; Liu et al. 2000; McKenna et al. 1998).

1.3.3 Versican

Versican is a widely expressed lectican. Although lacking the G2 domain, the overall molecular organization of versican is similar to that of aggrecan (Fig. 1.1), with an amino-terminal G1 domain that binds hyaluronan and link proteins, a central region carrying up to 23 CS chains, and a C-terminal G3 domain that binds multimeric ECM proteins like tenascins, fibulins, and fibrillin (Aspberg 2012; Zimmermann and Ruoslahti 1989; LeBaron et al. 1992). The CS region is encoded on two large exons, and alternative splicing gives rise to four different splice variants with both (V0), either (V1, V2), or none (V3) of these exons (Dours-Zimmermann and Zimmermann 1994).

Versican is important in the condensing mesenchyme preceding the formation of cartilage bone anlagen during skeletal development (Williams et al. 2005). In mice, versican was found present in the interterritorial matrix of the growth plate, at the sites of joint formation and superficially in articular cartilage (Choocheep et al. 2010; Matsumoto et al. 2006). Conditional versican gene targeting interfered with joint formation in a transforming growth factor (TGF)-β dependent mechanism (Choocheep et al. 2010). In adult human tissues, versican was found to be present in elastic and fibrous cartilage, but was not detectable in hyaline cartilage (Bode-Lesniewska et al. 1996). More recent quantitative proteomic studies show versican presence in a number of different human hyaline cartilages, with a preferentially superficial location in articular cartilage (Müller et al. 2014; Önnerfjord et al. 2012). In osteoarthritic cartilage, versican was upregulated and showed pericellular localization in chondrocyte clusters (Nishida et al. 1994; Cs-Szabo et al. 1997).

Versican activates myeloid cells in a TLR2 dependent manner, although the details of the interaction remain unclear (Kim et al. 2009). Furthermore, proteolytic cleavage of versican has been shown to produce a bioactive G1-containing fragment regulating interdigital web apoptosis (McCulloch et al. 2009; Nandadasa et al. 2014).

1.4 Small Leucine-Rich Repeat Proteoglycans (SLRPs)

The SLRPs are small ECM proteoglycans or glycoproteins of the leucine-rich repeat (LRR) protein superfamily (Fig. 1.2). They are characterized by a central region of LRR repeats, which is stabilized by N-terminal and C-terminal cysteine bridges. All SLRPs contain an N-terminal LRRNT cysteine knot motif with two disulfide bonds. The canonical SLRPs (see below) have a characteristic LRRCE motif, whereas noncanonical SLRPs have the LRRCT motif shared by many other types of LRR proteins (Park et al. 2008). Additional N-terminal or C-terminal protein extensions provide unique properties to the different SLRPs. Based on their primary structure, the SLRPs can be divided into five classes. Classes I to III constitute the canonical SLRPs and class IV and V the noncanonical SLRPs (Park et al. 2008; Iozzo and Schaefer 2015). Class V SLRPs have not been detected in cartilage and will not be discussed further in this chapter.

Many, if not all, SLRPs bind collagen, and some, like decorin, remain bound to the mature collagen fibrils. The SLRPs are in fact important regulators of collagen fibril assembly. Different SLRPs bind at different positions along the forming fibril and regulate the rate of polymerization as well as the diameter of the final fibril (Kalamajski and Oldberg 2010). Positioned at the fibril surfaces, the SLRPs can interact with other proteins present in the ECM. This allows them to function as linkers between different fibrils, and other supramolecular assemblies, as exemplified by biglycan bridging between collagen type II and type VI fibrils. Furthermore, SLRPs bound to collagen fibrils can protect the fibril against collagenase degradation (Geng et al. 2006). The functions of SLRPs are not limited to providing and regulating ECM structure. Thus, many SLRPs bind and regulate the activity of growth factors, or their receptors. Furthermore, some SLRPs, or SLRP fragments, regulate innate immunity through interaction with TLRs or the complement system.

1.4.1 Class I SLRPs

Class I SLRPs include decorin, biglycan, and asporin. Apart from asporin, these have an N-terminal extension carrying DS chain(s). They are synthesized as preproteins, with a propeptide of unknown function, and have the LRRNT cysteine knot motif, 12 LRR repeats, and the LRRCE-terminating cysteine bridge motif. Interestingly, the propeptides of biglycan and decorin are cleaved by the same bone morphogenetic protein-1/mTolloid proteases that remove fibrillar collagen propeptides (Scott et al. 2000; von Marschall and Fisher 2010).

Decorin is widely expressed and is present in cartilage, with one DS chain on its N-terminal extension (Krusius and Ruoslahti 1986; Rosenberg et al. 1985). It was named in reference to its appearance in electron micrographs, where it appears to decorate the surface of collagen fibrils (Ruoslahti 1988). Decorin also modulates collagen fibril assembly in vitro, resulting in slower fibrillar assembly (Vogel et al. 1984). Gene targeting of decorin led to disorganized collagen fibrils with irregular fibrillar diameters. This resulted in mechanically weaker skin of the mutant mice

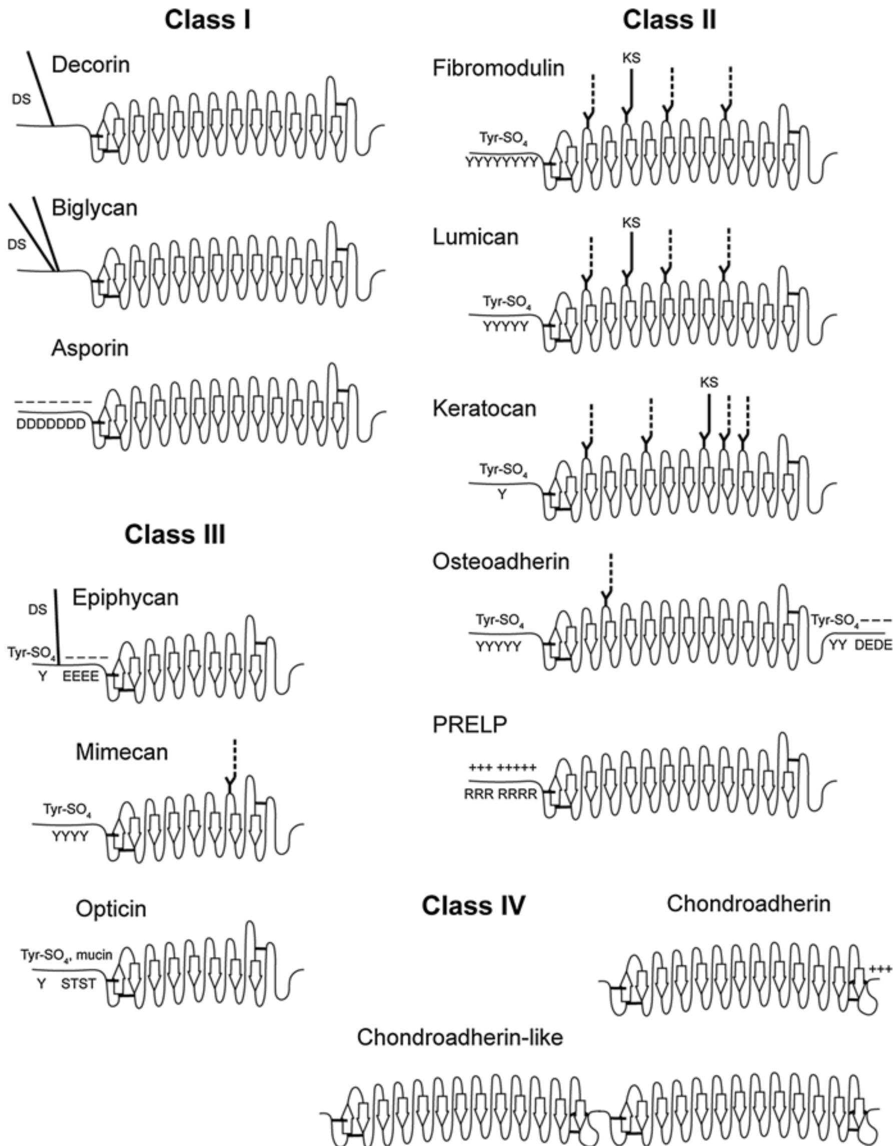

Fig. 1.2 Cartilage small leucine-rich repeat proteoglycans (SLRPs). The figure shows a generalized overview of the cartilage SLRPs grouped according to class (*I to IV*). Important structural properties are illustrated on a schematic core protein structure. Acidic and basic amino acid residue clusters providing negative or positive charges as well as dermatan sulfate (*DS*), keratan sulfate (*KS*), mucin-type O-glycosylation, and tyrosine O-sulfation (Tyr-SO4) posttranslational modifications are indicated. For details, see the main text

(Danielson et al. 1997). Decorin acts as a growth suppressor by interacting with TGF-β (Yamaguchi et al. 1990) and a number of different growth factor receptors; see (Iozzo and Schaefer 2015) for a recent review.

The structure of decorin was the first SLRP structure to be determined by X-ray crystal diffraction, which showed a curved solenoid structure with its concave face formed by a beta sheet and its convex face formed by loops and helices (Scott et al. 2004). This study also showed that decorin can form homodimers, which had been suggested from biophysical studies on decorin in solution (Scott et al. 2003) and was confirmed in later studies (Scott et al. 2006). The dimers formed by antiparallel binding of the concave surfaces of the monomers, which was difficult to reconcile with collagen binding through these surfaces. Although sites on the decorin monomer mediating interaction with collagen (Kalamajski et al. 2007; Svensson et al. 1995), as well as binding sites for decorin along the collagen molecule and fibril (Scott and Orford 1981), have been identified, the details of decorin binding to collagen remain unclear. Further mutational and biophysical studies have shown that decorin in solution exists in a monomer/dimer equilibrium and suggest that decorin binds collagen as a monomer through its concave face (Islam et al. 2013). Indeed, molecular modeling studies support decorin monomer binding across some four collagen triple helices on the fibril surface (Orgel et al. 2009).

Biglycan, as the name implies, carries two DS chains (Fisher et al. 1989). Biglycan is prominent in cartilage (Rosenberg et al. 1985) but is also found in other connective tissues, notably tendon and bone, where it was first identified (Fisher et al. 1983). Biglycan can form dimers in solution, and its crystal structure showed strong similarities with decorin, although in biglycan the monomers were somewhat more curved than decorin (Scott et al. 2006).

Like decorin, biglycan binds fibrillar collagens. Apart from fibrillar collagens, biglycan also binds collagen type VI, organizes this into a hexagonal network, and links collagen type VI molecules with type II fibrils (Wiberg et al. 2001, 2002, 2003).

Mice lacking biglycan show a bone phenotype similar to osteoporosis (Xu et al. 1998), with altered collagen fibril formation (Corsi et al. 2002), affecting bone fracture healing (Berendsen et al. 2014). Compound knockouts with other SLRP genes show additional and more pronounced phenotypes, such as increased skin fragility in decorin-biglycan double-knockout mice (Corsi et al. 2002) and increased osteoarthritis in fibromodulin-biglycan double-knockout mice (Ameye et al. 2002).

Biglycan interacts with TGF-β, BMP-4, and vascular endothelial growth factor (VEGF) and modulates Wnt signaling, osteoblast differentiation, and angiogenesis (Nastase et al. 2012; Berendsen et al. 2014). Biglycan has been shown to activate TLR2 and TLR4 signaling and thus regulate innate immunity (Schaefer et al. 2005). This suggests a function as a tissue-derived damage-associated molecular pattern, perhaps involved in driving inflammation in the joint. On the other hand, biglycan and decorin both bind C1q and inhibit complement activation (Sjöberg et al. 2009; Groeneveld et al. 2005; Krumdieck et al. 1992).

Asporin, the third member of the class I SLRPs, is actually not a proteoglycan. Instead, the N-terminal extension, where GAG chains are attached on decorin and

biglycan, shows a sequence of consecutive aspartate residues, providing a cluster of negative charges (Henry et al. 2001; Lorenzo et al. 2001). Although first identified in cartilage, asporin is in fact widely expressed. Asporin binds collagen through its LRR domain, competing for the same binding sites as decorin, and inhibits collagen fibril formation in vitro (Kalamajski et al. 2009). The asporin N-terminal polyaspartate sequence binds calcium, and asporin is a potent inducer of collagen mineralization (Kalamajski et al. 2009).

Asporin binds TGF-β (Kizawa et al. 2005; Nakajima et al. 2007; Kou et al. 2010) and BMP-2 and inhibits their actions (Tomoeda et al. 2008) while binding fibroblast growth factor (FGF)-2 and positively regulating its activity (Awata et al. 2015). In addition, recent studies in *Xenopus* show that asporin interacts with the insulin-like growth factor (IGF) receptor and activates IGF signaling while perturbing BMP, Wnt, and activin signaling (Luehders et al. 2015). Asporin also appears to be able to regulate innate immunity by binding TLR2 and TLR4 and decreasing cytokine release in response to lipopolysaccharide ligands (Yamaba et al. 2015).

No asporin knockout mouse has yet been published. Interestingly, the human asporin gene is polymorphic, with variable lengths of the polyaspartate stretch (Lorenzo et al. 2001). Genetic studies have reported an association of one of these alleles (D14) with risk for knee osteoarthritis and lumbar disk degeneration (Kizawa et al. 2005; Nakamura et al. 2007; Song et al. 2008), although this appears to be limited to specific Asian populations (Xing et al. 2013; Song et al. 2014).

1.4.2 Class II SLRPs

The class II SLRPs include fibromodulin, lumican, keratocan, osteoadherin, and proline/arginine-rich end LRR protein (PRELP). These SLRPs contain a twelve LRR core flanked by LRRNT and LRRCE motifs, carry KS chains on their LRR domain, and are often tyrosine O-sulfated.

Fibromodulin was first purified from cartilage (Heinegård et al. 1986) but is expressed in many connective tissues, including the tendon and skin (Oldberg et al. 1989). Cartilage fibromodulin carries up to four N-linked KS chains on its LRR domain (Plaas et al. 1990; Oldberg et al. 1989), and its N-terminal extension contains a number of tyrosine residues that can be modified by O-sulfation (Antonsson et al. 1991). Fibromodulin binds collagen fibrils at the gap region (Hedlund et al. 1994) and inhibits collagen fibril formation in vitro (Hedbom and Heinegård 1989). The fibromodulin knockout mouse showed irregular collagen fibrils and lower mechanical strength of tendons (Svensson et al. 1999). Interestingly, osteoarthritis incidence was increased in the fibromodulin knockout mouse, but whether this depends directly on changes in cartilage or on altered joint loading pattern due to changes in ligaments remains unclear (Gill et al. 2002). Both in tendons and cartilage of the fibromodulin knockout mouse, levels of lumican protein, another class II SLRP, showed compensatory upregulation. Lumican and fibromodulin were found to bind the same site on collagen type I, which is different from the decorin binding site (Svensson et al. 2000). Indeed, the collagen binding sites on fibromodulin and

lumican show homologous sequence (Kalamajski and Oldberg 2009). Recently, collagen type I cross-linking was found to be dysregulated in fibromodulin knockout animals (Kalamajski et al. 2014).

Fibromodulin binds complement C1q and is an activator of the classical complement pathway (Sjöberg et al. 2005) while also binding the complement inhibitors factor H and C4b-binding protein (Happonen et al. 2009; Sjöberg et al. 2007).

The N-terminal extension of fibromodulin is posttranslationally modified with up to nine O-sulfated tyrosine residues (Önnerfjord et al. 2004). This part of the protein mimics heparin and binds several growth factors and cytokines (Tillgren et al. 2009). The tyrosine O-sulfated domain can be released from the protein through MMP-13 proteolysis (Heathfield et al. 2004).

The expression of osteoadherin (Sommarin et al. 1998; Wendel et al. 1998), sometimes called osteomodulin, was initially thought to be restricted to mineralized tissues. In developing bone, osteoadherin is found in the primary spongiosa, enriched at the border between bone and hypertrophic cartilage (Sugars et al. 2013; Wendel et al. 1998). Quantitative proteomic studies have now clearly demonstrated osteoadherin presence also in articular cartilages (Önnerfjord et al. 2012; Wilson et al. 2012). Osteoadherin is a KS proteoglycan in the bone (Wendel et al. 1998), but whether this is the case in cartilage remains unclear. Like other SLRPs, osteoadherin binds collagen and affects collagen fibril formation (Tashima et al. 2015). Osteoblasts adhere to osteoadherin through integrin αvβ3 receptors (Wendel et al. 1998). Like fibromodulin, osteoadherin contains O-sulfated tyrosine residues in its N-terminal extension (Önnerfjord et al. 2004), which mediates interactions with heparin-binding motifs in other ECM components as well as with bioactive factors such as oncostatin M (Tillgren et al. 2009). Uniquely, osteoadherin also contains a C-terminal extension rich in aspartate and glutamate residues, which likely mediates osteoadherin interaction with hydroxyapatite (Sommarin et al. 1998).

In cartilage, the corneal KS proteoglycans lumican and keratocan exist as glycoproteins, carrying short, non-sulfated poly-(N-acetyllactosamine) chains (Corpuz et al. 1996; Rees et al. 2009). Likewise, although bovine cartilage fibromodulin is a KS proteoglycan (Oldberg et al. EMBO 1989), lumican and fibromodulin from human juvenile articular cartilage carry KS chains but appear to gradually shift to glycoprotein forms with age (Grover et al. 1995; Roughley et al. 1996). Like fibromodulin and osteoadherin, lumican and probably keratocan contain sulfated tyrosine residues in their N-terminal extensions (Corpuz et al. 1996; Önnerfjord et al. 2004). The functions of lumican and keratocan in cartilage have not been studied in detail, but likely overlap with those of other SLRPs. As mentioned above, lumican and fibromodulin have homologous collagen binding sequences in their LRR domains and bind the same site on collagen, and lumican is upregulated in fibromodulin knockout cartilage. Mice lacking lumican or keratocan also show abnormal corneal collagen fibril assembly and organization (Chakravarti et al. 1998; Liu et al. 2003). Lumican interacts with CD14, promoting TLR4-mediated innate immunity (Wu et al. 2007).

The SLRP PRELP, or prolargin, was first identified in cartilage (Heinegård et al. 1986) but is present in many connective tissues. PRELP is a glycoprotein rather than a proteoglycan, although short polylactosamine chains that may be sulfated to some

degree are present on cartilage PRELP (Bengtsson et al. 1995). Unlike other SLRPs, the atypical N-terminal extension of PRELP contains a number of arginine and proline residues, providing a cluster of positive charges (Bengtsson et al. 1995; Grover and Roughley 1998, 2001). This domain binds heparin and heparan sulfate proteoglycans in vitro (Bengtsson et al. 2000), whereas the PRELP LRR domain binds collagen (Bengtsson et al. 2002). In many tissues, PRELP is located adjacent to basement membranes, and as it binds the basement membrane HSPG perlecan, it may be involved in connecting the BM to the loose connective tissue (Bengtsson et al. 2002). The PRELP N-terminal peptide shows interesting bioactive properties. This peptide binds cell surface PGs on preosteoclasts, is internalized by the cells, and counteracts osteoclast differentiation (Rucci et al. 2009). In experiments in mice, the peptide showed efficacy in preventing bone mineral loss (Rucci et al. 2013). The PRELP heparin-binding peptide also shows antimicrobial activity (Malmsten et al. 2006, 2011).

1.4.3 Class III SLRPs

The class III SLRPs epiphycan, mimecan, and opticin are smaller than class I or class II SLRPs, with only seven LRRs, flanked by LRRNT and LRRCE cysteine motifs, and carry DS or KS chains.

Epiphycan (Johnson et al. 1997), also known as dermatan sulfate proteoglycan 3 (Deere et al. 1996) or Pg-Lb (Kurita et al. 1996), is expressed in the growth plate and articular cartilage of developing bones (Johnson et al. 1997). The protein levels in cartilage decrease with maturation (Wilson et al. 2012), and quantitative proteomic investigations of adult human tissues show epiphycan in rib and tracheal cartilages, but not in articular cartilages (Önnerfjord et al. 2012). This proteoglycan carries a DS chain in its N-terminal region. Mice lacking epiphycan appear normal at birth but fall behind wild-type controls in postnatal body weight and bone length growth (Nuka et al. 2010). The epiphycan-deficient mice also develop spontaneous osteoarthritis. In epiphycan/biglycan double-knockout mice, the phenotype is aggravated (Nuka et al. 2010).

Mimecan, also referred to as osteoglycin, is widely expressed in connective tissues, including cartilages. In cornea, it carries a KS chain on its N-terminal extension, whereas mimecan from other tissues appears to be glycoproteins with unsulfated oligolactosamine glycans (Funderburgh et al. 1997). Proteomic investigations have verified mimecan presence in various different cartilages (Önnerfjord et al. 2012), although its function in these tissues remains unclear. Mice lacking mimecan show no overt phenotype but have decreased skin tensile strength and increased skin collagen fibril diameter (Tasheva et al. 2002).

Opticin was identified in the eye (Friedman et al. 2000; Hobby et al. 2000; Reardon et al. 2000) and has been reported present in cartilage (Monfort et al. 2008; Tio et al. 2014). Unlike other SLRPs, opticin contains an N-terminal mucin-like domain carrying up to 16 sialylated O-linked glycans (Reardon et al. 2000). Opticin likely binds collagen as it was purified from vitreous collagen fibril preparations (Reardon et al. 2000) and also binds HS and CS chains (Hindson et al. 2005).

1.4.4 Class IV SLRPs

The class IV SLRPs chondroadherin and chondroadherin-like diverge from the canonical SLRPs in their LRR sequence and have LRRCT C-terminal cysteine motifs instead of the LRRCE of canonical SLRPs (Park et al. 2008). Class IV SLRPs do not carry GAG chains but nevertheless have important functions in the cartilage ECM.

Chondroadherin was identified as a cartilage ECM protein mediating cell adhesion (Sommarin et al. 1989). Chondroadherin has no N-terminal extension but contains a unique C-terminal end with an additional disulfide bond, a heparin-binding sequence, and a C-terminal loop structure (Neame et al. 1994). Chondroadherin binds fibrillar collagens (Månsson et al. 2001). In addition, chondroadherin is an integrin ligand (Camper et al. 1997), binding integrin α2β1 through the amino acid sequence WLEAK in its C-terminal loop structure (Haglund et al. 2011). Interestingly, the chondroadherin heparin-binding sequence is a syndecan ligand, and chondroadherin interaction with syndecan and integrin in concert is required for firm attachment, cell spreading, and formation of focal adhesion structures (Haglund et al. 2013). The heparin-binding sequence can be released from chondroadherin by proteolysis (Neame et al. 1994). Synthetic cyclic peptides corresponding to the chondroadherin integrin binding site have been shown to inhibit osteoclastogenesis and bone resorption (Capulli et al. 2014) and to inhibit bone metastasis of breast cancer cells (Rucci et al. 2015).

Mice lacking chondroadherin were viable and fertile without macroscopic phenotype, but the chondroadherin knockout mice showed thinner cortical bone, changed trabecular bone architecture, and decreased bone mechanical strength, and proteomic analysis revealed disturbed cartilage homeostasis (Hessle et al. 2013). Further nanomechanical analysis showed significant changes in the superficial zone cartilage of chondroadherin deficient mice (Batista et al. 2014).

Chondroadherin-like, a novel SLRP expressed in cartilage, was recently characterized (Tillgren et al. 2015). This atypical SLRP has a tandem arrangement of two LRRNT and LRRCT flanked LRR domains with homology to chondroadherin, linked by an arginine and proline-rich linker. Chondroadherin-like binds collagen and inhibits collagen fibril formation. In contrast to chondroadherin, chondrocytes do not adhere to chondroadherin-like. Chondroadherin-like appears to be involved in the regulation of chondrogenic differentiation. It is expressed in the cartilage anlagen and growth plate, and chondroadherin-like knockdown accelerated ATDC5 chondroprogenitor cell differentiation.

1.5 Conclusions and Perspectives

Detailed molecular and biomechanical studies combined with gene-targeted animal models have steadily improved our understanding of proteoglycan functions as structural components of the ECM, in regulation of ECM assembly, and as regulators of different growth factor systems. A growing number of studies now show that

proteolytic fragments of proteoglycans have bioactive properties. Proteoglycans present in cartilage also act as regulators of innate immunity through TLR and complement interactions and thus can regulate inflammatory responses in the joint and osteoarthritis progression.

Technological advances in mass spectrometry and proteomics are rapidly improving our ability to detect and quantify proteins including proteoglycans. This will allow quantitative study of spatiotemporal distributions and possibly interactions of proteoglycans in different tissue compartments, during development and in disease progression. The molecular architecture of cartilage is surprisingly complex, with different cellularity and collagen fibril dimensions and orientation at different depths from the joint surface and with the ECM organized into pericellular, territorial, and interterritorial zones around each chondrocyte (Heinegård and Saxne 2011). The SLRPs are key regulators of collagen matrix assembly, and determining the regional ECM composition will help to clarify the combinatorial effects of different regulators on collagen fibril formation. Proteomic approaches also allow identification of proteolytic cleavage sites and neoepitopes resulting from tissue degradation, as well as protein fragments that may show bioactivity. Indeed, novel cartilage proteoglycans or part-time proteoglycans may even be identified (Noborn et al. 2015). In parallel, genetic studies will likely reveal novel proteoglycan gene variants with linkage to hereditary skeletal disorders, which may be instructive in elucidating normal function as well as disease mechanisms in common with nonhereditary joint disorders. Taken together, the understanding of cartilage proteoglycan function in ECM molecular organization, proteoglycan turnover, and function as signaling molecules regulating, e.g., innate immunity, can be expected to increase substantially in the near future. This will most likely provide novel diagnostic tools and therapeutic targets in joint disease.

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Collagens in Hyaline Cartilage

2

Susanne Grässel

Abstract

Collagens are the most abundant proteins in mammals and mostly incorporated into the extracellular matrix (ECM) where they form complex supramolecular structures additionally incorporating different non-collagenous components. The ECM of articular cartilage comprises the majority of the tissue volume and thus determines the properties of this unique tissue. The fibrillar matrix – composed of cartilage collagens and associated macromolecules – is responsible for the integrity and stability of the cartilage ECM and determines the mechanical tissue properties, i.e., the load-bearing capacity. Besides the classical cartilage collagens II, IX, and XI, several other collagens were identified in the cartilage ECM during the past years. Among them are fibrillar collagens (collagens III, V, XXIV, and XXVII), network-forming collagens (collagens IV and X), filamentous collagens (collagen VI), and fibrillar-associated collagens with interrupted triple helices (FACITs XII, XIV, XVI, and XXII). Most of them are not specific for cartilage but are also components of many other connective tissue matrices and tissue junctions of the body, i.e., the skin, tendon, muscle, bone, and cornea, demonstrating a crucial redundancy of individual collagen function among different tissue types. This review summarizes information about the role of the different collagens in cartilage homeostasis and formation of fibrillar suprastructures. In addition, the impact of aberrant or loss of collagen expression in transgenic or collagen-knockout mouse models on cartilage matrix structure and physiology is described.

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2.1 Introduction

In the 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 articular 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 (for further information, see Chap. 1). Cartilage fibrils vary in their molecular organization, their width, and their orientation in the tissue in order to resist forces generated by external load. In adult articular cartilage, for example, thin fibrils near the joint cavity preferentially run parallel to the surface, since lateral forces predominate in this region. In the interterritorial regions of the deep zones, in contrast, wider fibrils are arranged perpendicularly to the surface to strengthen the tissue in the direction along the axis of the bones. A prominent feature of the fibrils in developing and in immature cartilage is their strictly uniform diameter of about 20 nm and their more random orientation.

The pioneering work of Benninghoff established the concept that collagen fibrils are oriented vertically in the deeper layers of articular cartilage (Benninghoff 1925), twisting into arches at the intermediate layers and assuming a horizontal disposition in the superficial layer. Benninghoff's concept has ever since received wide support especially by investigators using polarization microscopy.

Collagen accounts for about two-thirds of the dry weight of adult articular cartilage. The tissue's matrix strength depends on the extensive cross-linking of the collagen fibrils and the apparent zonal changes in fibrillar architecture with tissue depth. Once the fibrillar network is formed during development, there appears to be little capacity for articular chondrocytes to recapitulate the overall collagen architecture if the mature tissue is injured or undergoes degenerative changes as in osteoarthritis.

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, and Chap. 1). In order to warrant tissue stability, a mutual interaction of the fibrillar and extrafibrillar supramolecular compartments is required. The fibril surface is densely populated with several types of macromolecules 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 (for further information, see Chap. 3). Disruption of these macromolecular networks or the components that bridge them compromise the mechano-stability of cartilage and underlies a broad spectrum of inherited skeletal dysplasias (Newman and Wallis 2003; Warman et al. 2011) (Fig. 2.1).

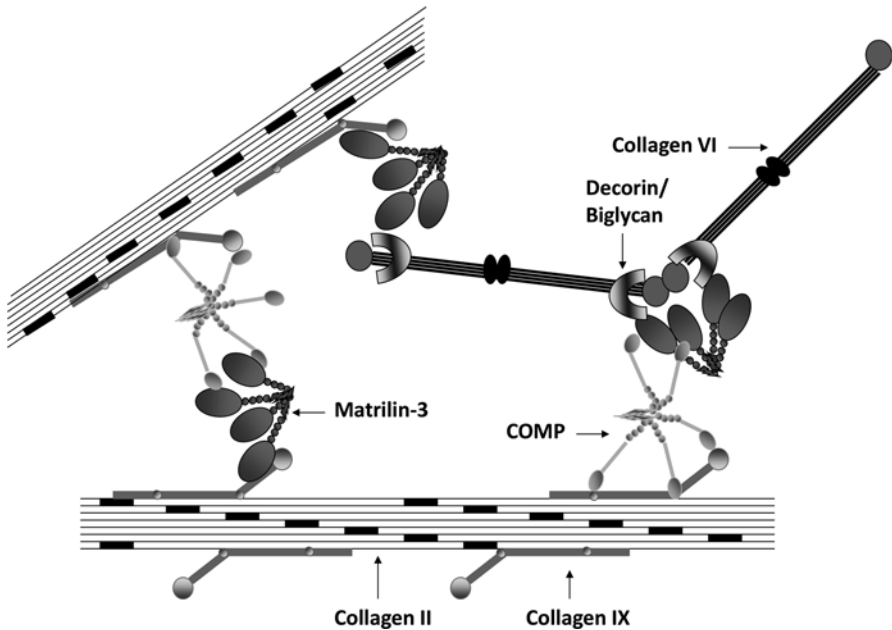

Fig. 2.1 Model for supramolecular assembly of cartilage fibrils and filaments into fibrillar networks. Matrilin-3 and COMP act as adapter molecules to interconnect D-periodically banded fibrils with each other and/or with collagen VI-beaded filaments to generate a heterotypic fibrillar network. The interaction may be mediated either by matrilin-3 binding directly to collagen IX or via COMP, which associates with the non-collagenous domains of collagen IX. Matrilin-3 contacts to collagen VI filaments are mediated by the small proteoglycans decorin and biglycan, which preferentially form complexes in the vicinity of the N-termini of collagen VI filaments themselves acting as adapter molecules (Adapted from Budde et al. (2005))

In mammalian articular cartilage, the primary classical collagen components (collagens II, IX, and XI) do not appear to alter dramatically in proportion between zones. (In birds, collagen I predominates at the articular surface and decreases with depth in an interchanging gradient with collagen II (Eyre et al. 1978).) The greatest quantitative difference occurs with maturation from the exclusively fine fibrils of young growth cartilages (≥10 % collagen IX, ≥10 % collagen XI, ≤80 % collagen II) to the thicker and more varied fibril diameters of mature articular cartilage (~1 % collagen IX, ~3 % collagen XI, ≥90 % collagen II) (Eyre 2002).

Many excellent recent reviews illustrate composition and diversity of the collagen family and their structural organization, biosynthesis, and degradation (Ricard-Blum 2011; Ricard-Blum and Ruggiero 2005; Exposito et al. 2010; Myllyharju and Kivirikko 2004; Heino 2007). For that this is not a major subject of this review which focuses on classical (collagens II, IX, X, and XI), nonclassical (collagens III, IV, V, VI, XII, XIV, and XVI), and novel (collagens XXII, XXIV, and XXVII) cartilage collagens and their impact on cartilage matrix structure, integrity, and function.

2.2 Classical Hyaline Cartilage Collagens

2.2.1 Collagen II

Collagen II, a fibrillar collagen, is a homotrimer of three α1(II) chains encoded by a single gene, COL2A1 (Figs. 2.1 and 2.2).

The α3(XI) chain of the heterotrimeric collagen XI is also a product of *COL2A1*, but it undergoes different posttranslational modifications (Furuto and Miller 1983). Collagen II is predominantly localized to cartilage, where its expression is high and maintained throughout life. It is, however, also expressed in several other tissues, including notochord, fetal brain, and developing heart and eye (von der Mark et al. 1977; Cheah et al. 1991; Wood et al. 1991). Complete absence of collagen II is not compatible with life, and most mutations in the human *COL2A1* gene lead to osteochondrodysplasias, a diverse group of disorders affecting not only cartilage homeostasis but also skeletal development, and cause severe bone dysplasias (for review, see Vikkula et al. (1994) and Kannu et al. (2012)). The phenotype of these diseases ranges from mild early-onset osteoarthritis to perinatal lethality. The mutations giving rise to the most severe disorders are inherited in a dominant fashion, cause disproportionate dwarfism (characterized by the disorganization of cartilaginous matrices and epiphyseal growth plates), and are often lethal. Chondrocytes from patients suffering from lethal achondrogenesis type II and hypochondrogenesis synthesize abnormal collagen II, which is not secreted into the extracellular matrix

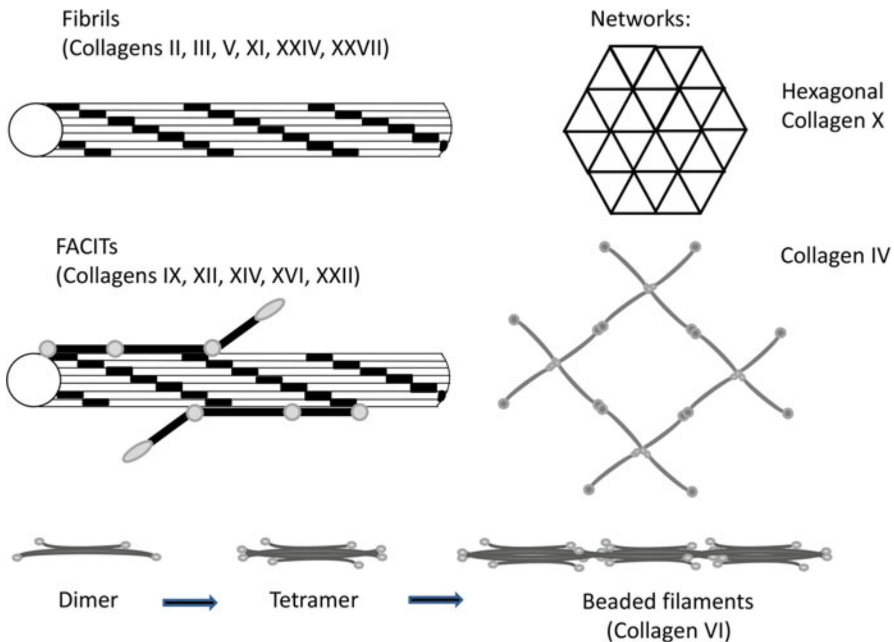

Fig. 2.2 Supramolecular assemblies formed by cartilage collagens

(Table 2.1). The absence of collagen II in cartilage tissue is associated with the deposition of collagens I and III, with collagen I not expressed by normal hyaline cartilage (Chan et al. 1993; Mundlos et al. 1996). In addition, simply the aging process plus degenerative diseases as osteoarthritis affect collagen II deposition into the articular cartilage ECM (Lorenz et al. 2014) (Fig. 2.3).

Cartilage fibrils are often referred to as collagen II fibrils, since this protein primarily occurs in the cartilage, where it is an abundant structural component. The

Table 2.1 Cartilage collagens

Collagen type	Alpha chains	Gene (mutations)	Hereditary diseases
Collagen II	α1(II)	*COL2A1*	Spondyloepiphyseal dysplasia Spondyloepimetaphyseal dysplasia Kniest dysplasia, Stickler syndrome, achondrogenesis, hypochondrogenesis
Collagen III	α1(III)	*COL3A1*	Ehlers-Danlos syndrome
Collagen V	α1(V), α2(V), α3(V), α4(V)[a]	*COL5A*, *COL5A2*	Ehlers-Danlos syndrome
Collagen IV	α1(IV) to α6(IV)	*COL4A1* *COL4A3* *COL4A4* *COL4A5* *COL4A6*	Familial porencephaly Alport syndrome Benign familial hematuria Alport syndrome Leiomyomatosis
Collagen VI	α1(VI), α2(VI), α3(VI), α4(VI)[b], α5(VI)[c], α6(VI)	*COL6A1* *COL6A2* *COL6A3*	Bethlem myopathy Ulrich congenital muscular dystrophy
Collagen IX	α1(IX), α2(IX), α3(IX)	*COL9A1* *COL9A2* *COL9A3*	Multiple epiphyseal dysplasia Multiple epiphyseal dysplasia Autosomal recessive Stickler syndrome
Collagen X	α1(X)	*COL10A1*	Schmid metaphyseal chondrodysplasia
Collagen XI	α1(XI), α2(XI), α3(XI)[d]	*COL11A1* *COL11A2*	Stickler syndrome, Marshall syndrome Stickler syndrome, Marshall syndrome Otospondylomegaepiphyseal dysplasia, deafness
Collagen XII	α1(XII)	*COL12A1*	Ehlers-Danlos like syndrome Anterior cruciate ligament ruptures
Collagen XIV	α1(XIV)	*COL14A1*	Punctate palmoplantar keratoderma
Collagen XVI	α1(XVI)	N/A	N/A
Collagen XXII	α1(XXII)	N/A	N/A
Collagen XXIV	α1(XXIV)	N/A	N/A
Collagen XXVII	α1(XXVII)	N/A	N/A

[a]The α4(V) chain is restricted to Schwann cells
[b]The α4(VI) chain is not expressed in humans
[c]The α5(VI) chain is known as α1(XXIX)
[d]The α3(XI) chain is identical to the α1(II) chain but is differentially processed posttranslational

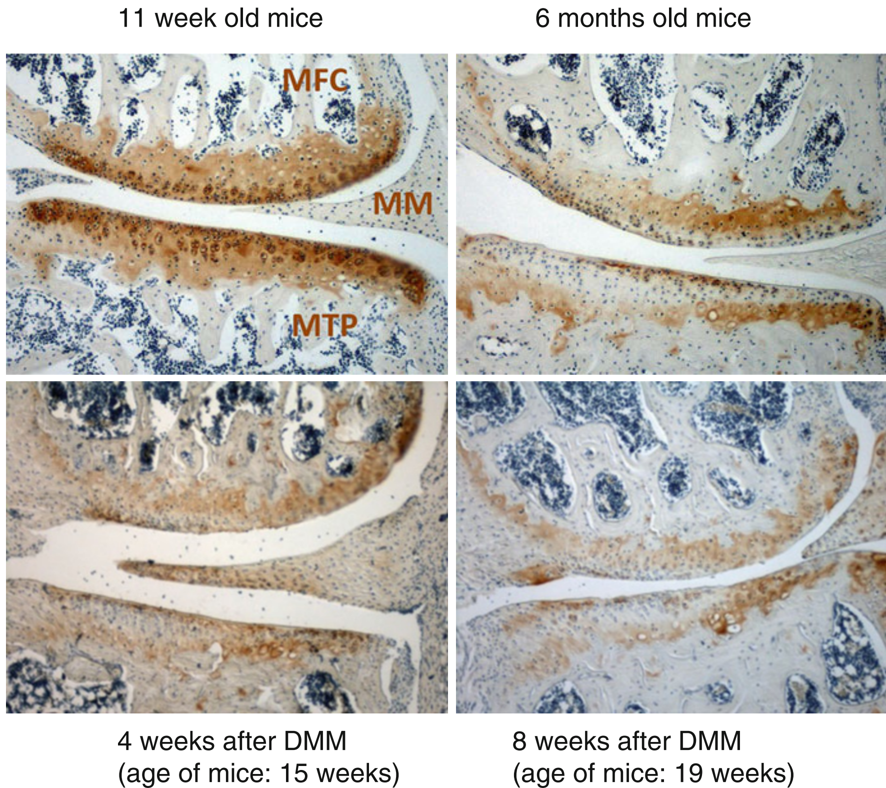

Fig. 2.3 Localization of collagen II in murine knee joints. Frontal sections of right knee joints from wild-type mice of 11 weeks and 6-month-old animals and of mice 4 and 8 weeks after surgical osteoarthritis induction using the DMM method were stained with an antibody against collagen II. Collagen II-positive cell numbers and collagen II-positive articular cartilage area (brown colour) decrease during aging and after surgical induction of OA. *MFC* medial femoral condyle, *MTP* medial tibial plateau, *MM* medial meniscus, *DMM* destabilization of the medial meniscus

biochemical composition of cartilage fibrils, however, is not only far more complex but also is nonuniform. Their molecular heterogeneity correlates well with morphological and functional diversity. Thinner fibrils, such as those of fetal hyaline cartilage, contain not only collagen II but also collagen XI (Mendler et al. 1989; Keene et al. 1995) as well as collagen IX (Hartmann et al. 1983; Muller-Glauser et al. 1986; Vaughan et al. 1988), which can occur in proteoglycan form carrying a single dermatan sulfate chain. Recently, the macromolecular alloy concept for cartilage fibril composition was developed in the Bruckner lab (Blaschke et al. 2000). Later, Hansen and Bruckner studied co-assembly of collagens I and XI, which coexist in fibrils of several normal and pathologically altered tissues, including fibrous cartilage and bone, or osteoarthritic joints. Collagen I/XI fibrils strikingly differed from the homogeneous fibrillar alloy generated by collagens II and XI, although the constituent polypeptides of collagens I and II are highly homologous. The authors

concluded that the mode of aggregation of collagens into vastly diverse fibrillar composites is finely tuned by subtle differences in molecular structures through formation of macromolecular alloys (Hansen and Bruckner 2003). Other matrix suprastructures also contain more than one molecular constituent. Thus, other extracellular matrix aggregates, including basement membranes and microfibrils containing fibrillins (Sherratt et al. 2001), collagen XVI (Kassner et al. 2003), or collagen VI (Wiberg et al. 2003; Wiberg et al. 2002), might be macromolecular alloys as well. It is even tempting to speculate that the concept applies to biological aggregates in general and, thus, may help to explain tissue-specific functions (Hansen and Bruckner 2003). It will be interesting to investigate from this point of view biological suprastructures such as chromatin, transcriptional machineries, the various forms of the cytoskeleton, ribosomes, proteasomes, multienzyme complexes, or lipid rafts.

Just over 100 different human *COL2A1* mutations have been described and are listed in the *Cardiff University Human Gene Mutation Database* (http://www.hgmd.org). Work with transgenic mouse models confirmed the importance of collagen II for endochondral ossification during embryonal skeletogenesis and its role in the pathology of heritable skeletal disorders (for review, see Aszodi et al. (1998b)). Mice overexpressing mutant forms of collagen II display severe or mild chondrodysplasias, depending on the nature of the mutation and the genetic background of the mouse strain. Skeletal development was severely compromised by overexpression of normal collagen II in mice. In young animals with this genotype, growth cartilage was highly disorganized and, notably, contained abnormally wide fibrils with a strong banding pattern. This pointed toward a crucial importance of the correct molar proportions of cartilage collagens in fibrillogenesis (Garofalo et al. 1993). A study of transgenic mice expressing a dominant-negative collagen II deletion mutation (Del1) described that these mice, along with the previously reported skeletal abnormalities, also suffered from abnormal spinal development (Savontaus et al. 1997). The most severe phenotype is observed in mice carrying a null mutation in the *Col2a1* gene (Li et al. 1995a). They develop a phenotype resembling human achondrogenesis type II, die around birth, have cleft palates, and have gross morphological and histological malformations in their endoskeleton. The long bones are shortened, contain a thickened cortical collar, and lack endochondral bone and epiphyseal growth plate; the vertebral arches are rudimentary and do not fuse. In addition, *Col2a1*-null mice are unable to dismantle the notochord. This defect is associated with the inability to develop intervertebral disks (IVDs) (Aszodi et al. 1998a). Previous studies showed that in both *Col2a1*-null mice and in a patient with achondrogenesis type II carrying a *COL2A1* mutation collagen II is completely absent in the cartilage, whereas collagen I was found to be ectopically expressed in the cartilage (Aszodi et al. 1998a; Chan et al. 1995). Recently, it was demonstrated that chondrocytes after collagen II knockdown via siRNA synthesized a substantial amount of collagen I. Analysis of single cells revealed that either collagen I or collagen II but never both collagen types were simultaneously expressed by individual chondrocytes (Xin et al. 2015). In general, this shift in the expression of

collagens is interpreted as an attempt to compensate for the loss of collagen II. However, the underlying mechanism for the collagen type switch is not fully clarified up to now.

The avascular nature of cartilage is a unique tissue property in mammalians, and consequently several anti-angiogenic and antitumorigenic molecules have been identified in the cartilage (see review Patra and Sandell (2012)). In this context, a cleavage product of collagen II with anti-angiogenic and antitumorigenic properties has been described recently. Collagen II is synthesized in two splice forms, IIA and IIB, which differ in the inclusion of exon 2, encoding a 69 amino acid von Willebrand factor C domain in the collagen IIA procollagen NH2-propeptide (Ryan and Sandell 1990). Procollagen IIA (PIIANP) is synthesized in many embryonic tissues, while procollagen IIB (PIIBNP) is primarily restricted to adult cartilage. In the course of investigating the functional roles of the collagen II NH2-propeptides, Wang et al. found that recombinant PIIBNP was able to eradicate selected types of cells, while PIIANP was never able to eliminate cells (Wang et al. 2010). Their initial studies focused on the ability of PIIBNP to destroy tumor cells. It was shown that PIIBNP inhibited tube formation in HUVEC cells, inhibited aortic outgrowth, and reduced bone resorption in a calvarial bone model by specifically killing osteoclasts but not osteoblasts (Sandell 2014). Binding to integrins avß3/ß5 is crucial for the function of PIIBNP, the main reason why cartilage is protected from the deleterious action of the propeptide as chondrocytes do not express these integrin subunits. These results suggest that PIIBNP may function to maintain the avascularity of cartilage and protect it from tumor cell invasion.

In studies with the *Col2a1*$^{+ex2}$ mice, it was shown that procollagen IIA was repressed in adult articular cartilage tissue (McAlinden 2014). Inhibition of *Col2a1* alternative splicing is apparently well tolerated in these mice during cartilage development, and procollagen IIA appears to compensate quite well for the absence of procollagen IIB. Chondrocytes in human and mouse osteoarthritic cartilage have been found to reexpress procollagen IIA; however, procollagen IIA expression by differentiated chondrocytes in normal mature murine articular cartilage suggests that IIA synthesis may not be associated with disease. Instead, it is possible that during normal matrix turnover in postnatal cartilage, rates of procollagen II synthesis are low enough to permit inclusion of exon 2 and synthesis of procollagen IIA. The author speculates that expression of procollagen IIA in non-diseased, mature articular cartilage may be a mechano-regulated response.

Recently, two novel splice variants of collagen II have been identified: isoforms IIC and IID which were found in chondroprogenitor cells (IIC, IID) and in differentiated chondrocytes (IIC only) (McAlinden 2014).

2.2.2 Collagen IX

Collagen IX, a heterotypic collagen belonging to the FACIT family, is referred to as a minor collagen comprising only 1 % of total collagen in adult articular cartilage (Martel-Pelletier et al. 2008). It is assembled from α1(IX), α2(IX), and α3(IX)

polypeptide chains and is a flexible molecule containing rodlike, 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 antiparallel fashion (Eyre et al. 2004). Its N-terminal triple helical domain 3 (COL3) and non-collagenous domain 4 (NC4) point away from the body toward 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) (Figs. 2.1 and 2.2). In adult tissues, collagen IX is present in a subpopulation of thin fibrils preferentially occurring 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 (Muller-Glauser et al. 1986; Hagg et al. 1998). Collagen IX stabilizes these 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 (Fässler 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). *Col9a1*-deficient mice develop an OA-like joint phenotype starting 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 α2β1 and α5β1, 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). The prenatal and perinatal organization of the growth plate is profoundly disturbed in collagen IX-deficient newborn and growing mice; 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 with profound irregularities in proteoglycan content. Furthermore, the formation of

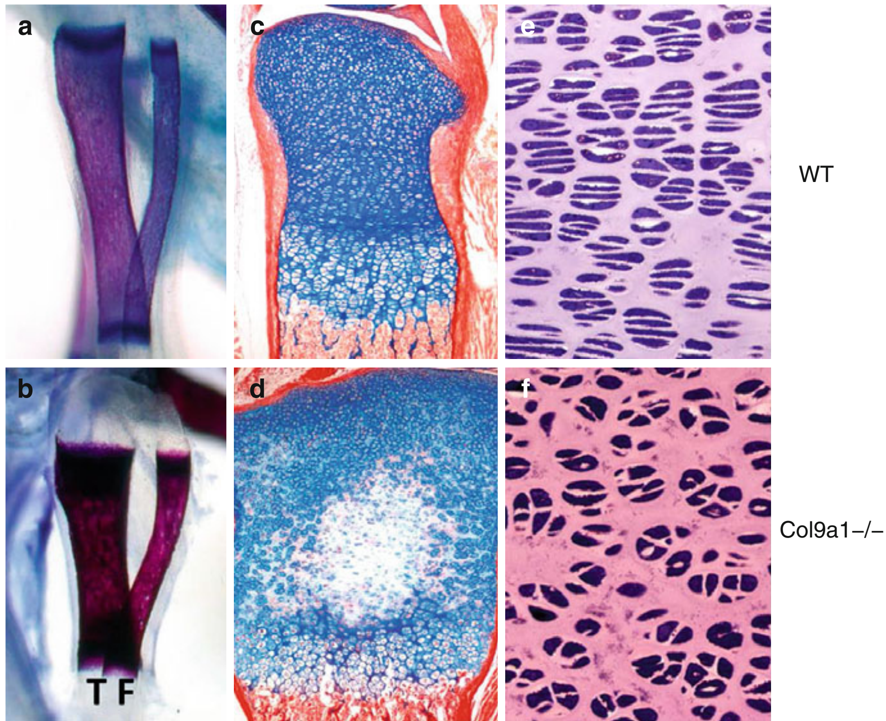

Fig. 2.4 Skeletal defects in collagen IX knockout mice. (**a**, **b**) show long bones of newborn *Col9a1*-deficient and WT mice stained with alizarin red. The tibia and fibula of *Col9a1*-deficient mice were shorter and broader compared to WT bones. (**c**, **d**) Weigert's hematoxylin/alcian blue/alizarin red-stained sections of tibia epiphysis from P0.5 newborn mice. The most prominent findings are (1) occurrence of a hypocellular area in the center of epiphyseal cartilage extending into the hypertrophic zone at later stages, (2) irregular alcian blue staining of the epiphyseal cartilage representing a disturbed glycosaminoglycan distribution, and (3) compromised formation of proliferation columns perpendicular rather than parallel to the long axis of the bone. All skeletal defects of knockout animals become attenuated with age. (**e**, **f**) Toluidine blue-stained semi-thin sections of tibiae from newborn wild-type (**e**) and *Col9a1*$^{-/-}$ mice (**f**) demonstrate profound changes in the organization of chondrocytes in the proliferative zone of *Col9a1*$^{-/-}$ animals. The chondrocytes occur in isogenous groups, indicating proliferation, but flattening of cells and arrangement to columns failed (Adapted from Dreier et al. (2008)). *T* tibia, *F* fibula, *WT* wild type

the typical proliferation columns in the growth plates is grossly disturbed. Within the hypertrophic zone, the cells are enlarged, but less densely packed and surrounded by more extracellular matrix with unusually irregular cell layers. Presumably for this reason, the border between proliferative and collagen X producing hypertrophic cells is poorly defined in older *Col9a1*-deficient animals. These abnormalities cause a markedly reduced length of long bones at early postnatal age, while the width is significantly increased (Dreier et al. 2008; Blumbach et al. 2008) (Fig. 2.4). A comparative proteomic analysis of costal cartilage from newborn *Col9a1*-deficient mice revealed 15 ECM proteins as differentially expressed to rib cartilage from WT controls (Brachvogel et al. 2013). Collagen IX deficiency is associated with dramatically reduced COMP and matrilin-3, consistent with known interactions. Matrilin-1,

matrilin-4, epiphycan, and thrombospondin-4 levels were reduced in collagen IX null cartilage, providing the first in vivo evidence for these proteins to belong to the collagen IX interactome. In addition, the more widespread expression of collagen XII in the collagen IX-deficient cartilage suggests an attempted compensatory response to the absence of collagen IX. The authors identified a cohort of differentially abundant proteins in mouse cartilage lacking collagen IX, a key component of the perifibrillar protein adaptor complex which underscores the importance of an intact fibrillar network to maintain matrix integrity.

In addition to the model, where the entire α1 (IX) chain is deleted, a transgenic model harboring 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 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 disk 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 disks and end plates 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-month-old *Col9a1*-deficient mice. The vertebral end plates 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 as comparable irregularities have not been described for the mice harboring a mutated collagen IX.

A recently accepted paper by Kamper et al. clearly demonstrated that vertebral bodies of newborn collagen IX-deficient mice were smaller and showed an increased mineral density compared to wild-type animals (Kamper et al. 2015). At birth, the lack of collagen IX led to a disrupted cellular organization in the cartilaginous end plate and to a smaller nucleus pulposus of the IVD. Expression levels and localization of other extracellular matrix proteins were strongly altered accompanied by a softening of cartilaginous tissues. In older animals, absence of collagen IX caused earlier and more pronounced disk degeneration with annular fissures. The absence of collagen IX induces early developmental, structural and biomechanical alterations in both vertebral body and intervertebral disk which eventually cause severe degenerative changes in the aging spine (Table 2.1).

2.2.3 Collagen X

Collagen X is a non-fibrillar, network-forming short chain collagen (Fig. 2.2) found predominantly in fetal hypertrophic cartilage in the epiphyseal growth zone of long bones, ribs, and vertebrae (Schmid et al. 1990, 1991; Schmid and Linsenmayer

1985). Its supramolecular structure is quite unique resembling hexagonal networks which are only described for collagen VIIII until yet. Initially thought that collagen X is restricted to the hypertrophic zone of the growth plate at the sites of endochondral ossification (Kirsch and von der Mark 1991, Reichenberger et al. 1991, and Chap. 5), collagen X transgenic and knockout models reveal a link between endochondral ossification and hematopoiesis (Sweeney et al. 2011). Surprisingly, collagen X has been identified in adult human and porcine articular cartilage. In articular cartilage, collagen X has been localized mostly to the zone of calcified, mature cartilage (Gannon et al. 1991), whereas expression of collagen X in noncalcified articular cartilage has been linked with osteoarthritis. There, collagen X was observed around proliferating and maturating chondrocytes in the upper and middle zones (Walker et al. 1995; von der et al. 1992) and during osteophyte formation (Hoyland et al. 1991). Osteoarthritic lesions often involve phenotypic changes into hypertrophic chondrocytes; however, in lesion-free cartilage areas expressing collagen X, the cells do not show signs of a hypertrophic phenotype. Similar findings have been reported in mice upon aging (van der Kraan et al. 2001) or after anterior cruciate ligament transection in rats (Stoop et al. 2001). This may be an indication that the increase in collagen X would be an attempt to modulate the properties of collagen fibrils, rather than being related to phenotypic conversion. Of note, Lammi et al. reported that collagen X appears to be a normal constituent in the canine articular cartilage and shows a site-dependent expression in the canine knee (stifle) joint. Its greatest presence, at 50–100-nm depth from the surface, may involve a modification of collagen fibril arrangement at the site of collagen fibril arcades, possibly providing extra support to the collagen network (Lammi et al. 2002).

Notably, increased deposition of collagen X in articular cartilage was observed during aging in mice (van der Kraan et al. 2001). In young mice, collagen X was contained in the noncalcified cartilages of the tibio-femoral joints, but collagen X expression is almost absent in the noncalcified cartilage of the patellofemoral joints at this age. In old mice, strongly increased expression is observed in the patellofemoral joints but not in the tibia-femoral joints. However, the authors concluded that collagen X expression and spontaneous OA in mice are not necessarily related since OA prone locations in the murine knee joint do not preferentially express collagen X.

2.2.4 Collagen XI

The cartilage version of collagen XI – which is almost specific for cartilage – is found in developing cartilage as a heterotrimeric molecule of two novel collagen gene products (α1(XI) and α2(XI)) and a third chain (α3(XI)) identical in primary sequence to α1(II)B, the common form of the splicing variant of the adult collagen II gene. Isolated from mature articular cartilage, the collagen XI fraction contains α1(V) and α1(XI) chains in roughly equal amounts. The α1(V) chain appears to occur in hybrid molecules together with α1(XI) and/or α2(XI) rather than in typical collagen V molecules found in non-cartilaginous tissues (Eyre 2002) (Fig. 2.2). The biological significance of this is unknown (see below under Sect. 2.3.3).

Overexpression of normal collagen II resulted in highly disorganized growth cartilage and abnormally wide fibrils with a strong banding pattern (Garofalo et al. 1993). A similar disorganization of the cartilage matrix with uncharacteristically thick fibrils as in collagen II overexpressing mice was seen in the cho/cho strain of mice (Seegmiller et al. 1971; Li et al. 1995b). These animals are deficient in the expression of α1(XI) polypeptides with a distinct cartilage phenotype containing abnormal fibrils with impaired diameter control. Comparable cartilage abnormalities were caused by point mutations in the human genes *COL11A1* and *COL11A2* in several families with Stickler syndrome, a severe form of chondrodysplasia (Table 2.1). The mutations resulted in amino acid substitutions for glycine residues in the α1(XI) or α2(XI) chains, respectively, that, in turn, led to an absence or a functional incompetence of collagen XI. An exon-skipping mutation in the *COL11A2* gene, presumably causing production of shortened α2(XI) chains, had similar consequences (Richards et al. 1996; Vikkula et al. 1995). However, chondrodysplasia (cho) is a 1-nt deletion mutation in the orthologous mouse *Col11a1* gene that causes a frameshift and premature termination, resulting in a severely truncated mutant α1(XI) collagen polypeptide (Li et al. 1995b). cho is thought to be a functional null allele since fibrillar collagens initiate triple helix assembly via their carboxyl propeptide domains, which is deleted in the cho polypeptide. Homozygous cho/cho mice are born with severe chondrodysplasia and cleft palate and die soon after birth.

Heterozygous cho/+ mice have a much less severe phenotype characterized by age-dependent osteoarthritis, demonstrating that *Col11a1* haploinsufficiency has a deleterious effect on articular connective tissue. The diameter of collagen fibrils in articular cartilage of knee joints from heterozygous cho/+ mice was increased relative to that in control cartilage, and histologic analysis showed OA-like degenerative changes in knee and temporomandibular (TM) joints, starting at the age of 3 months which became more severe with aging (Xu et al. 2003). At 3 months, protein expression for MMP-3 was increased in knee joints from cho/+ mice. Moreover, tensile stiffness in articular cartilage of knee joints from cho/+ mice was moderately reduced and was inversely correlated with the increase in articular cartilage degeneration. Heterozygosity for a loss-of-function mutation in *Col11a1* results in the development of OA in the knee and TM joints of cho/+ mice. Morphological and biochemical evidence of OA appears to be preceded by significant mechanical changes, suggesting that the cho mutation leads to OA through a mechanism that does not initially involve mechanical factors. Typically, recessive disorders of *COL11A1* are severe and lethal in the perinatal period, while dominant conditions are milder and nonlethal (Table 2.1). Recently, Hufnagel et al. described a novel heterozygous mutation in *COL11A1* and a severe skeletal dysplasia leading to a dominant disease that was lethal, yet not during the perinatal period (Hufnagel et al. 2014). Recently, genome wide association studies (GWAS) identified a single nucleotide polymorphism (SNP) rs2615977 to be associated with osteoarthritis (OA) and located in intron 31 of *COL11A1*, comprising a strong candidate gene for this degenerative musculoskeletal disease. However, a study by Raine et al. did not provide evidence for a correlation between the OA-associated SNP rs2615977 and *COL11A1* allelic expression imbalance (Raine et al. 2013). The lack of a correlation

between the OA-associated SNP rs2615977 and *COL11A1* expression implies that the association marked by this SNP is operating by a route other than an effect on the expression of the gene in mature cartilage.

The exclusive association of collagen XI with thin cartilage fibrils was observed by immunoelectron microscopy (Keene et al. 1995). Thus, several lines of circumstantial evidence suggest that collagen XI is essential for the regulation of the lateral fibril growth (Li et al. 1995b). Collagen XI could conceivably form an interconnecting, secondary filamentous network that provides links between fibrils and running within fibrils, not inconsistent with the current concept that collagen XI restricts the lateral growth of collagen II fibrils (see below). Clearly, the majority of the covalent links of collagen XI are type XI to type XI (Wu and Eyre 1995), and this fact needs to be accommodated in any workable model of fibril assembly (Eyre 2002). Key observation from a study in the Bruckner lab let to the proposal of a widely accepted model of heterotypic collagen II/IX fibrils as a biological alloy that is of help to visualize the structural requirements of lateral growth control (Blaschke et al. 2000). The authors demonstrated that heterotypic fibrils are limited to diameters of approximately 20 nm when formed from molar ratios of collagen II/XI of up to 8:1. This suggests that the bulky N-terminal domain of collagen XI is large enough to cover eight adjacent collagen II molecules on the fibril surface, thereby preventing further growth in diameter. In contrast to fibrils from immature cartilage, fibrils of adult joint cartilage are heterogeneous in diameter and composition. Blaschke et al. postulate that the control of lateral fibril growth is an intrinsic property of appropriate collagen mixtures not only during fibrillogenesis in vitro but also in cartilage tissue in vivo. The question then arises as to how the prototypic fibrils mature into the wider fibrils of the territorial zone of cartilage matrix. Conceivably, large banded fibrils could arise by accretion of collagen molecules onto 20-nm fibrils or by fusion of such fibrils. Evidence for fusion in situ may be seen in electron micrographs showing small fibrils merging into larger banded structures (Hunziker et al. 1997). Longitudinally sectioned collagen fibrils exhibit variations in thickness and kinking; they tend to align with their periodic banding in register and are frequently seen to split or fuse along their longitudinal course (Blaschke et al. 2000). The tendency of fibrils to form bundles is greater in deeper zones than in more superficial ones. This action could potentially be regulated by the presence of collagen IX on the surface of fibrils, which is consistent with the reduced content of collagen IX in mature fibrils.

2.3 Nonclassical Hyaline Cartilage Collagens

2.3.1 Collagen III

The fibril-forming collagen III is consistently detected by immunofluorescence in samples of normal and osteoarthritic human articular cartilage (Aigner et al. 1993; Wotton and Duance 1994) (Fig. 2.2). By electron microscopy, it has been found to co-localize with collagen II in the same banded fibrils and to retain its N-propeptide domain (Young et al. 2000b). Cross-linking studies confirm that collagen III is

copolymerized and linked to collagen II in human articular cartilage as a minor but regular component (Eyre 2002). This notion was corroborated in a study of the Aigner lab where collagen III was found in all cartilage subtypes partly co-distributed with collagens I and II (Wachsmuth et al. 2006). In articular cartilages, staining intensity was strongest in the transitional and the upper radial zones. In the deep radial zone, weak interterritorial staining co-occurred with a moderate pericellular/territorial staining. A recent study by Wu et al. confirms that significant amounts of collagen III are present in adult human articular cartilage cross-linked covalently to other collagen III molecules, suggesting their presence in the matrix as homotypic polymers of collagen III presumably in the form of fine filaments of head-to-tail cross-linked molecules (Wu et al. 2010). Their results show that collagen III molecules accumulate in mature human articular cartilage cross-linked to the surface of collagen II fibrils. The amount presumably varies between individual joints, anatomical location, and tissue microanatomy, perhaps dependent on the history of injuries and the wear and tear experienced by a normal joint during life. If so, the content will tend to increase with age. It has also been noted that as articular cartilage matures and ages, the collagen fibrils become thicker, and the content of collagens IX and XI decreases relative to collagen II (Eyre 2002).

In osteoarthritic cartilage, collagen III tended to be concentrated in the superficial and upper middle zones and to be synthesized by the chondrocytes in the absence of collagen I expression (Aigner et al. 1993, 1997). The covalent addition of collagen III to the polymeric matrix of articular cartilage of adult human joints suggests an active remodeling process. It occurs in both normal and osteoarthritic joints, but whether it signals pathological events or a healthy repair mechanism is not known (Eyre et al. 2006). However, it is tempting to speculate that collagen III is made by chondrocytes in addition to collagen II in response to matrix damage akin to the wound-healing role of collagen III in collagen I-based tissues. It is known that collagen III is prominent in fibrous repair tissue in the skin and other tissues (Lehto et al. 1985). Therefore, it seems likely that collagen III is synthesized also in the cartilage as a modifier of existing fibril networks in response to tissue and matrix damage.

2.3.2 Collagen IV

Collagen IV, a classical basement membrane network-forming collagen, has been recently identified in the pericellular matrix (PCM) of bovine and murine articular chondrocytes (Kvist et al. 2008) (Fig. 2.2). The authors identified the α1(IV) and α5 (IV) chains to be expressed particular in the pericellular matrix of chondrocytes in the superficial cartilage layer. Clusters of proliferating chondrocytes in human cartilage samples are positive for collagen IV which was rarely detected in the calcified cartilage zone (Foldager et al. 2014). These authors found diminished staining for collagen IV in degenerative human hyaline cartilage in contrast to its pronounced presence in healthy articular cartilage and meniscus chondrocytes. Both groups demonstrated strongest staining for collagen IV in the PCM of chondrocytes with

decreasing signals from the superficial chondrocyte layer to the calcified layer. In the light of these data, it is proposed that the chondrocyte, like several other cell types of mesenchymal origin, is surrounded by the functional equivalent of a basement membrane. However, it lacks the ultrastructural features of a typical basement membrane. This structure is presumably involved in maintaining chondrocyte phenotype and viability and may well allow a new understanding of cartilage development and provide clues to the progression of degenerative joint disorders (Kvist et al. 2008). Collagen IV is the only collagen detected in the cartilage which releases bioactive fragments by proteolytic cleavage of the NC1 domain as most matricryptins are derived from basement membrane collagens. All collagen chains α1(IV) to α6(IV) are involved in the production of different matricryptins. These fragments are involved in regulation of physiological and pathological processes such as development, angiogenesis, tumor growth and metastasis, and tissue repair (for review, see Ricard-Blum (2011) and Ricard-Blum and Ballut (2011)). However, it is not known up to now if cartilage collagen IV produces matricryptins and if so which types are released.

2.3.3 Collagen V

Collagen V belongs to the fibril-forming collagens comprising several isoforms that differ in chain composition (Fig. 2.2). They include the most abundant and widely distributed α1(V)α2(V)α3(V) isoform found mostly in placenta and the embryonic α1(V)3 homotrimer (Chanut-Delalande et al. 2004). Additionally, heterotypic collagen V/XI trimers have been identified in tissues like bone, vitreous, and cartilage. Collagen V/XI gene products are best considered as members of the same collagen subclass, although two gene clades contribute α-chains, clade A represented by α1(II) (i.e., α3(XI)) and α2(V) and clade B represented by α1(XI), α2(XI), and α1(V) (Boot-Handford and Tuckwell 2003).

Mutations in the genes encoding either the α1(V)4 or α2(V)5 chain can result in the human heritable connective tissue disorder, the classic Ehlers-Danlos syndrome (cEDS) (Table 2.1). Clinical hallmarks of which include skin hyperextensibility, atrophic scarring, and joint hypermobility, with patients also often presented with easy bruising and bleeding (see review De Paepe and Malfait (2012)).

In the cartilage, it was shown that the α1(V) chain becomes an integral fibril component, increasing in proportion at the expense of α2(XI) with increasing tissue maturity. In a reverse manner in bone, the α1(XI) chain accumulates with developmental age at the expense of α1(V) forming (α1(V)α1(XI)α2(V)) triple helical molecules (Niyibizi and Eyre 1989, 1994). Collagen XI from fetal cartilage consisted of (α1(XI)α2(XI)α3(XI)) heterotrimers, with little or no α1(V) detected (Wu and Eyre 1995). The collagen V/XI component of adult articular cartilage comprises four genetically distinct chains, α1(XI), α2(XI), α1(V), and α3(XI), assembled into at least two distinct heterotrimeric molecules, (α1(XI)α2(XI) α3(XI)) and (α1(XI)α1(V)α3(XI)). There is a shift in chain isotype usage as the

hyaline cartilage precursor matures postnatally. With increasing tissue maturity, the type XI collagen fraction contains more α1(V) and less α2(XI) in proportion to α1(XI) and α3(XI). In contrast, collagen V/XI from nucleus pulposus contains five genetically distinct chains, α1(XI), α2(XI), α3(XI), α1(V), and α2(V), which are distributed among several distinct heterotrimeric molecules. The findings support an evolving role for tissue-specific molecular variants of type V/XI collagen in regulating fibril form and function across vertebrate connective tissues during maturation (Wu et al. 2009).

2.3.4 Collagen VI

Collagen VI self-assembles into dimers and tetramers forming a characteristic and distinctive network of beaded microfilaments mostly found close around cells but also interspersed throughout the fibrillar matrix (Chang and Poole 1996). Collagen VI is formed by six genetically different chains (α1-α6 collagen VI) encoded by COL6A1-A6 genes (Lampe and Bushby 2005; Gara et al. 2008; Fitzgerald et al. 2008). In contrast to other collagens, collagen VI undergoes a complex multistep process of intracellular assembly into large aggregates before secretion (Furthmayr et al. 1983; Knupp et al. 2006) (Fig. 2.2).

Collagen VI is abundantly expressed in skin fibroblasts and has been extensively characterized in these cells and in skin tissue biopsies. However, collagen VI is also a component of cartilage tissues where it was first detected in costal and articular cartilage as well as in fibrocartilage where it is concentrated in the pericellular matrix of chondrocytes (Wu et al. 1987; Keene et al. 1988). Already at this time, it was suggested that collagen VI contributes significantly to the mechanical properties of connective tissues even though it constituted less than 1 % of the total cartilage collagen. The PCM is primarily characterized by the exclusive presence of collagen VI in normal cartilage, but it also possesses a high concentration of proteoglycans, fibronectin, and collagens II and IX (Poole et al. 1992, 1997). The functional role of the PCM in articular cartilage is still unknown, although the fact that it completely surrounds the cell suggests that it regulates the biomechanical, biophysical, and biochemical signals that the chondrocyte perceives (Guilak et al. 2006). In articular cartilage, collagen VI forms a network that anchors the chondrocyte to the PCM (Marcelino and McDevitt 1995; Sherwin et al. 1999) through its interaction with hyaluronan (Kielty et al. 1992), decorin (Bidanset et al. 1992), and fibronectin (Chang et al. 1997). A study by Wiberg et al. visualizes collagen VI microfibrillar networks, which are connected to collagen II fibrils by biglycan/matrilin-1 complexes as linkers (Fig. 2.1). These linkers also connect a number of procollagen molecules to the collagen VI scaffold. These may represent immobilized nucleation centers for collagen II fibril assembly. The authors suggest that it might be possible that a functional role of such collagen VI microfibril supramolecular assemblies is to act as scaffolds for the formation of the structurally critical fibrillar collagen networks. They assume a further role of the collagen VI network may be to present fibrillogenesis modulators such as *leucine-rich repeat* (LRR)

proteins in proximity to the growing fibrils. In this way, the formation of the collagen VI microfibrillar network in the early stages of tissue formation, or in repair processes such as wound and fracture healing, may play an important instructional role in tissue development, architecture, and homeostasis (Wiberg et al. 2003). Analysis of *Col6a1*-deficient mice revealed no gross morphologic differences between wild-type and collagen VI knockout chondrons other than reduced skeletal size of *Col6a1*$^{-/-}$ mice (Alexopoulos et al. 2009). Primarily, these findings show that mice lacking collagen VI exhibit accelerated development of hip osteoarthritis, as well as a delayed secondary ossification process and lower bone mineral density. Lack of collagen VI resulted in a loss of the stiffness (decreased modulus) of the PCM of the articular cartilage prior to any detectable histological changes. However, no differences in ECM properties were observed. These findings provide indirect evidence of a role for collagen VI in regulating the physiology of the chondrocyte, potentially due to alterations in the biological and mechanical environment of the chondrocytes in articular cartilage due to changes in biomechanical properties of the PCM or due to increased joint laxity associated with a deficiency in collagen VI.

With respect to novel functions, it was recently reported that soluble collagen VI stimulates chondrocyte proliferation in both adult and osteoarthritis chondrocytes. Additionally, collagen VI-expanded chondrocytes show a similar potential to untreated chondrocytes in engineered cartilage in 3D biomimetic hydrogel constructs. Soluble collagen VI might serve as a biological compound that can be useful for the expansion and utilization of scarce sources of chondrocytes, potentially for autologous chondrocyte implantation (Smeriglio et al. 2015). The observation by Alexopoulos et al. that collagen VI is ubiquitous present in the growth plate supports the hypothesis that collagen VI modulates cell proliferation as collagen VI deficiency seems to delay cell differentiation and proliferation, resulting in delayed skeletal development and decreased bone formation (Alexopoulos et al. 2009).

2.3.5 Collagens XII, XIV, and XVI

In addition to collagen IX, collagens XII, XIV, and XVI are members of the FACIT family (for review, see Ricard-Blum (2011) and Ricard-Blum and Ruggiero (2005)) of collagens.

2.3.5.1 Collagen XII

Collagen XII is widely expressed in collagen I containing mesenchymal tissues in the embryo, especially in developing bone, ligaments, tendons, fibrocartilage, smooth muscle, and skin (Walchli et al. 1994). Even though initially it was postulated that collagen XII, the largest member of the FACIT family, was absent from the cartilage, later collagen XII was detected in small quantities in bovine fetal cartilage (Watt et al. 1992) and human epiphyseal chondrocytes (Dharmavaram et al. 1998) (Fig. 2.2). Initially, collagen XII was shown to be associated with the

surface of collagen II fibrils in regions of cartilage where specific orientation of fibrils is observed (Keene et al. 1995). A mainly electron microscopic based study by Gregory et al. detected collagen XII in the growth plate located to hypertrophic chondrocytes and in developing articular cartilage (Gregory et al. 2001). Based on the distributions of collagens XI and XII, their data also suggested that during development, articular cartilage could be subdivided further into two zones. The superficial zone (one to three cells deep) is characterized by large amounts of collagen XII and little collagen XI, whereas the deeper zone (four to six cells deep) has more collagen XI and less collagen XII. In addition, collagen XII was distributed in areas of cartilage with more organized fibril orientation and may have a role in promoting alignment or stabilizing such an organization, thereby creating a matrix capable of withstanding load-bearing forces. Identification of collagen XII transcripts in mouse cartilage by in situ hybridization suggested that collagen XII protein localized in rat cartilage was synthesized by articular chondrocytes (Bohme et al. 1995). With its collagenous domain, collagen XII also binds decorin, fibromodulin (Font et al. 1996), and cartilage oligomeric matrix protein (COMP) (Agarwal et al. 2012), all of which are found on the surface of collagen fibrils. Hence, collagen XII and its binding partners could form flexible bridges between neighboring collagen fibrils that might function to absorb shear stresses upon loading (Chiquet et al. 2014).

A recently published in vitro study demonstrated that bovine chondrocytes secrete and accumulate collagen XII suggesting it may be necessary to provide a microenvironment that supports hyaline cartilage formation (Taylor et al. 2015). Using a collagen XII knockout mouse model, Izu et al. demonstrated that collagen XII regulates osteoblast differentiation and maturation, alignment, interaction, and polarization. The genetic deletion of collagen XII results in abnormal osteoblast differentiation, decreased bone matrix deposition, and decreased bone quality. This is related to impaired terminal differentiation of osteoblasts (Izu et al. 2011). Surprisingly, this mouse model has not been studied with respect to articular cartilage abnormalities up to now.

2.3.5.2 Collagen XIV

Studies of collagen XIV, a close homologue to collagen XII in chicken (Castagnola et al. 1992), bovine, and human tissues showed that collagen XIV is prevalent in the skin (Lethias et al. 1993; Berthod et al. 1997), tendon (Young et al. 2000a), cornea, and in minor quantities in articular cartilage (Watt et al. 1992). In addition, collagen XIV is often found specifically in areas of high mechanical stress at the ligament bone junction (Niyibizi et al. 1995). Localization to these areas indicates that collagen XIV may affect the mechanical properties of a tissue. Even though collagens XII and XIV share close structural homologies, they are not co-localized in the cartilage as often so in other tissues. Of note, the collagen XIV knockout mouse model was extensively studied with respect to tendon development and fibrillogenesis (Ansorge et al. 2009); however, as for the *Col12a1*-deficient mice, the impact of the absence of collagen XIV on articular cartilage physiology and matrix integrity was not studied up to now.

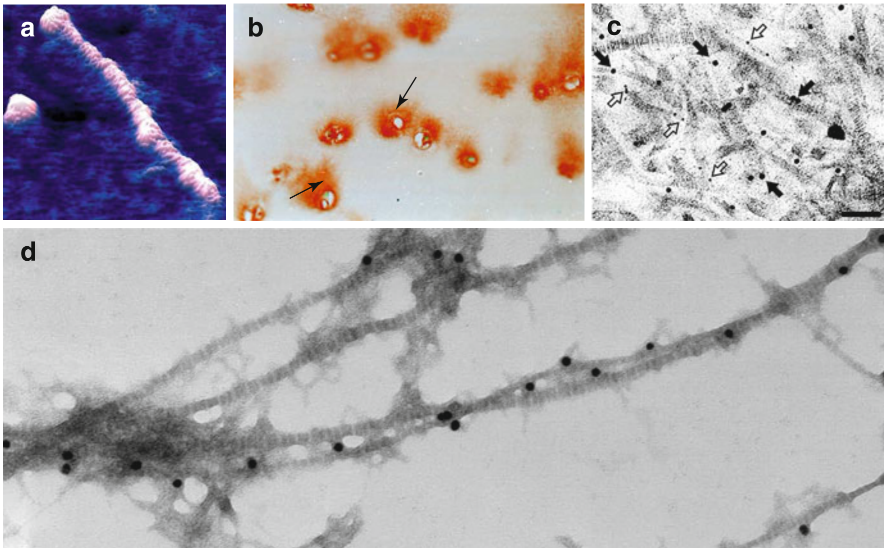

Fig. 2.5 Collagen XVI in articular cartilage. (**a**) Atomic force microscopy image of a recombinant collagen XVI molecule showing the rodlike structure of collagen XVI terminated by the nodule-like NC11 domain. (**b**) Immunohistochemistry of paraffin-embedded articular cartilage sections demonstrates localization of collagen XVI in the territorial matrix and in the PCM (*arrows*) around chondrocytes. (**c**) Immunogold electron microscopy of ultrathin sections of adult articular cartilage from the radial zone. Double labeling of collagen XVI (*large gold particles*, *closed arrows*) and collagen IX (*small gold particle*, *open arrows*) is found not to be co-localized; both collagens are associated to different D-periodically banded fibrillar fragments (From Kassner et al. (2003)). (**d**) Ultrastructural localization of collagen XVI in fibrillar extracts from adult articular cartilage by immunogold electron microscopy. A special subpopulation of thin D-periodically banded fibrils was labeled with collagen XVI; other fibril populations lack collagen XVI. *PCM* pericellular matrix

2.3.5.3 Collagen XVI

Collagen XVI, another member of the FACIT family, is expressed in various connective tissue cells and tissues without known occurrence of splice variants or isoforms and may act as a substrate for adhesion and invasion of connective tissue tumor cells (see review Grässel and Bauer (2013)) (Fig. 2.2). For skin and cartilage tissues, its suprastructure is known. Collagen XVI was identified in 2003 by Kassner et al. as a so far unknown component of cartilage fibrils, and it was demonstrated that in the territorial matrix, which is rich in 20-nm fibrils, collagens IX and XVI occur on similar small cartilage fibrils in a partly mutually exclusive manner (Kassner et al. 2003) (Fig. 2.5). These proteins share a carboxy-terminal FACIT domain, and therefore, the prototypic, thin cartilage fibrils may be stabilized either by collagen IX or collagen XVI. In addition, the choice of these proteins will influence the electrostatic surface charge properties of the fibrils since collagen IX often carries negatively charged glycosaminoglycan chains whereas collagen XVI does not. This provides an opportunity for modulation of interactions between the fibrils and the highly polyanionic

extrafibrillar matrix rich in aggrecan and hyaluronan. Presumably, there collagen XVI acts as an adaptor protein connecting and organizing large fibrillar networks and thus modulating integrity and stability of the extracellular matrix (ECM). Thick, well-banded fibrils of the interterritorial matrix contain neither collagen IX nor collagen XVI but maintain a polyanionic surface by inclusion of the proteoglycan decorin (Hagg et al. 1998). Removal of collagens IX and XVI is, thus, likely to be necessary for lateral growth of cartilage fibrils beyond 20 nm.

Beside some rare mutations in the *COL12A1* gene, a specific *COL12A1* gene polymorphism has been associated with anterior cruciate ligament ruptures in women (Posthumus et al. 2010). In addition, a rare autosomal dominant cutaneous disorder with variable penetrance has been associated with a heterozygous missense mutation in the *COL14A1* gene (Guo et al. 2012). Notably, no collagen XVI associated diseases are reported and no phenotype altering mutations in their genes are known as to now (Table 2.1).

2.4 Novel Hyaline Cartilage Collagens

Three recently described novel collagens, collagens XXII, XXIV, and XXVII, are expressed at tissue junctions, during endochondral bone formation and in adult articular cartilage.

2.4.1 Collagen XXII

The full-length cDNA and structure of collagen XXII was first described in 2004 by Koch et al. (2004) (Fig. 2.2). The overall structure of collagen XXII is similar to the FACIT or FACIT-related proteins. A striking observation was that collagen XXII is expressed only at sites of tissue junctions in the muscle, cartilage, heart, and skin. In articular cartilage, a small narrow band of collagen XXII was detected at the cartilage surface facing the synovial fluid. Similarly, ultrathin sections surface labeled for collagen XXII exhibited a narrow positive zone of gold particles close to the articular surface. Because collagen XXII belongs to the FACITs, its association with collagen-containing D-periodically banded fibrils was examined by immunoelectron microscopy of fragments of native cartilage fibrils isolated from tissue homogenates. However, their electron microscopic studies indicate that collagen XXII is not directly associated with collagen-containing fibrils. Rather, based on the observations on native fibril extracts from articular cartilage, it seems to interact with components of microfibrils, such as fibrillins or collagens VI. A similar situation prevails for collagen XVI which was recently shown to be associated with fibrillin-1 (Kassner et al. 2003). In the joints, the surface of the articular cartilage, i.e., the junction between cartilage and synovial fluid, was labeled with collagen XXII antibodies within a very thin, confined band (Koch et al. 2004). At the ultrastructural level, this region contains

highly organized thin collagen-containing fibrils and other suprastructural elements. In arthritic human joints, collagen XXII is still detectable with immunofluorescence staining, but the staining pattern is broadened and fuzzier. Future studies will show how collagen XXII is integrated into functional suprastructures of the articular surface and around the hair follicle and which the cellular origin of this protein is.

2.4.2 Collagen XXIV

Collagen XXIV, belonging to the fibril-forming collagen subfamily, is described as a marker of osteoblast differentiation and bone formation (Matsuo et al. 2008). Sequence analyses also revealed several features in the N-peptide, triple helix, and the C-propeptide of the α1(XXIV) chain which are unique to invertebrate fibrillar collagen chains (Koch et al. 2003). It is suggested that collagen XXIV is an "ancient" collagen which may contribute to the regulation of collagen fibrillogenesis. The most distinctive of these characteristics is a single imperfection in the collagenous domain. Collagen XXIV appears to be a marker for the process of bone formation in the embryonic mouse. It is first expressed at E14.5, concomitant with the appearance of the first ossification centers, and remains restricted to these, as well as to ossification centers that emerge later during subsequent stages of development. Notably, *Col24al* mRNA signals were absent in the cartilage even though in situ hybridizations detected strong *Col24al* mRNA expression in murine trabecular bone and periosteum (Matsuo et al. 2008).

2.4.3 Collagen XXVII

Contrary, collagen XXVII appears to be mainly restricted to cartilage in adulthood. It is associated with cartilage calcification and could play a role in transition of cartilage to bone during skeletogenesis. *COL27A1* is a member of the fibrillar collagen gene family and is expressed in cartilaginous tissues including the anlage of endochondral bone (Fig. 2.2). Phylogenetic analyses indicated that *COL27A1* forms a clade with *COL24A1* that is distinct from the two known lineages of fibrillar collagens (Pace et al. 2003). To begin to understand the role of collagen XXVII in skeletogenesis, the temporospatial distributions of its RNA message and protein product, were determined in developing human skeletal tissues. Laser capture microdissection and quantitative RT-PCR demonstrated that gene expression occurred throughout the growth plate and that it was higher in the resting and proliferative zones than in hypertrophic cartilage. Immunohistochemical analyses showed that collagen XXVII was most evident in hypertrophic cartilage at the primary ossification center and at the growth plate and that it accumulated in the pericellular matrix of chondrocytes. Synthesis of collagen XXVII overlapped partly with that of collagen X, a marker of chondrocyte hypertrophy, preceded the transition of cartilage to bone, and was associated with cartilage calcification. Immunogold electron microscopy of

extracted ECM components from mouse growth plate showed that collagen XXVII is a component of long non-banded fibrous structures, filamentous networks, and thin banded fibrils. The timing and location of synthesis suggest that collagen XXVII plays a role during the calcification of cartilage and the transition of cartilage to bone (Hjorten et al. 2007). Collagen XXVII is present in both mouse and human skeletal elements at similar developmental times and places. It was also shown that collagen XXVII is part of long, non-banded fibrils from which collagen II is excluded (Plumb et al. 2007). A study by Jenkins et al. has revealed a mechanism for the transcriptional regulation of *COL27A1* in chondrocytes. The *COL27A1* gene contains two enhancer elements that respond to SOX9, adding to the growing list of cartilage genes likely activated by this key regulator of chondrogenesis (Jenkins et al. 2005). Furthermore, this work has demonstrated that SOX9 must function as a dimer to activate the *COL27A1* enhancers which resemble enhancer elements from the classical cartilage collagen genes *COL2A1*, *COL9A2*, and *COL11A2*.

2.5 Perspectives

The prototype of a fibril-forming collagen besides collagen I is the first 1969 described collagen II by Miller and Matukas in the cartilage (Miller and Matukas 1969). Up to now, the collagen suprafamily comprises a total of 28 members of highly divergent collagens with respect to chain structure and suprastructure, tissue specificity and location. The common structural feature is the presence of a triple helix that can range from 96 % of the total structure (collagen I) down to less than 10 % (collagen XII). It is not to be expected to identify novel members of the collagen family in the future but rather novel functions of the individual collagens (i.e., splice variant PIIBNP of procollagen II) and the bioactive fragments (matricryptins) of various collagen family members generated via proteolytic cleavage mainly of the NC1 domain. These novel functions include regulation and modification of physiological and pathological processes in skeletal development, angiogenesis, tumor growth and metastasis, and tissue repair and turnover. In the meantime, 13 out of the 28 existing collagens have been detected in different types of cartilage tissue over the past years. It remains to see if yet other members of the collagen superfamily will be identified in the cartilage pointing to an increasingly different view of this formerly as mainly consisting of amorphous material characterized tissue.

In the view of articular cartilage repair and regeneration (i.e., de novo formation) by using novel tissue-engineering strategies, the zonal distribution of distinct articular cartilage collagens which populate pericellular, territorial, and interterritorial zones according to collagen type and its specific function is of crucial importance. In an adult joint, the complex heterogeneous collagen architecture and its unique macromolecular fibrillar phenotype are clearly a challenge to recreate and reconstruct after osteochondral trauma or thereof resulting joint tissue destruction as during pathogenesis of osteoarthritis.

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Cartilage Glycoproteins

3

Frank Zaucke

Abstract

The cartilage extracellular matrix consists of two major suprastructures, namely, the fibrillar collagenous network and gel-like aggrecan complexes. These suprastructures are interconnected by glycoproteins and small proteoglycans forming a kind of alloy. In addition to this role as so-called adaptor proteins, matrix proteins can directly bind to chondrocytes via cell surface receptors. The biological relevance of these numerous protein-protein interactions in the matrix is underlined by the fact that mutations in matrix components often lead to skeletal disorders. In addition, lacking integrity and stability of these interactions affects biomechanical properties, and softening of the tissue may predispose for degenerative diseases, e.g., osteoarthritis.

The structure of cartilage does change during development and aging, and, thus, the cartilage is more heterogeneous and dynamic as expected. Over the last years, it could be shown that matrix proteins can also play nonstructural roles, like modulation of growth factor activities and immune responses. This adds another layer of complexity to the tissue. This chapter summarizes general aspects of a standard cartilage extracellular matrix even though it has been demonstrated recently that different cartilage types vary substantially in composition and assembly.

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3.1 Introduction

The cartilage is a matrix-dominated tissue, and with regard to its abundance, the matrix is mainly composed of collagens (see Chap. 2) and proteoglycans (see Chap. 3). These two main components form suprastructures interconnected by plenty of proteins forming a kind of alloy (Bruckner 2010; Heinegård and Saxne 2011). The interconnecting molecules, sometimes also referred to as adaptor proteins, are from a biochemical point of view mainly non-collagenous glycoproteins and small proteoglycans. The glycosylation pattern may change during development and aging and can vary in different types of cartilage. Further, some of these adaptor proteins do not carry any carbohydrate modification. A subgroup of adaptor proteins is often located at the surface of cartilage collagen fibrils. These so-called perifibrillar proteins are not only simply connecting fibrils but are also involved in regulating their assembly and diameter.

This chapter summarizes general aspects of the composition and assembly of a standard cartilage extracellular matrix (ECM) even though it has been demonstrated recently that not only different cartilage types but also different zones in certain types of cartilage can vary substantially in their content and composition (Müller et al. 2014; Önnerfjord et al. 2012). Further, the cartilage ECM undergoes changes and remodeling during aging and progression of degenerative diseases, e.g., osteoarthritis (OA) (Peffers et al. 2014a, b). Over the last years, it became increasingly clear that cartilage is a much more heterogeneous and dynamic tissue than expected. The molecular architecture of a typical cartilage matrix is depicted in Fig. 3.1.

There is a huge body of literature indicating that one has to abandon the idea of cartilage matrix proteins being only structural components. In contrast, initiated by Paul Bornstein and his concept of matricellular proteins, there is accumulating evidence that matrix proteins and fragments thereof can also have regulatory functions (Sage and Bornstein 1991; Murphy-Ullrich and Sage 2014). In this chapter, the role of matrix proteins in growth factor binding, mediating inflammatory and immune responses, and their use as biomarkers in cartilage-associated diseases will also be discussed.

Chondrocytes directly interact with cartilage ECM proteins, and cell-matrix signaling, mostly mediated by integrins, regulates several chondrocyte functions, including differentiation, metabolism, matrix remodeling, responses to mechanical stimulation, and cell survival (Gao et al. 2014). Interactions of matrix proteins with surface receptors will not be touched as a later chapter will focus on chondrocyte integrins (further details in Chap. 4).

To unravel the function of individual matrix proteins, a huge list of animal models targeting the respective encoding genes has been generated over the last decades (Aszódi et al. 2006). The phenotypes of some models will be mentioned here but discussed in a broader context also in other chapters of this volume. Due to space constraints, it is not possible to discuss all cartilage components in detail, and therefore this chapter will focus mainly on some selected proteins that have been associated with diseases in humans without claiming that this

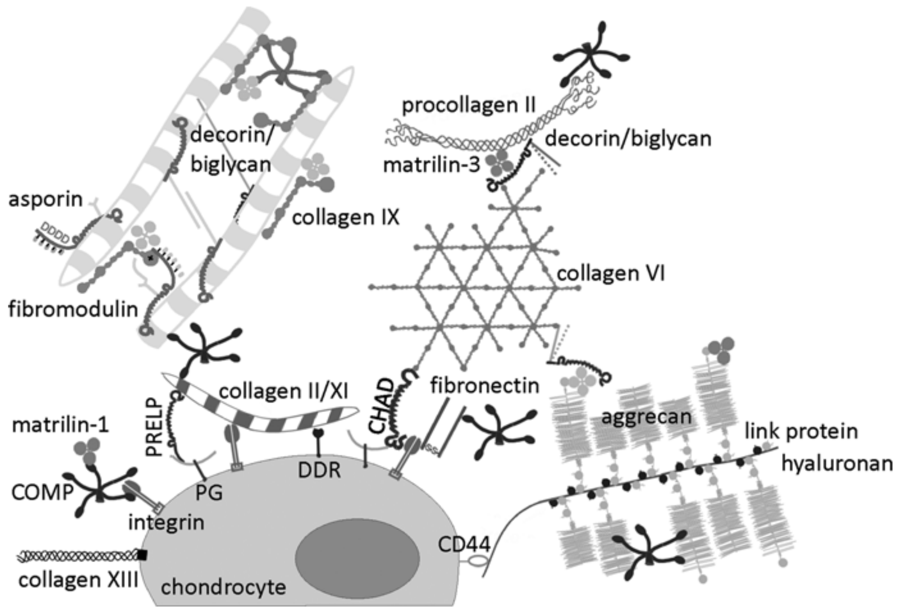

Fig. 3.1 Molecular organization of a typical cartilage matrix. A variety of protein-protein interactions stabilizes the cartilage ECM. Aggrecan complexes are interconnected with fibril and network forming collagens via adaptor proteins like COMP, matrilins, and small proteoglycans. The matrix is anchored at the cell surface via integrins, discoidin domain receptors (*DDR*), membrane-bound proteoglycans (*PG*), and CD44 (more details in the text; modified from Heinegård and Saxne (2011))

selection is complete and/or covers the most relevant constituents. The functions of these proteins and their relevance in skeletal disease are summarized in Table 3.1.

3.2 Thrombospondins

3.2.1 Thrombospondin-5/Cartilage Oligomeric Matrix Protein (COMP)

Thrombospondin-5 (also referred to as COMP) belongs to the thrombospondin family and is the major cartilage thrombospondin. It has been described originally in 1992 (Hedbom et al. 1992; Mörgelin et al. 1992) as homopentameric acidic oligomeric glycoprotein with a molecular weight of 524 kDa and a typical modular domain structure: an oligomerizing coiled-coil domain at the N-terminus is followed by four epidermal growth factor (EGF)-like domains, eight thrombospondin type 3 (T3) repeats, and a globular domain at the C-terminus. Visualized by electron microscopy, the five subunits of COMP form a bouquet-like structure with a central assembly domain and five flexible arms terminated by the globular domains. The

Table 3.1 Functions and disease relevance of selected non-collagenous cartilage matrix proteins in skeletal tissue

Protein	Function	Disease association
Thrombospondins		
Thrombospondin-1	Cell-cell and cell-matrix interactions, antiangiogenic properties	Not known
Thrombospondin-2	Collagen fibrillogenesis, chondrogenic differentiation	Not known
Thrombospondin-3	Not known	Highly expressed in osteosarcomas
Thrombospondin-5	Collagen fibrillogenesis Growth factor binding	Mutations cause chondrodysplasia, increased TSP5 serum levels in OA
Matrilins		
Matrilin-1	Adapter protein Cell attachment Inhibitor of neovascularization	Polymorphisms are associated with scoliosis and prognathism Autoantigen in relapsing polychondritis
Matrilin-3	Adapter protein Growth factor binding	Mutations cause chondrodysplasia; a polymorphism is associated with OA Increased serum levels in OA
Others		
WARP	Adapter protein	Not known
Chondroadherin	Cell-cell and cell-matrix interactions	Fragmented in degenerating discs Reduced in osteoporotic women
Asporin	Matrix protein and growth factor binding	Polymorphism is associated with OA
PRELP	Adapter protein Regulator of osteoclastogenesis	Not known
UCMA	Control of osteogenic differentiation	Carboxylation correlates with OA A polymorphism is associated with Paget's disease of bone
CILP	Binding of TGF-β	Associated with lumbar disc disease
Fibronectin	Cell-matrix interactions	Upregulated in OA Fragments enhance cartilage degeneration
Perlecan	Not known	Chondrodysplasia Upregulated in late-stage OA
Nidogens	Not known	Upregulated in late-stage OA

Details in the text
OA osteoarthritis

crystal structure of the so-called signature domain of COMP has been solved in 2009 and gave valuable insight into the interactions with collagens, glycosaminoglycans, and integrins (Tan et al. 2009). COMP is predominantly found in the extracellular matrix of cartilage, ligaments, and tendons (Hedbom et al. 1992). However, in the last years, COMP expression has been detected in a number of other tissues, including the skin, hair follicles, vasculature (Agarwal et al. 2012; Ariza de

Schellenberger et al. 2011; Du et al. 2011), and many different organs under fibrotic conditions (Andréasson et al. 2015).

The expression of COMP has been shown to be mechanosensitive (Giannoni et al. 2003), and after a pioneering study in marathon runners (Neidhart et al. 2000), several human in vivo studies show that mechanical loading rapidly leads to increased COMP serum levels. COMP fulfills its adaptor function in matrix assembly by interacting with a large number of cartilage ECM proteins, like collagens, proteoglycans including aggrecan, matrilins, fibronectin, and others (for review, see Acharya et al. (2014)). Interaction of COMP with several collagens was shown to be zinc dependent (Rosenberg et al. 1998), and, interestingly, COMP binding accelerates the formation of collagen fibrils with a rather uniform diameter in vitro (Halász et al. 2007). Its important role in fibrillogenesis and regulation of fibril diameter has been confirmed in COMP-deficient chondrocytes (Blumbach et al. 2009), and the effect of mutant COMP variants associated with chondrodysplasia in humans has been investigated both in vivo and in vitro (Hansen et al. 2011; Schmitz et al. 2008). The disruption of the collagen network seen in these studies provided important insights into the pathomechanism of these conditions.

Besides these more structural interactions, recent studies have shown that COMP directly binds members of the TGF-β superfamily of proteins, namely, TGF-β1 as well as bone morphogenetic proteins (BMP)-2, BMP-4, and BMP-7 (Haudenschild et al. 2011). COMP expression enhances several early aspects of chondrogenesis induced by BMP-2 (Kipnes et al. 2003), and COMP binding to BMP-2 promotes osteogenesis in human mesenchymal stem cells by enhancing BMP-2-induced intracellular signaling through Smad proteins and increased the levels of BMP receptors (Ishida et al. 2013). In the vasculature, COMP inhibited BMP-2 receptor binding and blocked BMP-2-mediated osteogenesis, thereby preventing the calcification of vascular smooth muscle cells (Du et al. 2011). *COMP* is also a direct response gene of TGF-β, and it was suggested that COMP is suitable for rapidly accessing the chondrogenic potential of stem cells (Li et al. 2011). The same group could show that COMP might act as a scaffold for growth factors and that a direct interaction with TGF-β enhances TGF-β-dependent transcription (Haudenschild et al. 2011). Nevertheless, it has to be determined in future studies what role the interaction between COMP and growth factors plays in vivo and, in particular, in skeletal development.

COMP is widely used as a diagnostic and prognostic marker for knee OA (Mobasheri 2012), but its utility in drug development to monitor and predict osteoarthritis is still under discussion (Das et al. 2015). However, it has been shown earlier that patients with rheumatoid arthritis display a specific fragmentation pattern in their synovial fluid (Neidhart et al. 1997). This idea was followed up using affinity chromatography and mass spectrometry to detect specific COMP neoepitopes in synovial fluids from patients with joint diseases (Åhrman et al. 2014). As mentioned above, COMP expression in different tissues is also elevated under fibrotic conditions and, at least in some cases, accompanied by increased COMP serum levels. This has to be considered in the future when using COMP serum levels as predictive or diagnostic marker as an increase might stem from rather different sources.

COMP degradation by matrix metalloproteases (MMPs) and ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) has been studied extensively in vitro. At least MMP-1, MMP-3, MMP-9, MMP-13, MMP-19, and MMP-20 are able to cleave COMP in vitro (for review, see Acharya et al. (2014)). Earlier studies demonstrated that also ADAMTS-4, ADAMTS-5, ADAMTS-7, and ADAMTS-12 degrade COMP (Liu et al. 2006a, b; Zhen et al. 2008). An increased expression of the latter two enzymes that directly bound to and degraded COMP was reported in cartilage and synovium of patients with arthritis supporting the idea that specific COMP fragments can be used as disease markers. Interestingly, COMP was shown to be arthritogenic, and both native and denatured COMP induced severe arthritis in selected rat strains (Carlsén et al. 1998).

COMP is also the first extracellular matrix protein for which an active role in inflammation has been described in vivo (Happonen et al. 2012). It can at the same time activate one complement pathway while it inhibits another. The authors speculated that the net outcome of these interactions depends on the nature of the released COMP fragments, which could be disease specific (Happonen et al. 2010). A recent study analyzed the potential of different COMP constructs to modify the expression of genes involved in cartilage homeostasis, e.g., MMP-1 and MMP-9, IL-6, and TGF-β. Surprisingly, and in contrast to several other cartilage proteins, COMP had no effect on primary human chondrocytes and cartilage explants, respectively (Ruthard et al. 2014). However, it might well be that only certain fragments presenting specific neoepitopes as mentioned above are potent inducers, while others remain inactive. The versatile roles of COMP in cartilage homeostasis are summarized in Fig. 3.2.

The fact that COMP-deficient mice have a rather normal skeletal development (Svensson et al. 2002) leads to the assumption that its loss might be compensated by other members of the TSP protein family. At least a partially redundant function was indeed reported in mice with compound deletion of different thrombospondins (Posey et al. 2008).

However, the importance of COMP in skeletal development is underlined by the fact that mutations in COMP cause moderate to severe disproportionate short-limb short stature with pronounced joint laxity in humans. Patients develop premature osteoarthritis and often require early joint replacement. The more severe form pseudoachondroplasia (PSACH) is caused exclusively by mutations in COMP (Briggs and Wright 1993), while the milder condition multiple epiphyseal dysplasia (MED) is also associated with mutations in other cartilage matrix proteins, like collagen IX and matrilin-3. COMP-associated chondrodysplasias are inherited in an autosomal dominant manner, and most individuals diagnosed with PSACH or MED have an affected parent, but the proportion of de novo pathogenic variants is unknown (Briggs and Wright 1993).

Electron microscopy of biopsies from PSACH patients show mutated COMP retained in typical granular or lamellar inclusions in the endoplasmic reticulum (ER) of chondrocytes (Stanescu et al. 1993). The accumulation of COMP leads to co-retention of other matrix components. Interestingly, the two binding partners collagen IX and matrilin-3 were also retained, while the trafficking and secretion of collagen II seem to be mostly unaffected (Dinser et al. 2002; Hecht et al. 2005; Maddox et al. 1997). The compromised ER function eventually leads to ER stress,

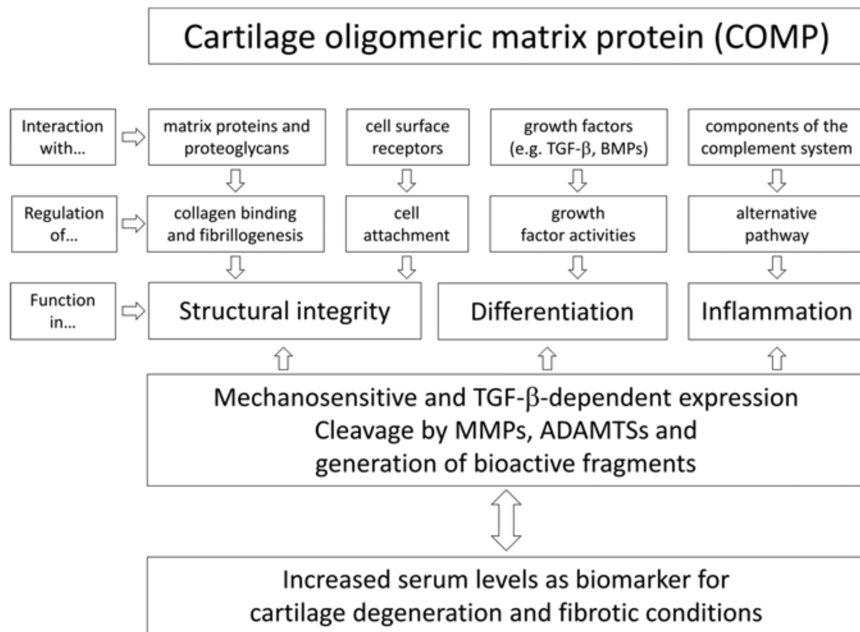

Fig. 3.2 Versatile functions of COMP in tissue homeostasis. COMP is not only important for the structural integrity of cartilage but also involved in differentiation processes and inflammatory responses. COMP expression is mechanosensitive and regulated by TGF-β. Proteolytic cleavage of COMP might generate bioactive fragments. Serum levels of full length but also fragmented COMP can be used as biomarkers for cartilage degeneration and fibrotic conditions (details and references in the text)

which in turn results in an increased apoptosis. This is in good agreement with the detection of many dead cells in the growth plate of affected individuals. Further, impaired cell viability might explain why chondrocytes are arranged in clusters rather than in columns (Hashimoto et al. 2003; Hecht et al. 2004). Based on these observations, it was suggested that chondrocyte depletion in the PSACH growth plate is the main reason for the diminished linear growth leading to disproportionate short stature. A set of genetically modified mouse models to mimic the human disease were generated and characterized over the last 10 years (reviewed in Briggs et al. (2015)). In such mouse models, not only ER stress but also oxidative stress and inflammation was identified as potential therapeutic targets (Posey et al. 2014), and a recent study in mice indeed suggested that antioxidant and anti-inflammatory agents might mitigate the pathology also in humans (Posey et al. 2015).

3.2.2 Other Members of the Thrombospondin Family

The structural and functional roles of other members of the thrombospondin family in cartilage are not very well understood even though their interaction with a variety of extracellular matrix proteins has been characterized (Tan and Lawler 2009).

Trimeric TSP-1 was purified together with pentameric COMP from bovine cartilage using EDTA-containing buffers. The two thrombospondins could be separated by heparin affinity chromatography as only TSP-1 has an N-terminal heparin binding domain. Interestingly, chondrocytes were not able to attach to TSP-1 but to COMP indicating a different function of these proteins in cartilage (DiCesare et al. 1994). In normal human articular cartilage, TSP-1 is expressed mainly in middle and upper deep zone chondrocytes. Its expression changes upon initiation of osteoarthritis. In mild and moderate stages, the number of TSP-1 expressing chondrocytes and the pericellular staining is increased. In severe osteoarthritic cartilage, a decrease in the number of TSP-1 synthesizing chondrocytes and a strong reduction in matrix staining were reported (Pfander et al. 2000). It has been shown that TSP-1 prevents excessive ossification in cartilage repair tissue by inhibiting hypertrophic chondrocyte differentiation induced by osteogenic protein 1 (OP-1) (Gelse et al. 2011). This observation might partially explain why an intra-articular gene transfer of TSP-1 was able to suppress the disease progression of experimental osteoarthritis in a rat model (Hsieh et al. 2010). Further, this study showed that TSP-1 exerts its antiangiogenic potential also in cartilage and a significant reduction of microvessel density was detected. As for COMP, the targeted deletion of TSP-1 had only very minor effects on skeletal development. However, it was suggested that TSP1 may help to define the timing of growth plate closure when other extracellular proteins are absent (Posey et al. 2008).

Mice deficient in each thrombospondin family member have been generated, but, if at all, only subtle skeletal abnormalities mainly in bone were identified. Both TSP-2 and TSP-3 knockout mice have thicker cortical bone, most likely due to an expansion of the osteoblast progenitor cell pool. However, a recent study has shown that TSP-2 deficiency leads to a brittle bone phenotype in cortical bone. Ultrastructural analysis of collagen fibrils by electron microscopy revealed an altered morphology of the ECM assembled by osteoblasts. This suggests that, like TSP-5 in cartilage, TSP-2 is required for optimal collagen fibrillogenesis in bone (Manley et al. 2015). A recent study suggested that TSP-2 might determine the chondrogenic differentiation potential of stem cells in an autocrine manner (Jeong et al. 2015). TSP-3 knockout mice show earlier ossification and more trabecular bone in the femoral head (Hankenson et al. 2000, 2005).

3.3 VWA Domain-Containing Proteins

3.3.1 Matrilins

The matrilins form a four-member family of multi-subunit matrix proteins with a closely similar domain structure. Except for matrilin-3, two von Willebrand factor A-like (VWA) domains are interconnected by a variable number of epidermal growth factor-like (EGF) domains. The subunits form mostly homotypic trimers or tetramers via a C-terminal coiled-coil domain. Matrilin-3 lacks the second VWA domain, and matrilin-2 has a unique domain which is present in N-terminal of the

coiled-coil domain. Matrilin-1 is the prototype member of the matrilin family and was originally named "cartilage matrix protein" (CMP). It was first identified as an aggrecan-associated protein (Paulsson and Heinegård 1979) and purified from bovine cartilage (Paulsson and Heinegård 1981). Matrilin-1 and matrilin-3 are found almost exclusively in cartilage, while matrilin-2 and matrilin-4 have a broader tissue distribution (Klatt et al. 2011; Wagener et al. 2005). Like COMP, matrilin-1 and matrilin-3 function as adaptor proteins connecting collagen fibrils with each other and with aggrecan (Mann et al. 2004; Wiberg et al. 2003a).

Matrilins oligomerize via their C-terminal coiled-coil domains, and a study using recombinant matrilin expression revealed that different matrilins are able to form hetero-oligomers (Frank et al. 2002). Even though seven different isoforms were described, so far, only hetero-oligomers consisting of matrilin-1 and matrilin-3 with a varying stoichiometry were detected in vivo and especially in cartilage (Klatt et al. 2000; Wu and Eyre 1998). The oligomerization of matrilins seems to have a functional impact as oligomer formation is changing during developmental processes like endochondral ossification (Zhang and Chen 2000). Matrilins are able to form both collagen-dependent and also collagen-independent filaments (Chen et al. 1995, 1999; Klatt et al. 2000). A collagen-independent network is often located in the pericellular matrix of chondrocytes (Klatt et al. 2000), and it has been suggested that matrilins are involved in the mechanotransduction in response to cyclic loading (Kanbe et al. 2007).

Via their VWA domains, matrilins interact with several other matrix components including COMP; collagens II, IX, and XI; and proteoglycans like decorin, biglycan, and aggrecan (Budde et al. 2005; Mann et al. 2004; Otten et al. 2010; Wiberg et al. 2003). However, the binding repertoire of matrilins goes beyond other structural matrix proteins. It has been demonstrated recently that matrilin-3 directly interacts with BMP-2, thereby suppressing Smad1 activity and chondrocyte hypertrophy (Yang et al. 2014). In addition, an interaction with TGF-β and other growth factors has been postulated but not systematically analyzed so far (Jayasuriya et al. 2014).

Some matrilin functions were suggested from in vitro studies, like mediation of cell attachment (Mann et al. 2007) and maintenance of chondrogenesis (Pei et al. 2008). However, their role in vivo and in particular during skeletal development remains elusive especially as most of the knockout strains display only a mild phenotype. Mice lacking matrilin-1 develop normally (Aszódi et al. 1999) even though in a second independent mouse line ultrastructural studies revealed an abnormal type II collagen fibrillogenesis and fibril organization in the matrix of growth plates (Huang et al. 1999). Matrilin-3-deficient mice were generated and characterized by two groups with a different phenotypic outcome. Ko et al. (2004) reported no obvious changes in skeletal development, while others found that at least aged matrilin-3 null mice were predisposed to develop osteoarthritis (van der Weyden et al. 2006). These authors suggested that matrilin-3 might modulate chondrocyte differentiation during embryonic development and control bone mineral density in adulthood. The apparent lack of a stronger phenotype in single matrilin-deficient mouse lines suggested a functional redundancy within the matrilin family, and therefore a

matrilin-1/matrilin-3 double-deficient mouse line was generated. However, even here the overall structure and integrity of the cartilage matrix remained mostly unaffected. Ultrastructural analysis using electron microscopy revealed moderately increased collagen fibril diameters and an increased collagen volume density in double-deficient animals (Nicolae et al. 2007). In summary, the analysis of matrilin-1- and matrilin-3-deficient mice implicates a role for both matrilins in the fine control of lateral collagen fibril growth. Interestingly, the knockdown of matrilin-1 in zebrafish lead to an impaired secretion and accumulation of collagens in the endoplasmic reticulum suggesting a novel intracellular function of matrilins in the secretory pathway (Neacsu et al. 2014). Recently, matrilin-1 was identified as an inhibitor of neovascularization in cartilage (Foradori et al. 2014).

As for COMP, extensive proteolytic cleavage has been reported for matrilins (Ehlen et al. 2009). ADAMTS-4 and ADAMTS-5, but not ADAMTS-1, are able to cleave matrilins. However, the function of the increased heterogeneity generated by this processing remains largely unknown. Interestingly, a matrilin-3-dependent feed-forward mechanism of matrix degradation has been described, in which proteolytically released matrilin-3 induces pro-inflammatory cytokines as well as ADAMTS-4 and ADAMTS-5 (Klatt et al. 2009). Further analysis revealed that the matrilin-3 von Willebrand factor A (VWA) domain oligomers are sufficient to induce interleukin-6 release from primary human chondrocytes (Klatt et al. 2013).

In human osteoarthritic cartilage, an increased expression of matrilin-3 was reported and interpreted as a cellular response to the modified microenvironment in the tissue (Pullig et al. 2002). Recently, increased levels of matrilin-3 were found in serum and synovial fluid of OA patients (Vincourt et al. 2008, 2012). Even though not restricted to cartilage, matrilin-2 has been proposed as a potential biomarker in early stages of osteoarthritis in articular cartilage (Zhang et al. 2014). Interestingly, one distinct mutation in matrilin-3 has been associated with the development of hand osteoarthritis and spinal disc degeneration (Eliasson et al. 2006; Min et al. 2006; Stefánsson et al. 2003) but not knee osteoarthritis (Pullig et al. 2007). Another striking association of matrilins with human disease is the causative role of several matrilin-3 mutations in the pathogenesis of autosomal dominant inherited multiple epiphyseal dysplasia (MED). Most of the disease-causing mutations identified so far are located in the VWA domain of the protein. Expression of such mutant variants in cell culture leads to impaired secretion and accumulation in the endoplasmic reticulum (Otten et al. 2005). In a murine model expressing a mutant matrilin-3, decreased chondrocyte proliferation and dysregulated apoptosis as a consequence of an unfolded protein response were reported in the cartilage growth plate (Leighton et al. 2007; Nundlall et al. 2010).

Other disease-causing mutations are located in the arginine-rich region at the N-terminus of matrilin-3 (Maeda et al. 2005) and within the first epithelial growth factor (EGF) domain in matrilin-3. The latter mutation causes spondylo-epimetaphyseal dysplasia (SEMD) (Borochowitz et al. 2004), an autosomal recessive inherited disease with some similarities to MED.

So far, there is no clear evidence that matrilin-1 mutations play a role in human disease. Matrilin-1 has been linked to osteoarthritis in a Dutch (Meulenbelt et al. 1997),

but not in a British population (Loughlin et al. 2000). There might be a linkage of the matrilin-1 gene to idiopathic scoliosis and (Bae et al. 2012; Chen et al. 2009) mandibular prognathism (Jang et al. 2010), but the pathogenic mechanisms remain completely unclear. The only clear disease association of matrilin-1 is its role as an autoantigen in relapsing polychondritis. This rare autoimmune disease is characterized by inflammation and progressive cartilage destruction, and patients typically have circulating antibodies against matrilin-1 (Buckner et al. 2000; Hansson et al. 2001). The detection of these antibodies is also used for diagnosis. Rats or mice immunized with matrilin-1 develop a disease with many similarities to human relapsing polychondritis (Hansson et al. 1999), supporting the idea of a causative role for matrilin-1.

3.3.2 von Willebrand Factor A-Domain-Related Protein (WARP)

WARP is a member of the von Willebrand factor A-domain protein superfamily. It consists of 415 amino acid residues and comprises a single N-terminal VWA domain containing a putative metal ion-dependent adhesion site motif, two fibronectin type III repeats, and a unique C-terminal segment (Fitzgerald et al. 2002). It can form disulfide-bonded multimers in cartilage tissue and is then located in the pericellular matrix of chondrocytes. Here, it interacts with domain III-2 of the perlecan core protein and the heparan sulfate chains of the perlecan domain I (Allen et al. 2006). Further it binds to collagen VI containing microfibrils (Hansen et al. 2012). WARP-deficient mice did not display any abnormalities in articular cartilage, intervertebral discs, and skeletal muscle. It is not essential for basement membrane formation or musculoskeletal development but seems to have a critical role in the structure and function of peripheral nerves (Allen et al. 2009). So far, there is no association to any human disease.

3.4 Leucine-Rich Repeat Proteins

3.4.1 Chondroadherin

Chondroadherin has been isolated by dissociative extraction of articular cartilage. It consists of 337 amino acids, has 11 leucine-rich repeats flanked by cysteine-rich regions, and exists in several isoforms (Neame et al. 1994). As a member of the small leucine-rich repeat protein (SLRP) family, it could regulate assembly and function of the collagenous networks in the cartilage extracellular matrix. It binds to collagen II and mediates cell-matrix interactions by bridging cell surface receptors with matrix components (Mansson et al. 2001; Mizuno et al. 1996). Integrin α2β1 has been identified as one such receptor (Camper et al. 1997; Haglund et al. 2011), but an interaction with cell surface proteoglycans like syndecans has also been described (Haglund et al. 2013).

In skeletal development, chondroadherin mRNA was found in femoral head and rib cartilage, as well as in the tendon, calvaria, and bone. The protein is present in

cartilaginous regions of the developing femoral head from postnatal day 14 to day 60. With increasing age, a restricted localization of chondroadherin was described in the territorial matrix surrounding late proliferative cells in the growth plate as well as in the developing articular cartilage in the maturing femoral head. Therefore, an important role for chondroadherin in the regulation of chondrocyte growth and proliferation was suggested (Shen et al. 1999).

A recent study provided evidence that chondroadherin plays a role in bone and cartilage homeostasis. A widening of the epiphyseal growth plate with an impaired hypertrophic differentiation of chondrocytes was detected. Nevertheless, the longitudinal growth was unaltered. A reduced number of bone sialoprotein expressing cells points to a decrease in proximal tibial metaphysis, while the osteoid surface was increased indicating a change in mineral metabolism. As mentioned above, chondroadherin interacts with collagens. However, the collagen organization and fibril diameter remained normal in chondroadherin-deficient animals (Hessle et al. 2014).

Interestingly, the relative amount and proteolytic degradation of chondroadherin have been associated with scoliosis in the human intervertebral disc. The abundance of chondroadherin was consistently lower on the concave side of patient discs indicating that synthesis and/or degradation of chondroadherin is mechanosensitive. First it was speculated that proteolytic fragmentation, which occurred only in some patients with scoliosis but never in control individuals, is most likely mediated by aggrecanases and MMPs (Haglund et al. 2009). However, the digestion of disc tissue with purified proteases revealed that ADAMTS-4 and ADAMTS-5 as well as several cathepsins and MMPs were unable to cleave chondroadherin but that the serine protease HTRA1 was the only protease capable. The activity of HTRA1 gave rise to a specific neoepitope that might be used as a biomarker for disc degeneration (Akhatib et al. 2013). The amount of chondroadherin was also reduced in biopsies of 50- to 65-year-old osteoporotic women. In search for a novel treatment strategy, a recent study demonstrated that preosteoclast motility and bone resorption could be prevented by a cyclic chondroadherin fragment that contained the integrin-binding domain (Capulli et al. 2014).

In 2015, a closely related protein, chondroadherin-like protein (CHADL), was first described and characterized (Tillgren et al. 2015). CHADL expression was detected mainly in skeletal tissues, particularly in fetal cartilage, and in the pericellular space of adult chondrocytes, CHADL binds collagen in cell culture and inhibits in vitro collagen fibrillogenesis. Knockdown of CHADL increased collagen II and aggrecan deposition in the cell layers. The authors suggested that CHADL might have a negative regulatory role in the formation of a stable extracellular matrix (Tillgren et al. 2015).

3.4.2 Asporin

Asporin belongs to the leucine-rich repeat family of proteins and consists of 380 amino acids, a putative propeptide, 4 amino-terminal cysteines, 10 leucine-rich

repeats, and 2 C-terminal cysteines. It was named asporin based on the presence of a polyaspartate stretch in the amino-terminal region. The number of consecutive aspartate residues can vary between 11 and 15. Its closest relatives within the leucine-rich repeat family of proteins are decorin and biglycan, but in contrast to these proteins, asporin is no proteoglycan. Asporin was originally purified from human articular cartilage and meniscus (Lorenzo et al. 2001). During mouse embryonic development, asporin mRNA expression was also detected primarily in the skeleton and other specialized connective tissues (Henry et al. 2001). However, in situ hybridization revealed the presence of asporin mRNA mainly in the perichondrium/periosteum of long bones, but its absence in articular cartilage and growth plates. Immunohistochemical analysis confirmed protein expression predominantly in the perichondrium/periosteum (Kou et al. 2007). It has been shown that asporin and decorin compete for binding to collagen I and that the polyaspartate in asporin regulates collagen mineralization (Kalamajski et al. 2009). Asporin directly interacts with fibroblast growth factor (FGF)-2 and positively regulates its activity (Awata et al. 2015).

Interestingly, a significant association between a polymorphism in this aspartic acid repeat of asporin and osteoarthritis was reported (Kizawa et al. 2005). Allele D13 of the repeat mediates OA protection, whereas allele D14 confers OA susceptibility. The 2 alleles cause differences in the capacity of asporin to inhibit the cartilage growth factor TGF-β, with the D14 allele being a particularly potent inhibitor. The interaction between asporin and TGF-β suppresses the expression of cartilage-specific genes like collagen II and aggrecan. In two independent populations of individuals with knee osteoarthritis, the D14 allele of asporin is overrepresented relative to the common D13 allele, and its frequency increases with disease severity (Kizawa et al. 2005). In Asians, the D14 polymorphism has been associated in addition with lumbar disc degeneration (Song et al. 2008).

However, in Caucasians the asporin polymorphism seems not to be a major influence on OA etiology (Mustafa et al. 2005). At present, there is no asporin-deficient animal model available that would help to understand disease mechanisms. Many issues must be addressed to clarify the association between asporin and OA to provide novel therapeutic strategies for OA, probably by controlling and modifying TGF-β-ECM interaction (Xu et al. 2015).

3.4.3 PRELP (Proline-/Arginine-Rich End Leucine-Rich Repeat Protein)

PRELP consists of 382 amino acid residues including a 20-residue signal peptide. The molecular mass of the mature protein is around 42 KDa. It comprises 10 leucine-rich repeats ranging in length from 20 to 26 residues. The N-terminal part is unusually rich in basic amino acids arginine and proline (Bengtsson et al. 1995). These clustered arginine residues confer a heparin and heparan sulfate binding capacity with an affinity in the low molecular range. PRELP binds to the

basement membrane heparan sulfate proteoglycan perlecan and to collagens I and II (Bengtsson et al. 2002). Since cells do also bind to PRELP in a heparin-dependent manner, it was suggested that also this leucine-rich repeat protein can act as a linker between the matrix and cell surface proteoglycans (Bengtsson et al. 2000). With regard to gene organization and protein structure, PRELP is closely related to fibromodulin and lumican but does not carry keratan sulfate chains (Bengtsson et al. 1995). At the mRNA level, expression levels are high in juvenile and adult cartilage in humans, but not in the fetus or neonate. The protein in the cartilage matrix was also less abundant in the neonate than in the adult (Grover et al. 1996). In contrast to this expression, Northern blot analysis of murine tissue indicates that PRELP mRNA is also expressed in the developing embryo prior to skeletogenesis (Grover and Roughley 2001). A gene expression analysis of rat growth plate cartilage revealed that *PRELP* is mainly expressed in the proliferative zone (Lui et al. 2010).

The exact function in skeletal development is not completely understood. It has been suggested that PRELP might contribute to the process of differentiation of progenitor cells to musculoskeletal tissue, e.g., ligaments (Tsuru et al. 2013). Another role in skeletal biology was uncovered only recently. It has been shown that the glycosaminoglycan-binding domain of PRELP can act via the NF-kappaB pathway to impair osteoclastogenesis (Rucci et al. 2009). This function as an osteoclast antagonist might become a novel strategy to treat bone loss (Rucci et al. 2013).

3.5 Other Cartilage-Specific Proteins

3.5.1 UCMA (Unique Cartilage Matrix-Associated Protein/Upper Zone of Growth Plate and Cartilage Matrix-Associated Protein)

UCMA is a cartilage-specific secreted protein with a molecular mass of 17 kDa. It is a highly conserved tyrosine-sulfated protein, and due to its high content of γ-carboxyglutamate (Gla), it is also referred to as Gla-rich protein (GRP). *Ucma* gene expression seems to be restricted to the developing mouse skeleton (Tagariello et al. 2008). It is principally expressed in resting chondrocytes in distal and peripheral zones of epiphyseal and vertebral cartilage (Surmann-Schmitt et al. 2008). In mouse chondrocytes, four alternatively spliced variants were identified (Le Jeune et al. 2010). The four transcripts are co-expressed during chondrogenesis, and the kinetics of their expression was found to be different. It was suggested that each isoform is finely regulated and that *Ucma* may play a role in early chondrogenesis. In the chondrocytic cell line MC615, *Ucma* expression parallels largely the expression of collagen II and decreases with maturation toward hypertrophic cells. The fact that recombinant UCMA does not affect expression of chondrocyte-specific genes or proliferation of chondrocytes but interferes with osteogenic differentiation of primary osteoblasts leads to the conclusion that

UCMA may be involved in the negative control of osteogenic differentiation of osteochondrogenic precursor cells (Surmann-Schmitt et al. 2008). The use of a lacZ cassette under the control of the *Ucma* promoter revealed that *Ucma* is also expressed in adult rib cartilage (Eitzinger et al. 2012). In addition to cartilage expression, *Ucma* was detected in the trabecular bone of adult rats, both in osteoblasts and osteocytes, suggesting that *Ucma* is not a cartilage-specific gene (Viegas et al. 2015). In search of genes regulated by the master transcription factors in bone formation, *Runx2* and *Osterix*, *Ucma* was indeed identified as a novel downstream gene (Lee et al. 2015). In this study it could be demonstrated that UCMA stimulates osteoblast differentiation and nodule formation which is contrast to earlier investigations.

To unravel the function in cartilage, an UCMA-deficient mouse strain was generated, but although previous in vitro studies implied a role for UCMA in calcification and ossification, skeletal development was completely normal (Eitzinger et al. 2012). In zebrafish, two *Ucma* genes, *Ucmaa* and *Ucmab*, are present due to duplication of the whole genome. Interestingly, knockdown of *Ucmaa* is sufficient to induce severe growth retardation and perturbance of skeletal development. The cartilage of the morphants has a decreased aggrecan and collagen II content (Neacsu et al. 2011). Inhibition of glutamate γ-carboxylation using warfarin leads to a very similar phenotype, indicating that γ-carboxylation is crucial for UCMA function. It was speculated that UCMA might also play a role in human skeletal diseases, and indeed a functional SNP located in the basal promoter of the *Ucma* gene – which provided a weak genetic association with Paget's disease of bone – was identified (Michou et al. 2012). More recently, a clear association of undercarboxylated UCMA with osteoarthritic cartilage and synovial membrane was reported (Rafael et al. 2014).

3.5.2 Cartilage Intermediate Layer Protein (CILP)

CILP is a cartilage-specific protein of 82 kDa that is particularly enriched in the middle to lower zones of articular cartilage (Lorenzo et al. 1998). This restricted localization indicates that it might play a role in regulating structural and biomechanical properties of these zones. It is upregulated in osteoarthritis and therefore discussed as a biomarker for this disease (Lorenzo et al. 2004). Several mouse strains immunized with the CILP fusion proteins developed chronic arthritis, and CILP was implicated in cartilage destruction in subsets of patients with osteoarthritis and rheumatoid arthritis (Tsuruha et al. 2001). Its molecular function is still unclear, but it has been shown that CILP inhibits transcriptional activation of cartilage matrix genes in nucleus pulposus cells by binding to TGF-β1 and inhibiting the phosphorylation of Smads (Wang et al. 2012). Overexpression of CILP in the nucleus pulposus promotes disc degeneration, indicating that CILP plays a direct role in the pathogenesis of lumbar disc disease (Seki et al. 2014). Interestingly, a functional SNP in CILP is associated with susceptibility to lumbar disc disease (Seki et al. 2005).

3.6 Other Widely Expressed Proteins That Are Found in the Cartilage

3.6.1 Fibronectin

Fibronectin occurs as a monomeric soluble molecule with an MW of 450 kDa, but it also has the ability to self-interact and form independent polymeric fibrillar networks (Schwarzbauer and DeSimone 2011). It is not a cartilage-specific component, but a specific splice variant has been described in this tissue (Scanzello et al. 2015). It contains the classical RGD sequence and cell binding is mainly promoted via α5β1 integrin. In the cartilage, fibronectin interacts with several collagens including collagen IX (Parsons et al. 2011), decorin (Gendelman et al. 2003), matrix Gla protein (Nishimoto and Nishimoto 2014), and COMP (Di Cesare et al. 2002). It was shown recently that cell condensation and induction of chondrogenesis early in development critically depend on fibronectin matrix assembly (Singh and Schwarzbauer 2014). The fact that fibronectin is upregulated in degenerative joint diseases, such as osteoarthritis, suggests that it may have a functional role in matrix homeostasis. Interestingly, proteolytic fibronectin fragments induce catabolic events that might enhance cartilage breakdown under pathological conditions (Xie and Homandberg 1993).

3.6.2 Components of the Basement Membrane

Chondrocytes are surrounded by laminin, collagen type IV, nidogen, and perlecan, the four major components that define a basement membrane. It was proposed that chondrocytes, like several other cell types of mesenchymal origin, are surrounded by a functional equivalent of a basement membrane (Kvist et al. 2008). The role of this structure in the cartilage remains elusive, but it might well be that it is involved in maintaining the chondrocyte's phenotype and viability. Analysis of perlecan-deficient mouse models revealed that perlecan is essential for maintaining cartilage integrity and cephalic development (Arikawa-Hirasawa et al. 1999; Costell et al. 1999). The importance of perlecan in humans is further underlined by a functional null mutation in the perlecan gene causing dyssegmental dysplasia, Silverman-Handmaker type (Arikawa-Hirasawa et al. 2001). Further, functional and structural mutations in perlecan were identified that cause the Schwartz-Jampel syndrome, with myotonic myopathy and chondrodysplasia (Arikawa-Hirasawa et al. 2002). In late stages of knee osteoarthritis, perlecan are found to be upregulated on both protein and mRNA levels especially in the area adjacent to the main cartilage defect (Tesche and Miosge 2004).

In human embryos, nidogen-1 and nidogen-2 were detected in condensed mesenchyme and in rib anlagen. In healthy articular cartilage, a homogeneous staining for nidogen-1 was observed, while nidogen-2 was localized pericellularly. Interestingly, both the localization and the expression levels change during development of osteoarthritis. In late-stage OA cartilage, expression of nidogen-1 was

decreased pericellularly around diseased chondrocytes, whereas nidogen-2 was increased. However, both nidogens had strongly increased levels around elongated chondrocytes, especially in areas of deep surface fissures (Kruegel et al. 2008). Based on these observations, it was concluded that nidogens might play a key role in OA pathogenesis, and either nidogen could be used as a diagnostic marker.

3.7 Future Perspectives

The composition of the extracellular matrix in different types of cartilage at different developmental stages has been extensively characterized using transcriptomic, proteomic, and mass spectrometry approaches. Nevertheless, the exact physiological function of distinct matrix components remains still elusive. Some insight was provided by the generation and detailed characterization of genetically modified mouse models. However, there are still a lot of open questions that have to be addressed in the future. A deeper knowledge of how proteins of the extracellular matrix (1) influence the differentiation of stem cells and chondrocytes; (2) regulate cartilage vascularization, mineralization, and endochondral ossification; and (3) determine mechanical properties of cartilage will contribute to a better understanding of cartilage biology and homeostasis. Hopefully, this will allow a more efficient targeting of pathomechanisms involved in degenerative cartilage diseases and an improvement of the poor intrinsic repair capacity of cartilage.

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The Cartilaginous Growth Plate

4

Attila Aszódi

Abstract

The cartilaginous growth plate is a well-organized and highly specialized tissue which drives the longitudinal elongation of bones developing through endochondral bone formation. A tightly regulated differentiation program of chondrocyte proliferation, maturation, and hypertrophy associated with extracellular matrix production, mineralization, and degradation culminates in the replacement of cartilage with bone. Chondrocytes within the growth plate are organized into anatomically well-defined horizontal zones, which reflect their morphologically, biochemically, and transcriptionally distinct differentiation stages. The differentiation process is accompanied by the establishment of cellular anisotropy and planar polarity that generates the unique spatial structure of the tissue. Proliferative chondrocytes acquire an elongated shape, align and divide orthogonally to the direction of the growth, and arrange into vertical columns that in most vertebrates direct the elongation process. Chondrocyte differentiation and polarity are essential and mutually interacting foundations of the normal growth pate function, and their disturbance results in chondrodysplasias with impaired longitudinal growth. This chapter will focus on the mechanisms responsible for the establishment and maintenance of the structural polarity of the cartilaginous growth plate.

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4.1 Introduction

The growth plate, or physis, is a narrow discoidal area positioned between the epiphysis and diaphysis of early postnatal long bones. The complex structure of the growth plate evolves during endochondral bone formation, an embryonic and adolescence morphogenetic process in vertebrates which largely replaces the initially cartilaginous template of most skeletal elements with bone (endochondral ossification). The essential physiological role of the physis is to support longitudinal and lateral growth of the developing bones in a complex unit composed of the cartilaginous growth plate and the associated peripheral fibrous structures (Brighton 1978; Ballock and O'Keefe 2003). The rate of linear long bone growth depends on the spatially and temporally coordinated activities of growth plate chondrocytes, namely, proliferation, volume increase (hypertrophy), and extracellular matrix (ECM) production. Chondrocytes of the cartilaginous growth plate undergo a differentiation process in the direction of the longitudinal growth as they proceed through a series of metabolic, molecular, and morphological changes. The orchestrated nature of this differentiation process is reflected by the zonal appearance of the growth plate, while the directed growth is largely attributed to the unique flattened shape and columnar organization of proliferative chondrocytes. Among the over 400 skeletal conditions affecting the normal growth of bones (Warman et al. 2011), many of them manifested in morphological changes of growth plate chondrocytes including shape change (e.g., rounding) and loss of tissue orientation. Numerous molecular networks have been identified that control the morphogenesis of long bones through local and systemic signaling processes. This chapter will summarize the major mechanisms regulating the specialized cellular architecture of the cartilaginous growth plate which is essential for the proper proximodistal elongation of endochondral bones.

4.2 Structure of the Growth Plate

The postnatal growth plate is a complex anatomical structure and functional unit which drives the three-dimensional expansion of endochondral bones of the axial and appendicular skeleton. Traditionally, it can be divided into three interacting parts: the cartilaginous growth plate, the bony metaphysis, and the peripheral ossification groove of Ranvier.

4.2.1 The Cartilaginous Growth Plate

The fully developed cartilaginous growth plate is a well-organized and spatially polarized tissue, which extends the primary center of ossification, thus mainly responsible for the postnatal linear bone growth (Fig. 4.1e). In human endochondral bone, the cartilaginous growth plate is transient and gradually resolved after the end of puberty, while in other vertebrates (e.g., rodents), it exists as a senescent plate

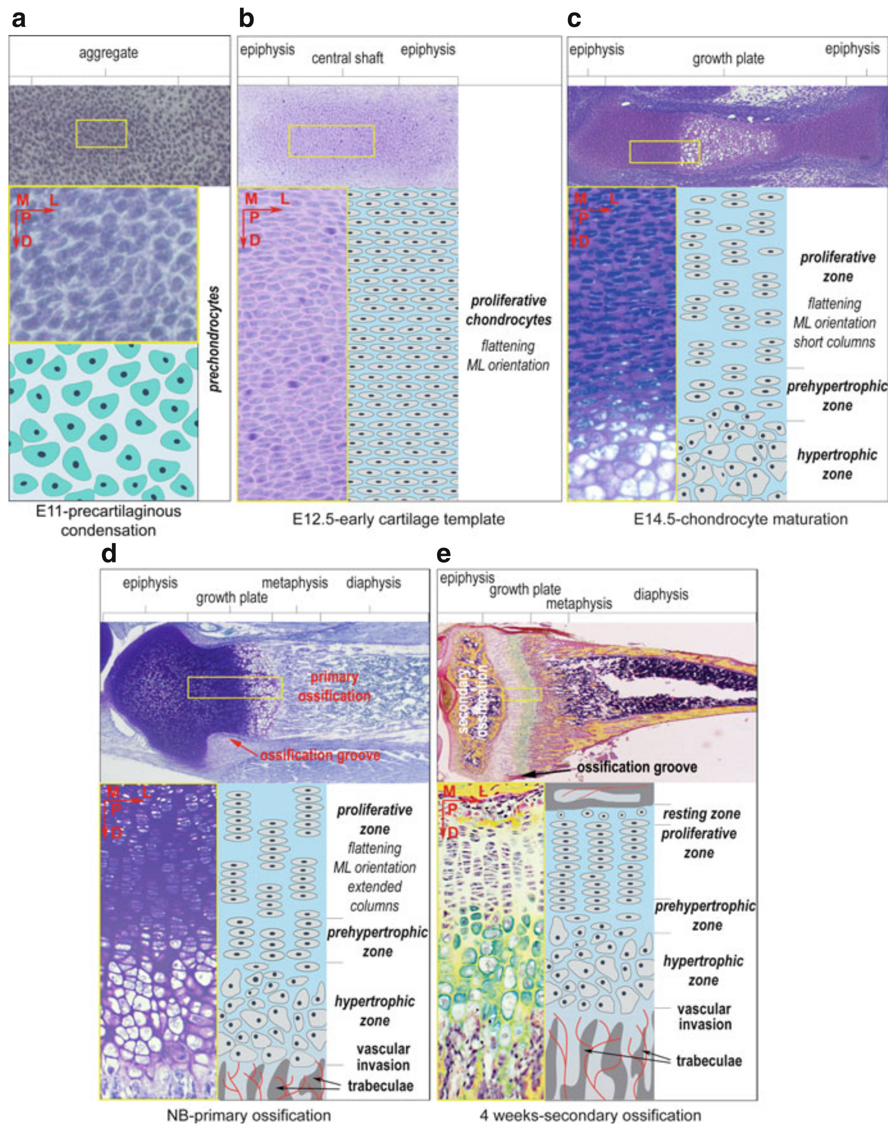

Fig. 4.1 Morphogenesis of the mouse growth plate during long bone development. (**a**) At embryonic day 11 (E11), mesenchymal precursor cells aggregate to form precartilaginous condensations. At this stage, the cells are polygonal in shape and show no apparent orientation relative to the mediolateral (*ML*) or proximodistal (*PD*) axes of the condensation. (**b**) By E12.5, the early cartilaginous template is differentiated. Chondrocytes in the central shaft are elongated along the ML axis of the cartilage anlage. (**c**) At E14.5, mirrored growth plates with a shared hypertrophic zone are visible. The flattened chondrocytes in the proliferative zone begin to form short columns along the PD axis, which is the direction of the longitudinal growth. (**d**) At the newborn stage (NB), the primary ossification center appears and the growth plate is anatomically separated into cartilaginous, bony (metaphysis), and peripheral (ossification groove) parts. Elongated vertical columns with mediolaterally elongated chondrocytes are prominent in the proliferative zone. (**e**) At 4 weeks (4w) of postnatal age, the secondary ossification center is formed. Columns in close vicinity may collide into a common, long stack of elongated chondrocytes

between the epiphysis and metaphysis throughout the life. Chondrocytes in the postnatal growth plate are organized into three basic horizontal zones, namely, resting, proliferative, and hypertrophic, which reflect their morphologically, metabolically, and transcriptionally distinct state in the differentiation program and into vertical columns which drives the elongation process via cell proliferation, hypertrophy, and matrix production (Hunziker et al. 1987; Hunziker and Schenk 1989; Hunziker 1994; Wilsman et al. 1996b). Frequently, a narrow transition area, the prehypertrophic zone is distinguished between the proliferative and the hypertrophic zones. The basic structure and dimension of the cartilaginous growth plate, and the relative contribution of its elongation activities to the growth, are slightly different from species to species, from site to site, and from postnatal stage to postnatal stage. For example, the cartilaginous avian growth plate is characterized by high cellularity and less matrix production resulting in less apparent columns, and its primary growth rate determining factor is the size of the proliferative zone (Howlett 1979; Kember et al. 1990; Farquharson and Jefferies 2000). In mammalians, due to the extensive matrix production, the columns are more distinct and the principal mechanism regulating linear growth rate is the volume change of hypertrophic chondrocytes (Breur et al. 1991; Hunziker 1994). Although mitotic activity continuously supplies the growth plate with new chondrocytes which produce cartilage matrix, mature, and undergo hypertrophy, the height of the physis is relatively constant owing to the dedicated balance between the intrinsic tissue growth and tissue resorption at the metaphyseal interface. Nevertheless, the postnatal growth plate is usually higher in fast-growing bones compared to slow-growing bones both timely (pubertal growth spurt versus end of puberty) and locally (tibia/femur versus vertebra/iliac crest). In general, the horizontal zones of resting, proliferative, prehypertrophic, and hypertrophic chondrocytes seem to be ubiquitous in all vertebrates; however, the columnar structure is not obligatory. In some amphibians such as the bullfrog *Rana catesbeiana* and the green frog *Rana esculenta*, the longitudinal extension depends on periosteal bone activity, and the cartilaginous growth plate, which lacks ordered columns, mostly contributes to lateral and not to the longitudinal bone growth (Dell'Orbo et al. 1992; Felisbino and Carvalho 2001).

The narrow region merging with the cartilaginous or bony epiphysis at the upper side of the cartilaginous growth plate (farthest from the diaphysis) is the resting zone (also called reserve or germinal zone). This zone is composed of small, single, or paired, usually rounded or slightly elongated, chondrocytes that are scattered randomly in an extensive extracellular matrix. Labeling experiments with nucleoside analogs, which incorporate into the DNA during the synthetic phase of the cell cycle, have indicated that reserve zone chondrocytes are slow cycling and divide very rarely (Kember 1971; Ohlsson et al. 1992; Candela et al. 2014). In adolescence mice, these slow-cycling cells reside in a tiny sublayer of the resting zone adjacent to the secondary ossification center (Candela et al. 2014). Based on these results, it has been long believed that the resting zone constitutes a pool of stem cell-like precursors for chondrocytes in the underlying proliferative zone. A direct, empirical evidence for the stem-like behavior of resting zone chondrocytes has been provided more than a decade ago. Using an elegant surgical procedure in rabbit, it was shown

that excision of the distal ulnar growth plate followed by the reimplantation of the resting zone to its original location resulted in complete or partial regeneration of the growth plate (Abad et al. 2002). Despite the doubtless proof of the resting zone as a germinal layer, the original source of the stem-like cells and the mechanisms responsible for their maintenance in the resting zone, however, are still unclear. It may be possible that these cells arrive from the bone marrow or from epiphyseal blood vessels of the secondary ossification center (Candela et al. 2014) or migrate from the perichondrial ring (see later) (Fenichel et al. 2006) or the zone retains epiphyseal cartilage with embryonic potential to generate the growth plate (Abad et al. 2002). Postnatal ablation of β-catenin in murine cartilage leads to the loss of labeled slow-cycling cells in the resting zone implicating that the Wnt/β-catenin signaling pathway plays an important role in the maintenance of the stem cell-like pool (Candela et al. 2014). Additionally, mice deficient for the receptor (Ppr1) of parathyroid hormone (PTH)/parathyroid hormone-related protein (PTHrP) in cartilage exhibit increased apoptosis of resting zone chondrocytes, while inactivation of the G-protein α-subunit, the stimulatory subunit of PPR-activated heterotrimeric G-proteins, induces proliferation of resting cells (Chagin et al. 2014). These results suggest that Ppr1signaling keeps stem cell-like chondrocytes in the resting zone quiescent and alive and that the activation of G-protein second message systems regulates the fate of those cells.

Surgical manipulations of the rabbit growth plate have also demonstrated that the resting zone produces a morphogen which directs the columnar arrangement of proliferative zone chondrocytes (growth plate orienting factor) and prevent their hypertrophic differentiation in the region adjacent with the resting zone (hypertrophy blocking factor) (Abad et al. 2002). Thus, the postnatal resting zone not simply separates the growth plate from the area of secondary ossification or stores nutrients for usage in lower zones, but it also actively participates in longitudinal growth by supplying precursors for proliferative zone chondrocytes and maintaining their columnar arrangements.

Chondrocytes in the proliferative zone, originated from progenitor cells of the resting zone, are strongly flattened, oriented with right angle to the direction of the growth, and undergo a clonal expansion along the longitudinal axis resulting in the characteristic columnar structure. Kinetic studies in rodent postnatal growth plates revealed that cells only in the upper half of the zone are mitotically active, whereas chondrocytes in the lower proliferative zone exit from the cell cycle and undergo a maturation process (Vanky et al. 1998). The average cell cycle time depends on the location of the growth plate and ranging between 24 and 76 h (Vanky et al. 1998; Wilsman et al. 1996a). The proliferative cells entering from the resting zone divide in average four times and then move down to replace the eliminated hypertrophic chondrocytes from the distal growth plate (Farnum and Wilsman 1993). Numerous growth factors and hormones regulate chondrocyte proliferation and maturation through the control of cell cycle and differentiation genes. The main signaling and transcriptional pathways interconnecting proliferation and differentiation are discussed in Chap. 8. In addition to the rapid proliferation, chondrocytes in the proliferative zone synthesize the bulk of cartilage matrix mainly composed of collagen II and aggrecan.

In the prehypertrophic zone, chondrocytes begin to increase their size and express specific markers such as Indian hedgehog (Ihh) and parathyroid hormone/parathyroid hormone-related protein receptor (Ppr1) which are involved in the regulation of chondrocyte differentiation (for further details, see Chap. 8). Most cells in this zone still deposit collagen II and aggrecan together with the hypertrophic marker collagen X. In the hypertrophic zone, the cells further enlarge in volume by a factor of 5–10 and become ovoid in shape (Hunziker 1994). Hypertrophic chondrocytes stop expressing collagen II and aggrecan and deposit collagen X instead. The fate of terminally differentiated chondrocytes at the chondro-osseous junction is dual: some cells undergo programmed cell death, while others are able to transdifferentiate into osteoblasts (Park et al. 2015; Zhou et al. 2014; Yang et al. 2014) and contribute to the formation of trabecular bones at the metaphysis (for further details, see Chap. 5). The matrix between the hypertrophic columns is mineralized, and chondrocytes in the last cell layers of the hypertrophic zone express vascular endothelial growth factor (VEGF) and matrix metalloproteinase 13 (Mmp13) which initiate vascular invasion (for details, see Chap. 8) and matrix degradation (Ortega et al. 2004), respectively. Vascular invasion is followed by the recruitment of endothelial cells, chondroclasts, and osteoblast precursors, which remodel the cartilage into trabecular bone.

4.2.2 The Metaphysis

At the level of the vascular invasion begins the metaphysis, usually a narrow area between the cartilaginous growth plate and the diaphysis, where the primary ossification is taking place (Brighton 1978; Ballock and O'Keefe 2003). The metaphysis can be divided into two distinct regions: the primary spongiosa continuous with the lower part of the hypertrophic zone and the secondary spongiosa just adjacent to the diaphysis. At the primary spongiosa, the forming trabeculae consist of mineralized cartilage spicules, which are the extensions of the partially degraded intercolumnar areas of the hypertrophic zone. These cartilage remnants serve as a scaffold for the deposition of a thin bone layer by osteoblasts. At the secondary spongiosa, the cartilage spicules are gradually resorbed, and the primary trabeculae are remodeled into lamellar trabecular bone. The metaphysis besides being the site of active bone remodeling provides continuity between the cartilage and bone and gives support to the growth plate.

4.2.3 The Ossification Groove of Ranvier

At the periphery, a fibro-chondro-osseous structure surrounds the cartilaginous growth plate which consists of the wedge-shaped groove of Ranvier and the ring of LaCroix (Brighton 1978; Langenskiold 1998). Anatomically, the groove of Ranvier is associated with the upper half of the growth plate and continuous with LaCroix's perichondrial ring which merges with the periosteum of the metaphysis. Functionally,

the zone of Ranvier is a reservoir of progenitor cells and contributes for the circumferential (or latitudinal, diametric) growth of bones (Karlsson et al. 2009; Shapiro et al. 1977), while the ring of LaCroix predominantly provides mechanical stability at the cartilage-bone junction of the growth plate (Rodriguez et al. 1985). The groove histologically can be divided into three regions: (1) the innermost, densely packed cells, which are likely progenitors for osteoblasts that form the bony bark of the perichondrial ring; (2) a more dispersed area with chondrogenic progenitors that may differentiate into chondrocytes and which is responsible for the appositional growth of the cartilaginous growth plate; (3) and the outermost fibrous sheet with oriented collagen fibers which is continuous with the outer layer of the periosteum and the perichondrium (Shapiro et al. 1977).

The importance of the peripheral structures in bone growth has been well documented. Fractures within the groove of Ranvier (Riseborough et al. 1983; Ilharreborde et al. 2006) or removal of the ring of LaCroix (Rodriguez et al. 1985) results in severe growth disturbances. Stem cell-like, slow-cycling progenitor cells have been identified in both the resting zone of the cartilaginous growth plate and the ossification groove of Ranvier (Karlsson et al. 2009; Candela et al. 2014; Peltomaki and Hakkinen 1992). Although some studies have suggested that progenitors migrate from the resting zone toward the groove, today there is a consensus that the ossification groove of Ranvier can be regarded as a specific stem-cell niche, from where the progenitor cells migrate toward the growth plate cartilage and, possibly, toward the articular cartilage (Candela et al. 2014; Karlsson et al. 2009; Fenichel et al. 2006; Robinson et al. 1999). Mesenchymal progenitor markers including CD90, CD105, STRO-1, and Jagged1 were identified in the zone of Ranvier (Karlsson et al. 2009; Walzer et al. 2014) but not in apical cells just above. These cells are distinct from the cells of surrounding tissues and likely act as transient amplifying cells which later terminally differentiate and disperse throughout the cartilage (Walzer et al. 2014; Karlsson et al. 2009).

4.3 The Morphogenetic Sequence of Growth Plate Development

The polarized and striated appearance of the postnatal growth plate is established during the multistep process of endochondral bone formation, which lays down most bony elements via a cartilaginous intermediate in vertebrates (Lefebvre and Bhattaram 2010). This paragraph will focus primarily on the development of the cartilaginous growth plate in the context of chondrogenesis and chondrogenic differentiation. For deeper insight of additional aspects of endochondral bone formation, the reader should refer to Chaps. 5, 6, 7, and 8 in this volume.

The formation of the cartilaginous anlagen is well studied in the developing limbs of mice and chicken. The process begins with the migration of skeletogenic mesenchymal stem cells to the sites of the future bones, where they aggregate and form precartilaginous condensations (Fig. 4.1a). At this stage, the condensed mesenchymal cells are isotropic in appearance. The rounded or polygonal cells do not

show an obvious orientation; however, the Golgi-nucleus axis, as a measure of internal polarity, is biased toward the longitudinal axis of the aggregates (Holmes and Trelstad 1980) suggesting that early condensations form along a central line. In the next step, prechondrocytes differentiate into chondrocytes which start depositing cartilage-specific molecules such as type II collagen and sulfated proteoglycans. The early chondrocytes are highly proliferative (therefore, they are also named chondroblasts) and exhibit clear flattening in the central, cylinder-like part of the cartilaginous template (Fig. 4.1b). The chondrocytes show a tendency to elongate along the mediolateral axis of the cartilaginous mold and thus orient their long cell axis and their Golgi-nucleus axis (Trelstad 1977), nearly perpendicular to the direction of the longitudinal growth. The special arrangement of any cells or cell structures in a given tissue with respect to the tissue/body axes is generally referred to as tissue/planar cell polarity (PCP) (Wang and Nathans 2007; Gao and Yang 2013). Thus, cell anisotropy and PCP appear simultaneously in the early cartilaginous templates. At the next developmental stage, most central chondrocytes stop to proliferate, increase in volume, and become hypertrophic (Fig. 4.1c). Now club-shaped expansions at the ends of the anlage are clearly visible (the epiphyses) which are separated by an extended middle shaft (the diaphysis). The diaphysis is fully occupied by the proximal and the distal growth plates which merge in a common hypertrophic zone. The mediolaterally elongated proliferative chondroblasts of the plates are continuous with the rounded, non-oriented chondroblasts of the epiphyses. In the embryonic epiphyseal cartilage, the chondrocytes are frequently referred to as resting, but due to their relatively high proliferative activity, this is misleading and it may be more correct to use the terminology "non-columnar chondrocytes." In the proliferative zone of the growth plate, chondrocytes undergo the next level of organization forming short, longitudinally arranged stacks of 2–3 discoid chondrocytes. In subsequent steps, the hypertrophic core is mineralized and invaded by vessels, and the cartilage is gradually replaced by bone. At around birth, the primary ossification center is flanked with the growth plates which have now elongated vertical columns containing strongly flattened, mediolaterally oriented chondrocytes in the proliferative zone (Fig. 4.1d). Postnatally, secondary ossification centers form in the epiphyses, and the typical growth plate structure with resting, proliferative, prehypertrophic, and hypertrophic zones appears. The columns may further extend through internal elongation or by the collision of shorter columns (Fig. 4.1e) (Johnstone et al. 2000).

The internal elongation of the column in the proliferative zone occurs through oriented cell division followed by extensive intercalation movements of the daughter chondrocytes (Fig. 4.2) (Ahrens et al. 2009; Li and Dudley 2009). In contrast to most cell types in the body which round up during mitosis, proliferative chondrocytes stay flattened and divide according to Hertwig's rules postulating that (1) the axis of the mitotic spindle takes "the direction of the greatest protoplasmic masses" and (2) "the plane of division always cuts the axis of the spindle perpendicularly" (Hertwig 1893). Dictated by the unique cell geometry, column extension follows the classical Dodds model, which was named after the American anatomist who first described the behavior of dividing proliferative chondrocytes in 1930 (Dodds 1930).

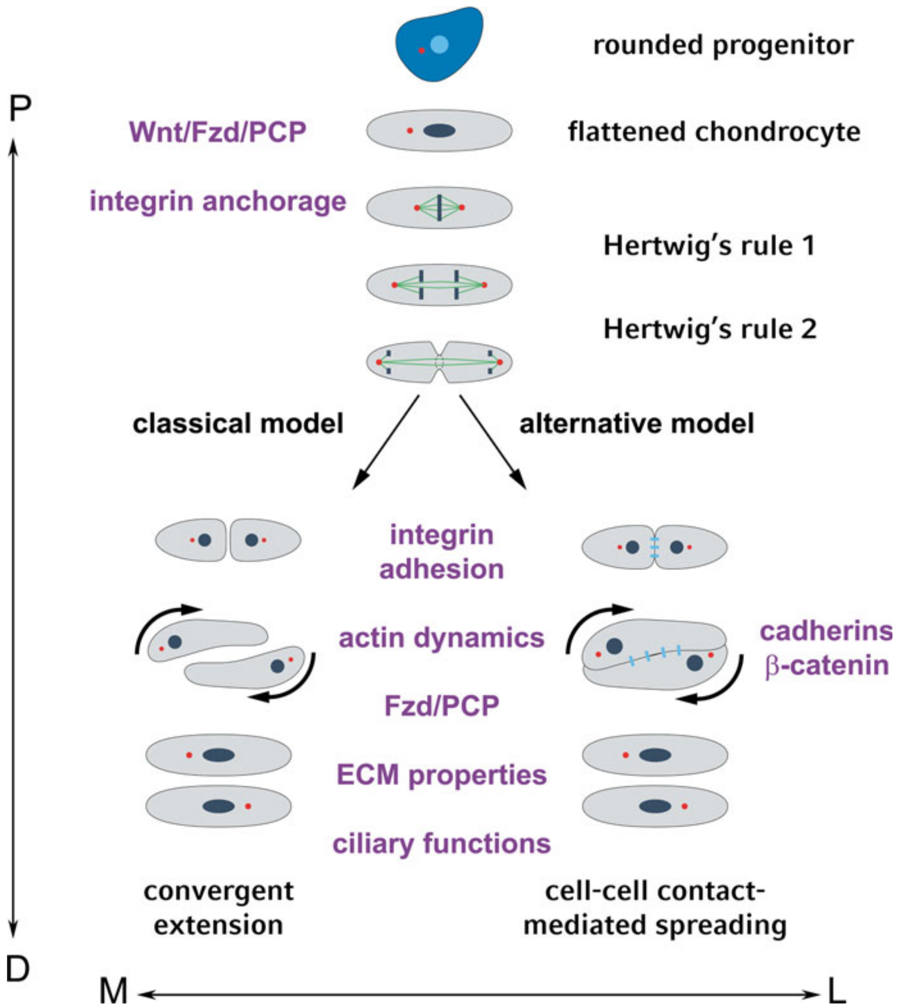

Fig. 4.2 Models of column formation. Column progenitor cells differentiate into proliferative chondrocytes which elongate along the mediolateral (*ML*) axis, perpendicular to the longitudinal, proximodistal (*PD*) direction of the growth. Mitotic figures align along the long cell axis, while the division plane cut the spindle axis perpendicular (Hertwig's rules). In the classical convergent extension model, after cell division, the daughter chondrocytes undergo ML intercalation movements to extend the column in the PD direction. In an alternative model, post-division chondrocytes rotate back to the column through a transient, cell-cell contact-mediated spreading. Regulatory mechanisms controlling chondrocyte polarity and postmitotic rearrangements are indicated

The model states that (1) mitotic figures in the mediolaterally elongated chondrocytes lie perpendicular to the longitudinal, proximodistal direction of the growth; (2) the cell division plane is parallel to the columns; (3) semicircular daughter cells lie in a plane orthogonal to the longitudinal columnar axis; and (4) through a gradual increase of length-to-width ratio, the daughter cells intercalate back to the same

vertical raw. This model of oriented chondrocyte division and mediolateral intercalation is highly similar to convergent extension, a morphogenetic process which leads to tissue narrowing (convergence) and elongation (extension) (Keller et al. 2000) during the development of many organs.

Taken together, three levels of structural organization can be observed in the proliferative zone of cartilaginous growth plate: (1) flattening of the chondrocytes (cell anisotropy), (2) their alignment and division are oriented at right angles to the longitudinal direction of the growth (tissue/planar cell polarity), and (3) their columnar stacking along the long axis.

4.4 Regulation of Growth Plate Polarity

4.4.1 Morphogens and Transcription Factors

Extensive studies in the last decade have revealed that growth plate polarity primarily depends on the morphogenetic signals provided by the Wnt signaling network (Romereim and Dudley 2011; Gao and Yang 2013; Yang and Mlodzik 2015). Wnt signaling is composed of numerous components including secreted Wnt ligands, specific receptors, antagonists, and intracellular effectors. Classically, two major arms are activated: the canonical pathway acting through the stabilization of its core component β-catenin and the β-catenin-independent noncanonical pathway. This latter branch is further splitted into the Wnt/Ca^{2+} cascade which regulates intracellular Ca^{2+} release and into the Wnt/PCP cascade involved in diverse morphogenetic processes controlling polarized cellular behavior, oriented cell division, and convergent extension movements. Genetic studies have revealed that all the three Wnt pathways are involved in cartilage development, but only the Wnt/PCP pathway is implicated in the regulation of growth plate polarity (for further details for Wnt signaling in cartilage function, see Chap. 10). PCP in vertebrates is operated by a set of proteins including the Wnt receptor Frizzled (Fzd) seven-pass transmembrane family members, the Wnt coreceptor Ror1/2 kinases (receptor tyrosine kinase-like orphan receptor 1/2), the Fzd-interacting four-pass transmembrane protein Vangl1/2 (Van Gogh-like 1/2), and the cytosolic components Dishevelled (Dvl) and Prickle (Pk). In order to generate planar chondrocyte alignment in the developing cartilage, PCP signaling should involve, similar to other tissues, (1) a tissue level orientation cue, (2) asymmetric cellular distribution of PCP proteins, and (3) translation of polarity information into polarized outputs (Devenport 2014). In the mouse limb bud, cartilage templates of the digits elongate in the proximodistal direction along a distal-to-proximal morphogenetic gradient of Wnt5a (Gao et al. 2011; Parr et al. 1993). The Wnt5a signaling gradient is sensed by a Ror2-Vangl2-Fzd receptor complex, which induces the gradient of Vangl2 phosphorylation in a Wnt5a dose-dependent manner. Changes in Vangl2 activity switch the membrane localization of Vangl2 from symmetric (on non-oriented mesenchymal cells) to asymmetric on newly differentiated, mediolaterally elongated chondrocytes (Gao et al. 2011). The genetic interaction between Wnt5a/Ror2 signaling and the PCP pathway in the

control of cartilage polarity has been demonstrated in knockout mice. Mice lacking *Wnt5a* or *Ror2* lose the asymmetric localization of Vangl2 to the proximal side of chondrocytes (Gao et al. 2011) and display short limbs associated with altered growth plate morphology (DeChiara et al. 2000; Takeuchi et al. 2000; Yamaguchi et al. 1999; Yang et al. 2003). Wnt5a-, Ror2-, or Vangl2-deficient chondrocytes fail to flatten and stack into columns suggesting that the reduced proximodistal elongation of limbs is caused by disrupted PCP in chondrocytes.

Supportive evidence for the involvement of Wnt5a-/PCP-mediated regulation of cartilage morphogenesis stems from observations of craniofacial morphogenesis in zebrafish. During development, cartilaginous head elements have flattened chondrocytes forming stacks by intercalation (Kimmel et al. 1998). *Wnt5* (*pipetail*) or *Knypek* mutants display rounded and disorganized chondrocytes indicating defective PCP (Piotrowski et al. 1996; Topczewski et al. 2001). *Knypek* encodes a zebrafish glypican, a member of glycosyl-phosphatidylinositol (GPI)-anchored cell surface heparin sulfate proteoglycans, which has been implicated in functional interactions with the noncanonical Wnt11 and Wnt5 (Topczewski et al. 2001) and with trilobite/Vangl2 in movements of convergent extension (Marlow et al. 1998). Ablation of *Piga* in mice, which codes for an essential enzyme of GPI biosynthesis, also leads to rounding and the lack of intercalation movements of proliferative growth plate chondrocytes (Ahrens et al. 2009).

In addition to the essential role of PCP signaling in the establishment of cellular orientation in the differentiating cartilage anlage, further studies showed its importance in maintaining polarity in the growth plate. Retroviral-mediated downregulation of *Frzd7* and *Dvl* or overexpression of *Frzd7* and *Vangl2* in chicken growth plate resulted in the failure of orientation and cell division of proliferative chondrocytes along the mediolateral axis, which in turn disrupted the longitudinal columnar arrangement (Li and Dudley 2009). Interestingly, it has been recently shown that activation of the Wnt/PCP pathway in chondrocyte pellet culture in vitro promotes chondrocyte elongation and columnar organization (Randall et al. 2012). While normal pellet culture did not exhibit any specific cellular orientation, the combined administration of Wnt5a with the forced expression of *Fzd7* and *Ror2* in chondrocytes led to columnar architecture reminiscent of the native growth plate. Together, these findings demonstrated that the Wnt/PCP cascade has a central role for column formation, and when it is disrupted, growth plate polarity is severely impaired.

Additional studies have identified molecular players upstream of Wnt5a which potentially modulate the PCP pathway during cartilage morphogenesis. The naturally occurring *spdh* (synpolydactyly homolog) mice, which carry a loss-of-function mutation of the *Hoxd13* gene, display an elongation defect of their metacarpal bones. HOXD13 is a master transcription factor of skeletal morphogenesis at the distal part of the limb and its absence in humans leads to synpolydactyly. Both *Wnt5a* null and *spdh/spdh* mice are characterized by defective polarity in metacarpal tissues including the lack of flattening and columnar arrangement of proliferative chondrocytes in the growth plate (Kuss et al. 2014). Besides the phenotypic similarities of the mutants, *spdh/spdh* mice show downregulation of *Wnt5a* in the hand plates and mislocalization of the PCP components Dvl2 and Prickle1 in

proliferative chondrocytes. Hoxd13 and Wnt5a are partially coexpressed in the hand plate mesenchyme, and in *spdh/spdh* limb explants, the implantation of *Hoxd13* or *Wnt5a* expressing cells partially rescues the polarity phenotype (Kuss et al. 2014). Asymmetric distribution of the "PCP indicator" Vangl2 in proliferative growth plate chondrocytes is also disturbed in mice with conditional ablation of SoxC genes encoding the transcription factors Sox4, Sox11, and Sox12 (Kato et al. 2015). SoxC genes are required for perichondrial expression of Wnt5a and for the expression of PCP components *Frzd3* and *Csnk1e* in chondrocytes. Deletion of SoxC in skeletogenic mesenchymal cells results in the lack of differentiated growth plates, whereas SoxC ablation in chondrocytes impairs the formation of elongated columns and leads to premature rounding up of proliferative cells.

4.4.2 The Extracellular Matrix

The cartilaginous growth plate has an elaborated and specialized extracellular matrix, which is entirely produced by the chondrocytes. The components of the ECM can be divided into three major molecular classes which are detailed in the previous chapters: proteoglycans (Chap. 1), collagens (Chap. 2), and non-collagenous glycoproteins (Chap. 3). These molecules are organized into specific macromolecular assemblies providing the tissue with its unique properties. The basic structural and mechanical functions of the matrix are determined by the highly hydrophilic proteoglycan gel, composed of mainly the chondroitin sulfate proteoglycan (CSPG) aggrecan-link protein-hyaluronan aggregates (for details, see Chap. 1), which are embedded into an insoluble, heterotypic collagen fibrillar network containing collagens II, IX, and XI (for details, see Chap. 2). The high density of negatively charged sulfated glycosaminoglycan side chains of aggrecan attracts cations and water to the tissue generating an internal hydrostatic pressure which withstands compressive forces. The collagen meshwork reinforces proteoglycan swelling and is responsible for the resistance against tensile and shear forces. The two macromolecular networks are extensively interconnected and regulated through small proteoglycans (see Chap. 1) and adhesive/nonadhesive glycoproteins (see Chap. 3). Chondrocytes interact with ECM structural molecules via surface proteins including integrins (receptors for collagens and fibronectin), CD44 (receptor for hyaluronan), and discoidin domain receptor 2 (DDR2, receptor for collagen II). The matrix, besides giving structural support to the cells, allows diffusion of nutrients; sequesters growth factors, morphogens, and cytokines; and presents them to the corresponding receptors upon inductive stimuli. Furthermore, an increasing body of evidence indicates that the macro- and micro-mechanical environment determined by the ECM dictates chondrocyte fate and morphology in the cartilage. Thus, the ECM plays indispensable roles in instructing chondrocytes through mechanical and chemical signals.

The ECM in the growth plate, similar to the chondrocytes, undergoes a differentiation process and displays zone-specific anisotropy of the collagen network (Hunziker 1994; Eisenstein et al. 1971; Eggli et al. 1985). In the resting zone, the

matrix is relatively nonstructured, while in the proliferative and hypertrophic zones, the ECM is organized into longitudinal (in between the columns) and transverse (inside the columns) septa. In each zone of the mature growth plate, ECM molecules are arranged into various compartments with distinct biochemical composition and, depending on the visualization methods, with distinct structural intricacies (Hunziker et al. 2014). The narrow envelope immediately surrounding the chondrocytes is the pericellular matrix (PCM) which is rich in proteoglycans and type VI collagen. On classical transmission electron micrographs, this compartment is largely devoid of fibrils; however, cryopreservation techniques or atomic force microscopy which preserve better the native composition and structure of the ECM revealed that this area is filled up with very fine collagen fibrils (Prein et al. 2015; Hunziker et al. 1996; Nicolae et al. 2007). The adjacent territorial matrix (TM) compartment encompassing the PM has a collagen meshwork with fine fibrils which are randomly oriented in the transverse septa, but they tend to align along the direction of the growth in the longitudinal septa. The PM and TM together define the chondron, the functional unit of the cartilage, which is practically corresponding to the individual, stacked chondrocyte column in the proliferative zone (Hunziker 1994). The interterritorial matrix (ITM) compartment constitutes the bulk of the longitudinal septa characterized by thick fibrils oriented parallel to the columns (Eggli et al. 1985). Atomic force microscopy, which is capable to simultaneously monitor structural and mechanical properties of the cartilage matrix, has revealed that the stiffness in the proliferative zone of the murine growth plate gradually increases both in the longitudinal and the transverse septa during development. The gradually elevated stiffness of the PM/TS compartments was correlated well with the timely coordinated flattening of the proliferative chondrocytes suggesting that the increasing pressure exerted by the matrix may contribute to the change from roundish to elongated shape of chondrocytes and could deform the columnar chondrocytes along the mediolateral direction (Prein et al. 2015; Gould et al. 1974). In addition, it has been also found that the stiffness of the longitudinal septa always exceeds the stiffness of the transverse septa. This spatially defined mechanical heterogeneity of the matrix compartments led to the hypothesis of a simple model which can partially explain intercalation movements of columnar chondrocytes after cell division (Fig. 4.3) The higher matrix stiffness in the longitudinal septa may mechanically resist chondrocyte migration and elongation into the intercolumnar region and eventually force daughter cells to rotate and subsequently flatten in the less stiff intracolumnar matrix (Prein et al. 2015).

Evidence obtained from heritable human chondrodysplasias and from mice with naturally occurring or induced genetic mutations suggests that all ECM molecular classes contribute to the establishment and/or maintenance of normal growth plate structures. Mutations in genes coding for the components of the heterotypic type II/IX/XI collagen fibrils cause chondrodysplasia with severity ranging from lethal to moderate. Ablation of the *Col2a1* gene encoding the α1 chain of the homotrimeric collagen II in mice (Aszodi et al. 1998; Li et al. 1995a; Talts et al. 1998) or functional null mutations of the corresponding *COL2A1* gene in human achondrogenesis type II patients (Chan et al. 1995) lead to lethality at birth due to a structurally and

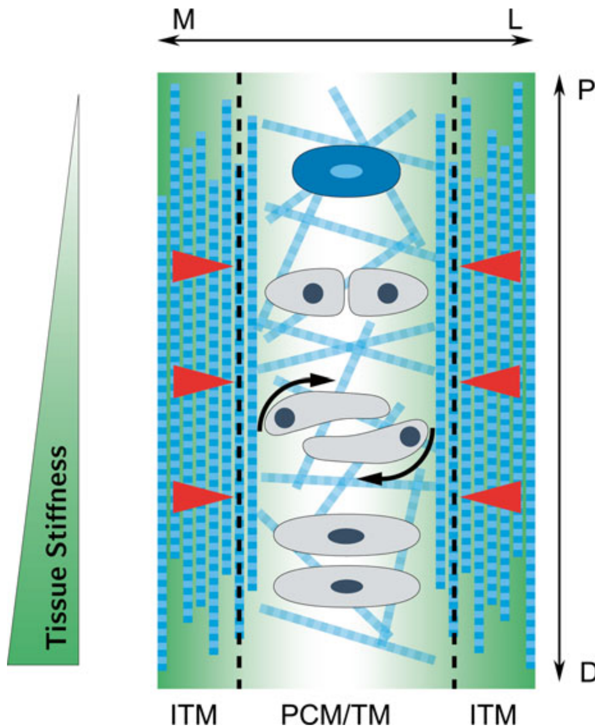

Fig. 4.3 Model for the role of extracellular matrix biomechanics for chondrocytes rearrangement. The anisotropic organization of extracellular matrix generates differential stiffness distribution. The higher stiffness in the intercolumnar interterritorial matrix (*ITM*) mechanically resists chondrocyte expansion along the mediolateral (*M-L*) axis. Postmitotic daughter cells are forced to intercalate back into the softer intracolumnar area. *PCM* pericellular matrix, *TM* territorial matrix, *P-D* proximodistal axis (Modified from Prein et al. (2015))

functionally destroyed cartilaginous skeleton. Collagen II-deficient templates lack a discernable collagen network resulting in very soft cartilage. The mutant cartilage is unable to elongate properly owing to misshaped and misoriented chondrocytes which never form columns. The naturally occurring *cho/cho* mice, which have fewer and thicker collagen fibrils in the cartilage matrix due to a premature stop codon at the N-terminal region of the α1(XI) chain of collagen XI, exhibit a similar growth plate phenotype and perinatal lethality (Li et al. 1995b). Collagen IX deficiency does not cause lethality but results in abnormal collagen fibrils and proliferative chondrocyte rounding in the growth plate of young animals associated with significant softening of the cartilage (Kamper et al. 2015; Dreier et al. 2008; Blumbach et al. 2008). Impaired columnar arrangement, chondrocyte shape change, and softer ECM in the proliferative zone are also characteristics for mice with mutations in genes encoding collagen prolyl 4-hydroxylases, which catalyze formation of 4-hydroxyproline residues required to form triple-helical collagen molecules (Aro et al. 2015). These mouse mutants imply that the proper collagen network is

essential for growth plate morphogenesis and underlie the importance of a mechanically functional ECM in proper growth plate polarity and endochondral bone formation.

The lack of the functional CSPG aggrecan in spontaneous mouse strains carrying recessive mutations in the *Acan* gene leads to perinatal lethality owing to cleft palate and severe impairment of endochondral bone formation (Watanabe et al. 1994; Krueger et al. 1999). In contrast to collagen II mutants which display laterally expanded and soft cartilage, the absence of the major proteoglycan aggrecan in the ECM is characterized by compression of the cartilage and a greatly increased chondrocyte-to-matrix ratio (Lauing et al. 2014). The growth plates devoid any organization, lack columns, and contain rounded cells accompanied by defects in hypertrophic differentiation and alterations in the expression pattern of cartilage-specific genes. The binding of aggrecan to hyaluronan is stabilized by the link protein, a glycoprotein showing structural similarities to the G1 domain of aggrecan (for further details, see Chap. 1.) Cartilage link protein null mice develop lethal chondrodysplasia and have abnormal growth plates with a proliferative zone lacking columnar structures and intermingled prehypertrophic and hypertrophic zones (Watanabe and Yamada 1999). Perlecan is a heparan sulfate proteoglycan (HSPG) which is present in the growth plate cartilage. Perlecan null mice die at two stages: at E 10.5–12.5 due to basement membrane defects and perinatally due to respiratory failure caused by severe chondrodysplasia. The growth plate of perlecan null mice is disorganized associated with impaired chondrocyte proliferation, differentiation, and reduced density of the collagen network in ECM (Costell et al. 1999; Arikawa-Hirasawa et al. 1999). Many mouse strains carrying mutations in genes which affect biosynthesis of the glycosaminoglycan side chains of cartilage proteoglycans exhibit chondrodysplasia and growth plate polarity defects by modulating morphogen signaling and/or ECM organization (Kluppel et al. 2005; Mizumoto et al. 2014; Hiraoka et al. 2007; Koziel et al. 2004).

Glycoproteins act as adaptors interconnecting collagen-aggrecan complexes or directly bind surface receptors to fine-tune the ECM structure and modulate chondrocyte behavior, respectively. Members of the thrombospondin (Tsp) and matrilin (Matn) families of glycoproteins are present in cartilage (for further details, see Chap. 3) suggesting functional roles of these molecules in endochondral bone formation. The analysis of single or multiple knockout mice for thrombospondins revealed that *Tsp1*, *Tsp3*, *Tsp5* (also named *cartilage oligomeric protein*, *Comp*), *Tsp3/Tsp5*, and *Tsp1/Tsp3/Tsp5* mutants all show, albeit very mild growth plate disorganization in agreement with the apparently normal skeletal growth in these mouse strains (Posey et al. 2008). Similarly, mice deficient in *Matn1*, *Matn3*, and *Matn1/Matn3* have no skeletal abnormalities despite the mild disorganization of the collagen fibrillar network in the growth plate of double mutant mice (Nicolae et al. 2007). The phenotype is more dramatic in compound mutants of thrombospondins and collagen IX (Posey et al. 2008). *Tsp3/Tsp5/Col9a1* mutants display significant reduction of limb growth and more severe disruption of the columnar structure than the single mutants. Interestingly, all *Col9a1* mutant strains incorporate very little matrilin-3 into the cartilage matrix; thus the absence of rather complex molecular

associates involving Tsp3, Tsp5, Col9a1, and Matn3 in the ECM could significantly perturb growth plate organization.

4.4.3 Integrin-Mediated Cell-Matrix Interactions

Integrins are heterodimeric, transmembrane cell adhesion molecules composed of α and β subunits (Legate et al. 2009). There are 18 α and 8 β subunits, which can combine into 24 different heterodimers and bind ECM components such as collagens, fibronectin, and laminins or interact with counterreceptors on adjacent cells. The twelve β1 integrin-containing receptors form the biggest integrin subfamily and ablation of the *Itgb1* gene coding for the β1 integrin in mice leads to peri-implantation lethality. In general, integrins connect the ECM to the intracellular actin cytoskeleton providing the mechanical basis for anchorage, cell shape determination, force transmission, and migration. Integrins transmit extracellular chemical and mechanical signals into the cells (outside-in signaling) through multiprotein adhesion complexes associated with integrin cytoplasmic tails and trigger various signaling cascades which, in cooperation with growth factor signaling, control cellular differentiation, proliferation, and survival. To effectively sense changes in the cellular microenvironment, integrin-ligand binding and subsequent signal transduction are dynamically regulated via the control of integrin activation involving a series of events referred to as inside-out signaling. Thus, integrins are central molecular hubs which utilize a bidirectional signaling machinery to modulate cellular differentiation and tissue organization.

Growth plate chondrocytes express various integrin receptors for cartilage matrix ligands such as α1β1, α2β1, and α10β1 for collagen II; α5β1, αvβ3, and αvβ5 for fibronectin; and α6β1 for laminin. In vitro experiments have revealed that β1 integrins may play an essential role in various aspect of cartilage development. Thus, β1 integrin-blocking antibodies prevented the formation of cartilaginous nodules from limb bud mesenchymal cells in micromass culture, reduced chondrocyte growth, increased apoptosis, disrupted actin cytoskeleton, and impaired hypertrophic differentiation in chicken sternal organ culture. During monolayer culture, chondrocytes lose their phenotype and dedifferentiate into fibroblasts which can be monitored through the relative expression of the chondrocyte-specific α10β1 integrin and fibroblast-specific α11β1 integrin (16). Culturing chondrocytes in the presence of bone morphogenetic protein-2 stabilizes the chondrogenic phenotype and high levels of α10 expression (16). In line with this observation, chondrocytes maintain their polygonal shape and differentiated phenotype when they are cultured in tissue culture dishes coated with collagen II implicating that collagen-binding β1 integrins are important for maintaining the chondrogenic phenotype (17).

Although the importance of β1 integrin for cartilage development and function was clearly demonstrated in vitro, its precise in vivo role during endochondral bone formation was only revealed in mice with conditional inactivation of *Itgb1* in chondrocytes using the Cre transgene driven by the collagen II promoter (*Col2a1cre*) (Aszodi et al. 2003). The majority of mutant mice died at birth due to severe

chondrodysplasia caused by multiple defects in growth plate polarity and function. Most importantly, the columnar organization and mediolateral orientation of proliferative chondrocytes were completely diminished in the mutants accompanied by cell rounding. In the absence of collagen-binding heterodimers, mutant chondrocytes could not adhere to the collagenous matrix which likely accounts for rounding of the normally flattened chondrocytes and the lack of intercalation movements. As integrins are crucial linkers between the ECM and intracellular cytoskeletal networks, it is not surprising that the absence of β1 integrins on chondrocytes also led to the disruption of the cortical actin cytoskeletal ring. Actin organization and dynamics in chondrocytes are regulated through the family of Rho GTPases (for details, see Chap. 11). Interestingly, the lack of Rac1 (Wang et al. 2007) or Cdc42 (Suzuki et al. 2015) in chondrocytes results in phenotypes which partially resemble those seen in the β1 integrin-deficient growth plate including chondrocyte shape change and disorganized columnar structure. β1 integrin-deficient growth plate chondrocytes also displayed frequent binucleation indicating a cytokinesis defect. It has been shown that integrin trafficking mediated with the small GTPase Rab21 to and from the cleavage furrow is necessary for normal cell division (Pellinen et al. 2008). As β1 integrin has been localized to the furrow in chondrocytes (Aszodi et al. 2003), it is tempting to speculate that β1 integrins may play a direct role for actomyosin ring constriction in the furrow to complete chondrocyte division. Additionally, the analysis of the β1 integrin mutant growth plate revealed a chondrocyte proliferation defect associated with increased levels of the cell cycle inhibitor p16 and p21 and a disorganized collagen network characterized by reduced fibrillar density and irregular fibril diameters. Essentially the same growth plate defects were observed in mice where the deletion of *Itgb1* was achieved in limb mesenchymal progenitor cells using the *Prx1cre* transgene (Raducanu et al. 2009). Importantly, the lack of β1 integrin in the skeletogenic mesenchymal stem cells did not affect chondrogenic differentiation demonstrating that β1 integrin-mediated cell-matrix interactions and signaling processes are not required for early stages of endochondral bone formation.

Whereas the deficiency of all β1-containing heterodimers on the surface of chondrocytes results in severe growth plate phenotypes, murine-specific deletion of most genes encoding the corresponding α subunits has no apparent consequence on cartilage development. However, constitutive ablation of the collagen-binding α10 integrin gene (*Itga10*) in mice results in a mild, nonlethal chondrodysplasia characterized by postnatal dwarfism, moderate chondrocyte shape change, and slight disorganization of the growth plate (Bengtsson et al. 2005). Similar to the β1-deficient chondrocytes but to at lower extent, the lack of *Itga10* also impairs proliferation and collagen fibrillar assembly in the cartilage matrix. Recently, a truncating mutation in exon 16 of the canine *ITGA10* gene was identified in Nordic hunting dogs with disproportionate short-limbed dwarfism (Kyostila et al. 2013). The phenotype of the diseased growth plate is similar to that of the mouse α10-deficient cartilage and caused by the lack of α10 protein in the affected dogs. These observations suggest that although α10β1 is an important integrin heterodimer which modulates growth plate function, the absence of multiple β1 integrins is required for the most severe

cartilage defects indicating partial compensation among β1 integrin-containing heterodimers.

Integrin focal adhesion complexes integrate a plethora of adaptor and signaling proteins, and among them, a few has been identified as regulators of growth plate function. Integrin-linked kinase (ILK) is a ubiquitously expressed protein which forms a ternary complex binding to the cytoplasmic tail of β integrins and to two adaptor proteins called PINCH and parvin (Ghatak et al. 2013). ILK is an essential linker between integrins and the actin cytoskeleton and regulates the activities of small Rho GTPases such as Rac1 and RhoA. Therefore, it is not surprising that *Col2a1cre*-driven cartilage-specific deletion of the *Ilk* gene in mice displays mild "integrin-like" growth plate abnormalities like reduced proliferation, disorganization of the actin cytoskeleton, and chondrocyte shape change (Grashoff et al. 2003; Terpstra et al. 2003). *Prx1cre*- or *Col2a1cre*-driven ablation of the gene encoding Kindlin-2, a member of the kindlin family of key regulators of integrin-mediated cell-matrix interactions (Meves et al. 2009), leads to lethality after birth and severe skeletal defects which most resemble to the phenotype of the β1 integrin mutant mice. These abnormalities include the complete loss of all levels of growth plate polarity and reduced chondrocyte proliferation (Wu et al. 2015). It is worth mentioning that while cartilage-specific ablation of *Ilk* had very minor effects in chondrocyte adhesion (Grashoff et al. 2003), Kindlin-2-deficiency reduced integrin activation already in mesenchymal progenitors (Wu et al. 2015) suggesting that mutant chondrocytes may have significantly impaired integrin-mediated anchorage to the cartilage extracellular matrix.

4.4.4 Cell-Cell Interactions

Cadherins are Ca^{2+}-dependent cell-cell adhesion molecules which have been implicated in diverse tissue morphogenetic processes such as regulation of cell shape, movement, and sorting. Members of the large cadherin family (e.g., E, N, P, T, M cadherins) establish weak interactions between the two opposing cell surfaces which can be strengthened by multiple molecular associates including catenins. Cadherin/catenin complexes, similar to integrins, are connected to the actin cytoskeleton through dynamic interactions. In this scenario, β-catenin directly binds to the cytoplasmic domain of cadherins and enhances their clustering, while α-catenin mediates the linkage to the actin network via actin-binding proteins (Nelson 2008). Most components of the cadherin/catenin complex have been identified in the mouse growth plate (Sampson et al. 2007), but because chondrocytes are normally separated with an abundant ECM and direct surface contact between neighboring cells is believed to be negligible, the importance of cell-cell interactions in growth plate function has been questioned. This view was recently challenged by investigating the dynamic process of column formation in mouse growth plate explants combining fluorescence membrane labeling and time-lapse confocal microscopy (Romereim et al. 2014). High-resolution live-cell imaging revealed that daughter cells in the proliferative zone remain in an intimate contact after cell division until

the end phase of the rotational movement. This observation and the finding that cadherins and β-catenin are immunohistochemically localized to the junctions strongly suggest the involvement of cell-cell interactions in columnar alignment of chondrocytes. Indeed, interfering with calcium-cadherin binding partially disrupted the transient adhesion interface and prematurely abolished chondrocyte rotation. Based on these data, a novel two-step model can be drawn for column elongation in the proliferative zone: (1) cell-cell adhesion surfaces form between opposing chondrocytes concomitant with the division; (2) daughter chondrocyte rearrangement is mediated via the expansion of the adhesion interface in a process more similar to spreading than migration (Fig. 4.2).

4.4.5 The Primary Cilium and Growth Plate Function

Primary cilia are specialized sensory organelles that extend from the surface of most vertebrate cells. These appendages originate from the plasma membrane-associated basal body and are composed of an axoneme surrounded by a soluble compartment (cilioplasm) and a bilayer lipid membrane. A primary cilium can be classified as "nonmotile" characterized by an axoneme which consists of nine doublets of microtubules but lacks a central microtube pair typical for "motile" cilia. Primary cilia are generated and maintained through the process of intraflagellar transport (IFT), a bidirectional transport system mediating the movements of large protein complexes (ITF particles) along the axoneme. The anterograde transport directs components from the basal body to the tip of the cilium driven by kinesin motors, whereas the retrograde transport returns proteins driven by a dynein motor (Yuan et al. 2015; Muhammad et al. 2012; Prasad et al. 2014). In general, a primary cilium acts as a cellular antenna that senses extracellular chemical or mechanical signals and translates them into intracellular signaling processes. Ciliary dysfunction in human leads to ciliopathies, a broad range of pleiotropic syndromes which include skeletal dysplasias with disturbed bone growth and patterning (Haycraft and Serra 2008).

Primary cilia on chondrocytes are regarded as polarity markers of the growth plate as they protrude randomly on resting zone cells, while in the proliferative and hypertrophic zones, they align parallel with the direction of the columns and the longitudinal growth (Fig. 4.4a) (Ascenzi et al. 2007; de Andrea et al. 2010). Ultrastructural and confocal microscopic studies have indicated the close association of Golgi apparatus, primary cilium, and the ECM in chondrocytes that may be important for directed secretion of matrix molecules (Fig. 4.4c) (Jensen et al. 2004; Poole et al. 1997). Thus, the specific orientation in the growth plate suggests that primary cilia are required for establishing and/or maintaining normal chondrocyte and matrix anisotropy by orchestrating chemical and mechanical signals during development. This view has been supported by numerous mouse mutants showing abnormal columnar organization of chondrocytes upon ablation of genes associated with IFT and cilia formation (Geister et al. 2015; Haycraft et al. 2007; Kolpakova-Hart et al. 2007; McGlashan et al. 2007; Song et al. 2007). Mice with conditional ablation of the genes encoding Ift88 (intraflagellar transport protein 88/Tg737/

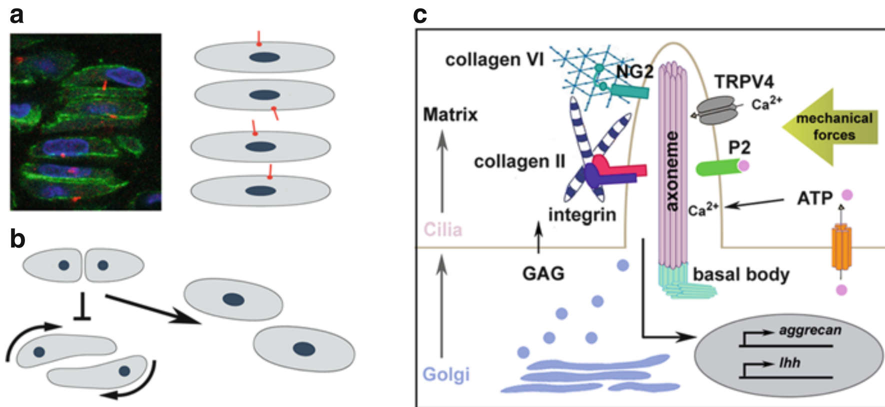

Fig. 4.4 Ciliary functions in the growth plate. (**a**) In normal growth plate, primary cilia, represented by red immunofluorescence signals, align along the longitudinal axis of the column in the direction of bone growth. (**b**) In the absence of cilia, chondrocyte rearrangement movements are impaired and the columnar structure is disrupted. (**c**) Primary cilia on growth plate chondrocytes interact with the extracellular matrix through cell surface receptors such as integrins and NG2 and sense mechanical forces. Cilia are required to mediate ATP-induced Ca^{2+} signaling via P2 purinergic receptors in compressed chondrocytes to upregulate aggrecan expression and secretion of sulfated glycosaminoglycans (*GAG*). The transient receptor potential vanilloid 4 (*TRPV4*) receptor may also mediate Ca^{2+} signaling induced by mechanical loading. Hydrostatic pressure and cyclic tensile strength increase Indian hedgehog (*Ihh*) expression and the activation of downstream hedgehog signaling in cilia. A physical continuum between the mechanosensory cilia and the polarized Golgi apparatus may modulate the production of the extracellular matrix

polaris, a core component of IFT particles) or Kif3a (a subunit of the heterotrimeric kinesin-2 motor) exhibit distinct bone defects depending on the time of gene deletion. When *Ift88 or Kif3a* was ablated in skeletogenic mesenchymal progenitors of the early limb bud using the *Prx1cre* transgene, mice had polydactyly and impaired embryonic bone development characterized by improperly formed growth plate with accelerated hypertrophic differentiation, delay of vascular invasion, and the lack of a bone collar (Haycraft et al. 2007; Kolpakova-Hart et al. 2007). In contrast, *Col2a1cre*-dirven deletion of *Ift88* or *Kif3a* in differentiated chondrocytes resulted in growth plate degeneration only postnatally accompanied by chondrocyte shape change and impaired chondrocyte rearrangement leading to disorganization of the columnar structure in the growth plate (Fig. 4.4b) (Ochiai et al. 2009; Song et al. 2007). Chondrocyte-specific ablation of the *Ift80* gene, which code for another IFT protein, led to shortened limbs and mildly disorganized chondrocyte columns (Yuan and Yang 2015). Since the mutant progenitor cells or chondrocytes largely lack cilia, these studies demonstrate that ciliary functions are essential for normal differentiation and organization of the growth plate cartilage.

Much evidence revealed that the ciliopathies of mutant mice are connected to abnormal mechanotransduction, hedgehog (Hh), and Wnt signaling in the cartilaginous growth plate. Chondrocytes that are exposed to mechanical forces transmit the signals via various mechanotransducers including stretch-activated ion

channels, integrins, and Ca^{2+}- and cyclic adenosine monophosphate (cAMP)-mediated second messenger signaling (Fig. 4.4c) (Bader et al. 2011). Mechanical loading activates ATP-induced Ca^{2+} signaling through P2X and P2Y purinergic receptors, aggrecan mRNA expression, and secretion of glycosaminoglycans in normal chondrocytes but not in Ift88 mutant chondrocytes, which lack cilia (Wann et al. 2012). Both wild-type and mutant chondrocytes showed mechanically induced ATP release; however, cilia-deficient cells failed to elicit a Ca^{2+} response to exogenous ATP implicating that cilia may function as a controlling organelle of ATP reception and do not act as initial mechanoreceptors. Depending on the mechanical stimulation, chondrocyte primary cilia also change in number and length implicating an adaptive mechanism for the biomechanical microenvironment (McGlashan et al. 2010). Several transmembrane receptors and membrane channels are localized to the cilia of chondrocytes. Integrins β1, α2, and α3 and the transmembrane proteoglycan NG2 were found on primary cilia (McGlashan et al. 2006). Integrins are vital for mechanotransduction, cell migration, and ECM secretion/assembly; thus, on chondrocytes cilia, they may have multiple functions by linking matrix biomechanics and ECM organization to cellular behavior such as chondrocyte rotation. Transient receptor potential vanilloid 4 (TRP4), an osmotically sensitive Ca^{2+} channel which is required for normal cartilage development, and connexin 43, a mechanosensitive ATP-release channel, were also found on chondrocyte cilia and implicated as putative mechanoreceptors (Knight et al. 2009; Phan et al. 2009).

Since IFT protein- and cilia-deficient cartilage show remarkable similarities to the phenotype of Indian hedgehog mutant mice (St-Jacques et al. 1999), and components of Hh signaling (for details, see Chaps. 8 and 9) were identified in the cilia (Oro 2007; Robbins et al. 2012), it has been suggested that the primary cilium mediates Hh signaling during endochondral bone formation (Haycraft and Serra 2008). In the absence of Sonic hedgehog (Shh) or Ihh, the signaling molecule Smoothened (Smo) is localized in intracellular vesicles at base of the cilia, and its function is repressed by the Hh receptor Patched-1 (Ptc1). At the same time, Sufu (Suppressor of fused) turns Gli (glioma) proteins into transcriptional repressor forms which move from the cilium into the nucleus and silence gene transcription. In the presence of Hh proteins, Ptc1 leaves the cilia allowing the accumulation of active Smo in the cilium, which in turn activates gene transcription through the transcriptional activator forms of Gli proteins. When *IFT88/Kif3a* was deleted in the chondroprogenitors of the early limb bud, the expression of *Ptc1* and *Gli1* was reduced in embryonic chondrocytes indicating disrupted Hh signaling (Haycraft et al. 2007; Kolpakova-Hart et al. 2007). Similar downregulation of *Ptc1* and *Gli1* expression was observed in primary cilia-depleted postnatal columnar growth plate chondrocytes (Chang and Serra 2013). Additionally, *Smad1/Smad5* double knockout mice had disrupted Ihh pathway and misoriented primary cilia on growth plate chondrocytes accompanied by disorganized growth plates further potentiating a strong connection between ciliary Hh signaling and the regulation of chondrocyte rotation (Ascenzi et al. 2011). It has been also shown that the Hh machinery in chondrocytes is mechanosensitive and disruption of cilia blocks Ihh expression and signaling

induced by hydrostatic compression or cyclic tensile strength (Shao et al. 2012; Thompson et al. 2014).

Several studies have attributed a role for cilia in Wnt signaling. In the postnatal growth plate of *Ift88-Col2a1ce* mutants, the expression of the Wnt antagonist *Sfrp5* (secreted frizzled-related protein-5) was also lower, while the activity of the Wnt/β-catenin pathway was increased in proliferative chondrocytes indicating a link, likely indirectly through reduced Hh signaling, between ciliary function and the canonical Wnt pathway (Chang and Serra 2013). The loss of Ift80 in chondrocytes results in similar overactivation of the canonical Wnt signaling (Yuan and Yang 2015). The inactivation of two PCP effectors encoded by the *Inturned* and *Fuzzy* genes resulted in defective ciliogenesis and compromised Hh signaling in the frog *Xenopus laevis* (Park et al. 2006). Importantly, the PCP-dependent convergent extension movements of chondrocytes in Meckel's cartilage were disrupted leading to the loss of columnar structures. The corresponding mouse null mutants die during embryogenesis and exhibit abnormal limb patterning; however, growth plate architecture was not investigated in these mice. A hypomorphic mutant of *Inturned* shows moderate loss of cilia associated with retarded growth and limb polydactyly (Chang et al. 2015). Despite impaired endochondral ossification, mutant cartilage has apparently normal PCP signaling demonstrated by the proper flattening, orientation, and columnar organization of the proliferative growth plate chondrocytes. Thus, species-specific differences may exist in the role of *Inturned* in controlling PCP of cartilaginous tissues.

4.4.6 External Mechanical Stress

Biomechanical cues during development are essential for tissue morphogenesis by controlling the fate, differentiation, proliferation, shape, position, and gene expression of cells (Heisenberg and Bellaiche 2013). Mechanical stimuli generated by muscle constriction drive chondrogenesis and shape the skeleton (Shea et al. 2015). Prolonged inhibition of fetal movements in humans leads to fetal akinesia deformation sequence (FADS), a rare condition characterized by a spectrum of growth abnormalities including defects in bone and joint development. Pharmacologically or genetically induced immobilization of vertebrate embryos affects numerous aspects of skeletogenesis through the modulation of growth plate function. Paralyzed mouse or chicken embryos have been characterized by shorter cartilaginous rudiments (Hosseini and Hogg 1991a, b; Rot-Nikcevic et al. 2006; Hall and Herring 1990), a smaller proliferation zone and a reduced proliferation rate (Germiller and Goldstein 1997; Roddy et al. 2011), a shortened hypertrophic zone, and delayed formation of the primary ossification center (Nowlan et al. 2010). As cyclic mechanical stress applied on immature primary chondrocytes in vitro (Wu and Chen 2000) or on rabbit premaxillae in vivo (Wang and Mao 2002) enhances chondrocyte proliferation, it is likely that the lack of muscle constriction-generated mechanical forces directly inhibits proliferation by preventing the operation of mechanical signal transduction pathways inherent to chondrocytes. In addition to the control of

proliferation, fetal mechanical forces are important for column elongation through the modulation of chondrocyte intercalation. In paralyzed zebrafish, chondrocyte stacks of the craniofacial cartilage were severely disrupted due to chondrocyte rounding and impaired rotational movements (Shwartz et al. 2012). The impact of muscle-induced mechanical forces on chondrocyte rotation is evolutionarily conserved, as muscle-less mouse embryos show similar, although milder, defects in columnar organization in the proliferative zone of the growth plate (Shwartz et al. 2012).

4.5 Perspectives

Our understanding of the complex mechanisms that regulate the spatial and temporal behavior of growth plate chondrocytes has advanced substantially but is still far not complete. The body of gathered data on the role of morphogenetic signals, extracellular matrix properties, chondrocyte-matrix interactions, ciliary functions, and biomechanical forces for cartilage development provides fundamental cues to establish a link between molecular pathways and coordinated alignment of chondrocytes across the plane of the growth plate. Further progress is needed to decipher the intricate interaction among polarity determinants to draw a clearer picture of directional activities of the growth plate. Transcriptional profiling has identified numerous novel genes which are expressed in spatially or temporally regulated manner in the growth plate to modulate human adult height (Lui et al. 2012) paving the way to discover molecules and mechanisms that have not previously implicated in the columnar architecture of chondrocytes. Animal models and recent advantages of high-resolution, time-lapse imaging will shed light on how molecular mechanisms are coupled to the dynamic process of chondrocyte rearrangements during endochondral bone formation.

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Cell Fate of Growth Plate Chondrocytes in Endochondral Ossification: Cell Death or Reprogramming into Osteogenic Lineage?

5

Klaus von der Mark and Xin Zhou

Abstract

The cell fate of hypertrophic growth plate chondrocytes at the chondro-osseous junction has been a subject of discussion for several decades: On the one hand, there is ample evidence for programmed cell death by apoptosis or other mechanisms in the lower hypertrophic zone; on the other hand, several studies have indicated that some hypertrophic chondrocytes may not be "terminally differentiated" but are able to further differentiate into osteoblasts. Recent lineage tracing studies from four laboratories using genetic markers have now unequivocally demonstrated that a progeny of growth plate chondrocytes is able to give rise to osteoblasts which contribute substantially to trabecular, endosteal, and cortical bone formation during fetal and postnatal long bone development. This shows that not all "terminally" differentiated cells at the chondro-osseous junction are eliminated by programmed cell death but have the option to survive and enter the osteogenic lineage. The ability of chondrocytes to transdifferentiate into bone cells was also confirmed in bone injury healing experiments which demonstrated that the majority of newly formed osteoblasts were derived from chondrocytes of the fracture callus. The molecular mechanism of chondrocyte reprogramming into the osteogenic lineage, however, remains to be elucidated. A detailed analysis of the lower hypertrophic growth plate zone by confocal microscopy revealed small, 4–6 μm reporter gene-positive cells, i.e., hypertrophic chondrocyte-derived cells, which were mitotically active and differentiated to

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osteoblasts in vitro. They seem to be derived from hypertrophic chondrocytes by condensation, possibly through autophagy, and may represent intermediate stemlike cells that have the option to enter the osteogenic or other bone marrow cell lineages.

5.1 Introduction

The skeletal body plans of all vertebrates are laid out as cartilaginous models, but only in few species such as sharks or rays the skeleton remains cartilaginous throughout lifetime. In most vertebrates, the cartilage models of long bones, ribs, and vertebrae are subsequently replaced by bone during fetal and postnatal development, while only specialized cartilaginous tissues such as articular cartilage and nasal or tracheal cartilage persist in the adult (Hall 1987, 2005). Cartilage replacement by bone occurs in a dynamic and highly complex process called endochondral ossification, which is one of the most intriguing puzzles in vertebrate development with important implications on our understanding of bone fracture healing, skeletal dysplasias, and degenerative joint diseases (for reviews, see Poole (1991), Mackie et al. (2008), and Ballock and O'Keefe (2003)).

The cartilaginous elements of the vertebrate appendicular skeleton develop by condensation of limb mesenchyme. In the limb bud, hyaline chondrocytes in the center of the blastema proliferate and undergo a series of distinct differentiation steps to proliferating, prehypertrophic, and hypertrophic chondrocytes. The zone of mature hypertrophic cartilage in the diaphysis of long bones is then invaded by vascular sprouts from the perichondrium, resorbed by osteoclasts, and filled with bone marrow and trabecular bone, forming the primary ossification center (POC). A sheath of cortical bone develops around the diaphysis by endesmal ossification, while trabecular bone is formed in the POC by osteoprogenitor cells which invade with endothelial cells from the perichondrium or – as we will see below – are derived from hypertrophic chondrocytes. Both perichondrium-derived and chondrocyte-derived osteoprogenitor cells deposit osteoid on the surface of remaining cartilage spicules which after mineralization provide the bone trabeculae of the primary spongiosa (Olsen et al. 2000; Karsenty et al. 2009). At both the distal and proximal sides of the primary ossification center (POC), growth plates develop in which the process of chondrocyte maturation and replacement by bone proceeds toward both epiphyses (for reviews, see Ballock and O'Keefe (2003), Mackie et al. (2008), Provot and Schipani (2005), Poole (1991), Amizuka et al. (2012), and Tsang et al. (2014)).

Pace and extent of skeletal growth are tightly controlled by multiple, coordinated mechanisms regulating proliferation, differentiation, and maturation of chondrocytes and osteoblasts. The role and the cell fate of hypertrophic chondrocytes at the cartilage–bone marrow interface, also called chondro-osseous junction, in endochondral ossification are complex and controversially discussed. According to common view, late hypertrophic chondrocytes are eliminated by programmed cell death

during the cartilage resorption process (for reviews, see Gibson (1998) and Shapiro et al. (2005)). On the other hand, numerous morphological and experimental studies going back almost a century have indicated that hypertrophic chondrocytes may be able to convert into osteoblast-like, bone-forming cells in vitro and in vivo (Yang et al. 2014a, b; Park et al. 2015; Zhou et al. 2014). The relevance of these studies has, however, not been widely accepted. Recently, however, four independent lineage tracing studies in transgenic mice have provided conclusive evidence by using genetic recombination in chondrocytes that hypertrophic growth plate chondrocytes can survive and give rise to a progeny of osteoblasts which contribute substantially to trabecular, endosteal, and cortical bone formation. Furthermore, in two of these studies, it was shown that also in regenerating bone tissue after experimental bone fracture callus formation, the majority of osteoblasts are derived from chondrocytes (Zhou et al. 2014; Yang et al. 2014b). These findings challenge current textbook views on the origin of trabecular, endosteal, and cortical osteoblasts constituting endochondral bone and on the cell fate of hypertrophic growth plate chondrocytes. The new data indicates that hypertrophic chondrocytes have the option to undergo programmed cell death or to survive by reentering the cell cycle and undergoing reprogramming into osteoprogenitor cells and other bone marrow cells.

In this chapter, the authors will present new concepts of the cell fate of *hyp*ertrophic growth plate chondrocytes in light of the new genetic lineage tracing studies and discuss the relevance of the results with respect to our understanding of the origin of osteoblasts not only in development but also in bone repair, chondrodysplasias, and degenerative joint diseases. Overall, previous and recent findings indicate that programmed cell death and reprogramming of hypertrophic chondrocytes to osteoblasts are not mutually exclusive cell fates, but two options which are both realized at the chondro-osseous junction, although the mechanism regulating entry into these different pathways is still open. At this point, the authors would like to refer the detailed descriptions of the developing growth plate and the regulation of differentiation and maturation of chondrocytes in Chaps. 4, 8, and 10 in this volume.

5.2 Chondrocyte and Osteoblast Differentiation in the Growth Plate

5.2.1 Chondrocyte Differentiation

In the growth plate, the chondrocytes in the resting, proliferating, prehypertrophic, hypertrophic, and late (“terminal”) hypertrophic zone have each specific roles in endochondral ossification: In the “resting” zone – or better called reserve zone – chondrocytes proliferate and expand to shape the epiphysis which is later also ossified by the secondary ossification center. In the proliferating zone, chondrocytes divide most rapidly and align into vertical columns of flattened, lens-shaped cells which finally differentiate into prehypertrophic and hypertrophic chondrocytes. Proliferating and hypertrophic chondrocytes are primarily responsible for the

longitudinal growth of long bones, as cells increase not only in number but also in their volume up to 20-fold, and deposit substantial amounts of extracellular cartilage matrix (for review, see Ballock and O'Keefe (2003), Provot and Schipani (2005), Mackie et al. (2008), and Chap. 4). Maturation into hypertrophic chondrocytes is also characterized by the development of a granular cell surface and protrusion of numerous microvilli which release matrix vesicles that serve as nuclei of cartilage mineralization (Anderson 1985; Wuthier 1988).

Maturation of proliferating to hypertrophic chondrocytes is associated with fundamental matrix remodeling (summarized in Ortega et al. (2004)): The hyaline cartilage matrix which is composed predominantly of aggrecan and collagens II, VI, IX, and XI, deposited by resting and proliferating chondrocytes, is substituted by a calcifying matrix which contains type X collagen, a network forming collagen synthesized only by hypertrophic chondrocytes (Schmid and Conrad 1982; Gibson et al. 1984), as well as alkaline phosphatase (AP), osteopontin, bone sialoprotein (BSP), osteocalcin, and proteoglycans (Ballock and O'Keefe 2003; Cancedda et al. 1995; Mackie et al. 2008). In their late stage of differentiation, hypertrophic chondrocytes secrete MMP13 (collagenase 3), a matrix metalloproteinase which breaks up the matrix of calcified cartilage in preparation for the resorption by osteoclasts (Stickens et al. 2004; Inada et al. 2004; Johansson et al. 1997), and the vascular endothelial growth factor (VEGF) A, which induces capillary invasion and bone marrow formation (Gerber et al. 1999; Zelzer et al. 2004). The remarkable similarity of the gene expression pattern of hypertrophic chondrocytes to that of osteoblasts which includes AP, osteopontin, bone sialoprotein, and osteocalcin (Gerstenfeld and Shapiro 1996) strongly supports the concept of a continuous chondrocyte-to-osteoblast lineage.

Growth and differentiation of chondrocytes in the growth plate are regulated in a complex, synergistic manner by growth factors, hormones, developmental growth factors, and their receptors (for reviews, see, Goldring et al. (2006), Cancedda et al. (2000), Lefebvre and Smits (2005), Kronenberg (2003), and Nishimura et al. (2012)). Most important for growth and proliferation are insulin-like growth factors (IGFs) and thyroxin, growth factors of the fibroblast growth factor (FGF), and bone morphogenetic proteins (BMPs). The critical step of chondrocyte maturation into hypertrophic chondrocytes is also under the control of the regulatory cycle of parathyroid hormone-related protein (PTHrP) and Indian hedgehog (IHH) (see Chap. 8 and reviews by Lanske and Kronenberg (1998), Kronenberg (2006), and Vortkamp (2000)), while various WNT factors regulate chondrogenic and osteogenic differentiation in the growth plate through canonical and noncanonical pathways (see Chap. 10 and reviews by Day and Yang (2008) and Hartmann (2007)).

5.2.2 Osteoblast Differentiation

Concomitant with the maturation of chondrocytes in the mid diaphysis into hypertrophic cells, osteoprogenitor cells in the perichondrium surrounding the diaphysis differentiate into osteoblasts which produce the calcified cortical bone sheath

consisting of type I collagen as major organic constituent, as well as osteocalcin, bone sialoprotein, osteopontin, matrix GLA protein, alkaline phosphatase, proteoglycans, and other bone constituents (for reviews, see Olsen et al. (2000), Karsenty et al. (2009), and Reddi (1981)). Differentiation of osteoprogenitor cells to osteoblasts is strongly enhanced by factors of the TGFß/BMP family, in particular by BMP-3, BMP-7, and TGFß1, and by vitamin D (1,25$(OH)_2D_3$), PTH/PTHrP, glucocorticoids, prostaglandin E2, IGF-I, and IGF-II (Aubin et al. 1995; Karsenty et al. 2009; Lian et al. 2006; Komori 2006). Osteoblasts are characterized by an epithelioid cell shape and cell polarity, directing secretion of bone matrix components to the basal surface facing the bone matrix, and by the development of long cell protrusions (canaliculi) into the bone matrix which are essential for intercellular communication, mechanotransduction, and exchange of metabolites. When epithelioid osteoblasts are embedded into bone matrix, they differentiate into postmitotic osteocytes which terminate bone matrix production, acquire stellate cell shape, and develop numerous canaliculi (Komori 2013). During longitudinal bone growth in the fetus, further osteoprogenitor cells are self-renewing in Ranvier's groove (Langenskiold 1998) and migrate along the periosteum to produce further cortical bone; some migrate into the bone marrow with onset of capillary invasion though the cortical bone sheath along with endothelial cells (Colnot et al. 2004; Maes et al. 2010). In the primary spongiosa, they differentiate into osteoblasts on the surface of remaining, unresorbed calcified cartilage spicules and form bone trabeculae by deposition of osteoid, the organic part of the bone matrix, which consists of type I collagen and other bone matrix proteins. Recent evidence strongly indicates that further osteoblasts in the primary spongiosa are generated by transdifferentiation of hypertrophic chondrocytes (see below).

5.3 Sox9, Runx2, and Osterix as Key Transcription Factors Regulating Chondrogenic and Osteogenic Differentiation

A continued chondrocyte-to-osteoblast lineage during endochondral ossification becomes plausible when considering the common origin of limb chondrocytes and osteoblasts from Sox9 expressing chondro-osteoprogenitor cells in the limb mesenchyme (Akiyama et al. 2005). Continued Sox9 expression promotes chondrogenic differentiation and maintains the chondrogenic phenotype, whereas RUNX2 and osterix (Osx) drive the progenitor cells into the osteogenic lineage (for reviews, see de Crombrugghe et al. (2001), Lefebvre and Smits (2005), Tsang et al. (2014), Karsenty et al. (2009), Komori (2010), and Lefebvre et al. (2001)). Spatial and temporal aspects of chondrogenic versus osteogenic differentiation in skeletal development are primarily controlled by Wnt/ß-catenin signals (Hartmann 2007; Day and Yang 2008) (see also Chap. 10).

SOX9 is a transcription factor of the SRY family which is essential for sex determination and development of cartilage and neural tissues (Lefebvre and de Crombrugghe 1998; Bi et al. 1999; Tsang et al. 2014). The existence of

Sox9-positive chondro-osteoprogenitor cells in the undifferentiated limb mesenchyme was demonstrated by inactivation of *Sox9* before mesenchymal condensation under the control of the *prx* promoter, which abolished not only cartilage formation but also endochondral bone formation (Akiyama et al. 2002). Sox9 controls all steps of chondrocyte differentiation until chondrocytes reach hypertrophy (Leung et al. 2011; Lefebvre and de Crombrugghe 1998). Together with the related Sox5 and Sox6 transcription factors, SOX9 activates the expression of cartilage matrix genes C*ol2a1*, *Col9a1*, *Col11*, *Agc1* (the gene of aggrecan) and others (Lefebvre et al. 1997; Lefebvre and Smits 2005; Ng et al. 1997). In the growing limb, Sox9 is expressed in resting, proliferating, and early hypertrophic cartilage, but interestingly, it is absent from late hypertrophic chondrocytes (Zhao et al. 1997; Hattori et al. 2010). The complete loss of SOX9 from the late hypertrophic zone is noticeable in light of the enhanced expression of osteogenic genes in this zone, including *Runx2*, *osterix* (*Osx*), *osteopontin* (*Spp1*), and *bone sialoprotein* (*BSP*), and compatible with the concept of a conversion of hypertrophic chondrocytes into osteoblasts.

RUNX2 (cbfa1, Osf2) is a transcription factor of the runt family which is essential for osteogenic differentiation and bone development (Ducy et al. 1999; Karsenty et al. 1999; Komori 2008). It activates the expression of osteoblast-typical genes such as osteocalcin (*Bglap*), type I collagen (*col1a1 and col1a2*), *Bsp*, *Spp1*, and *Runx2* itself by binding to OSE2 consensus sequences in their promoters (Komori 2010; Ducy et al. 1996, 1997). Inactivation of Runx2 gene allows the development of the cartilaginous anlagen of the skeleton but blocks completely both endesmal and endochondral ossification (Ducy et al. 1997; Komori et al. 1997; Otto et al. 1997). RUNX2 also activates expression of Osx (*SP7*), a zinc finger transcription factor which also acts as master gene of osteogenic differentiation (Nakashima et al. 2002).

Interestingly, although both Runx2 and Osx are primarily essential for osteogenic differentiation, they are also involved in the regulation of chondrocyte maturation and endochondral ossification. Both *Runx2* and *Osx* are expressed in prehypertrophic chondrocytes (Takeda et al. 2001; Nakashima et al. 2002; Hattori et al. 2010), and Runx2 promotes expression of type X collagen and maturation to hypertrophic chondrocytes (Kim et al. 1999; Wang et al. 2004; Stricker et al. 2002). As a consequence, Runx2-deficient mice lack hypertrophic chondrocytes in growth plates (Komori et al. 1997). Furthermore, Runx2 induces the expression of *Vegfa* chondrocytes of late hypertrophic cartilage, which is required for capillary invasion into hypertrophic cartilage (Carlevaro et al. 2000; Gerber et al. 1999; Zelzer et al. 2001), and *Mmp13* (Hess et al. 2001; Jimenez et al. 1999; Porte et al. 1999; Inada et al. 2004), which loosens up the matrix of hypertrophic cartilage in order to allow invasion of bone marrow sprouts (Inada et al. 2004; Johansson et al. 1997). Thus, while early stages of chondrocyte differentiation are strictly controlled by Sox9, with onset of *Runx2* and *Osx* expression in prehypertrophic chondrocytes, the cell fate of growth plate chondrocytes seems to be successively directed into an osteogenic lineage as heralded by cellular hypertrophy, matrix calcification, and onset of osteogenic gene expression.

5.4 The Cell Fate of Hypertrophic Chondrocytes

5.4.1 Programmed Cell Death

Morphological studies dating back more than a century ago have reported on alterations in hypertrophic growth plate chondrocytes of different species which pointed to cellular degeneration and death (Brachet 1893; Fell 1825; Ham 1952). Also in more recent ultrastructural studies, degenerative changes in growth plate chondrocytes were interpreted as signs of programmed cell death reminiscent of apoptosis (Farnum and Wilsman 1989a; Gibson et al. 1995; Gibson 1998; Bronckers et al. 1996; Aizawa et al. 1997; Zenmyo et al. 1996; Roach et al. 2004; Roach 1997). Apoptosis, the most common form of programmed cell death, is an evolutionary conserved process that serves to eliminate unwanted or superfluous cells during normal tissue development and regeneration of organs and tissues in almost all species (for reviews, see Jacobson et al. (1997) and Fuchs and Steller (2011)). The term apoptosis was coined first by Kerr et al. (1972) in an ultrastructural study where they defined a series of distinct morphological changes in cells dying under physiological conditions. In contrast to cell necrosis in which cells swell and become ruptured in response to stress or tissue injury, during apoptosis, cells retain membrane integrity but shrink by genetically controlled intracellular degradation of organelles, proteins, and DNA, leaving condensed nuclei. The dead cells or cell fragments are rapidly phagocytosed and therefore are visible mostly inside macrophages or other phagocytic cells. Apoptosis is primarily an intrinsic, cell-autonomous process but can be also induced by extrinsic signals, e.g., by phosphate (Mansfield et al. 2001) and NO (Teixeira et al. 2001).

Extensive studies on the cellular and molecular changes in nematodes, flies, and vertebrae led to the discovery of reliable molecular tools suitable to characterize stages of apoptotic cell death, in addition to ultrastructural criteria. Intracellular degradation involves a cascade of caspases, a group of proteases detected originally in *C. elegans* (Ellis and Horvitz 1986). Today, the immunohistological identification of caspase 3 and of cleaved caspase 7 is commonly used to detect apoptotic cells in tissues, besides the detection of DNA fragmentation using the DNA nick end labeling method (TUNEL method) (Gavrieli et al. 1992; Hatori et al. 1995).

Reports on programmed cell death of hypertrophic chondrocytes were partially divergent, with respect to type, stage, and extent of cell death (for reviews, see Gibson (1998), Shapiro et al. (2005), and Tsang et al. (2014)). For example, Gibson et al. (1995) demonstrated by light microscopy condensation of chondrocytes in resorbing chick sterna at the interface with the invading bone marrow, and electron microscopy demonstrated a range of morphological alterations including retraction from the pericellular matrix, cytoplasmic and nuclear condensation, and vesiculation, suggestive of sequential steps of apoptosis. Similarly, Farnum and Wilsman (1987, 1989b) described condensed hypertrophic chondrocytes in the lower lacunae adjacent to the cartilage–bone marrow interface in Yucatan swine and suggested that these cells might be dying by apoptosis. They reported that only about 12 % of the lowest hypertrophic chondrocytes revealed condensed morphology, whereas in

the chick sterna, more than 50 % of hypertrophic chondrocytes at the interface with invading bone marrow showed signs of apoptosis owing to the more intensive vascular invasion of chick cartilage (Gibson et al. 1995).

Evidence for apoptotic death of hypertrophic chondrocytes was also supported by the detection of DNA breaks using the TUNEL method (Hatori et al. 1995; Aizawa et al. 1997; Ohyama et al. 1997; Gibson 1998), but again with diverging results. For example, Hatori et al. (1995) reported that only 9 % of chondrocytes isolated from the hypertrophic region chick growth plate at the vascular interface showed fragmented DNA, whereas other studies suggested that DNA fragmentation occurred earlier in differentiation and was much more extensive (Aizawa et al. 1997), with up to 44 % TUNEL-positive cells in the hypertrophic and 15 % in the proliferative zone (Ohyama et al. 1997).

5.4.2 Regulation and Induction of Apoptosis

Several in vitro studies indicate that programmed cell death of hypertrophic chondrocytes in the growth plate is activated by microenvironmental factors (Shapiro et al. 2005). For example, Mansfield et al. (2001, 2003) have shown that inorganic phosphate (Pi) and Ca^{2+} which are released during resorption of hydroxyapatite of the calcified cartilage during endochondral ossification are able to induce apoptotic cell death of chick chondrocytes in vitro. Texeira et al. (2001) provided evidence that this process is mediated by nitrogen oxide (NO) since Pi induced a threefold increase in NO concentration, which can kill chondrocytes in vitro; it may activate cell death by causing loss of thiol in the growth plate (Teixeira et al. 2003) and loss of mitochondrial membrane potential (Teixeira et al. 2001).

Important factors controlling survival and differentiation of growth plate chondrocytes are the hypoxia-induced factors HIF1α and HIF2α (Schipani et al. 2001). HIF factors are essential for chondrocyte survival in the growth plate by activating energy generation through anaerobic glycolysis. They are enhanced under the hypoxic conditions in the center of the hypertrophic zone, but an increase under the oxygen levels at the cartilage erosion zone due to capillary invasion may render hypertrophic chondrocytes to become sensitive to apoptogens (reviewed in Shapiro et al. (2005) and Toledo-Pereyra et al. (2004)).

5.4.3 Paralysis and Chondroptosis, Dark and Light Cell Death

In most tissues, apoptotic cells are normally phagocytosed by macrophages or other adjacent cells, whereas growth plate chondrocytes (with the exception of terminally differentiated chondrocytes in opened lacunae and the chondro-osseous junction) are surrounded by extracellular cartilage matrix and cannot be phagocytosed. Owing to the distinct lack of typical apoptotic morphological changes in the terminal hypertrophic chondrocytes, some authors concluded that chondrocyte death in the growth plate does not follow the classical mechanism of apoptosis (Farnum and

Wilsman 1989a; Roach and Clarke 2000; Carames et al. 2010). Instead, the presence of autophagic vacuoles and the expression of autophagy-regulating genes by growth plate chondrocytes suggest that these cells undergo processes more similar to autophagy than apoptosis (Roach and Clarke 1999, 2000; Shapiro et al. 2005; Staines et al. 2013). Roach et al. (1995, 1997) defined special forms of chondrocyte cell death such as *paralysis* (Roach and Clarke 1999) or *chondroptosis* (Roach et al. 2004). In an electron microscopic study using an improved fixation technique with ruthenium hexamine trichloride (RHT; Hunziker et al. 1983), Roach and Clarke (2000) distinguished between two forms of cell death in the chick growth plate, one leading to “dark” condensed chondrocytes containing a convoluted nucleus with patchy chromatin condensations and dark cytoplasm with excessive amounts of endoplasmic reticulum. Other cells defined as “light” hypertrophic chondrocytes showed inconsistent chromatin condensation and varying nuclear appearance from pale to uniformly condensed, with progressively disintegrating cytoplasm and organelles, while cell membranes and nuclear membranes were largely preserved. These findings were confirmed in a study on fetal horse growth plates by Ahmed et al. (2007) who distinguished “dark” cell death, marked by a condensed nucleus with progressively convoluted nuclear membranes and cytoplasm fragmented into membrane-bound apoptotic bodies, whereas in “light” cell death, the nucleus was condensed into irregular patches with a convoluted nuclear membrane.

In conclusion, all experimental evidence available supports the notion that hypertrophic chondrocytes undergo some form of programmed cell death at the cartilage erosion front which may be different from the classical apoptotic cell death. But the studies show diverging results with respect to the rate of cell death, morphology, and extent of cellular changes in the growth plate; some of the observed morphological alterations may be indicative of autophagy which may lead to cell death, but in other cases to cell shrinkage, to cell survival, and to reprogramming into a progeny of osteogenic or other stemlike cells with future functions in the primary ossification center.

5.4.4 Evidence for Transdifferentiation of Chondrocytes to Osteoblasts

5.4.4.1 Hypertrophic Chondrocytes Proliferate and Are Metabolically Active

There is ample evidence that hypertrophic chondrocytes have the ability to proliferate, although slowly, and are metabolically active, expressing distinct sets of genes including *Col10a1*, *Opn*, *ALP*, *PTHrP*, *IHH*, *Osx*, *Runx2 HIF1a*, *FGFR3*, *LOX4*, and others in the upper hypertrophic zone and *Mmp13*, *Vegfa*, *Rankl*, *BMP6*, and *Col10a1* in the lower hypertrophic zone (see Chap. 8). Also ultrastructural studies are indicative of a high metabolic activity of chondrocytes even in the lower hypertrophic zone (Holtrop 1972; Hunziker et al. 1984; Farnum and Wilsman 1993). The proliferation rate, however, declines with increasing hypertrophy, and the general view is that the late hypertrophic chondrocytes located in the lower growth plate

adjacent to the cartilage erosion zone have left the cell cycle (Farnum and Wilsman 1993). They were therefore defined as "terminally" differentiated, although there is experimental evidence that some of these cells are still mitotic. For example, Crelin and Koch (1967) showed that hypertrophic chondrocytes in the mouse pubic bone cartilage incorporated ^{3}H-thymidine. Evidence that hypertrophic chondrocytes may resume proliferation was also obtained by BrdU labeling of hypertrophic chondrocytes in the chick growth plate (Galotto et al. 1994).

5.4.4.2 Previous Evidence for Chondrocyte-to-Osteoblast Transformation

In their thymidine labeling experiment, Crelin and Koch (1967) observed that not only chondrocytes but also chondroclasts, osteoblasts, and osteocytes had incorporated ^{3}H-thymidine and thus concluded that hypertrophic chondrocytes of the calcified cartilage may transform into chondroclasts and bone-forming cells. A number of further cell and organ culture experiments have supported the concept of a continuous chondrocyte-to-osteoblast transition in the growth plate, indicating that hypertrophic chondrocytes are not terminally differentiated (Fell 1825; Holtrop 1972; Bentley and Greer 1970; Kahn and Simmons 1977). For example, Thesingh et al. (1991) described organ cultures of fetal mouse bones that were stripped of periosteum and observed that the chondrocytes differentiated to hypertrophic cells and further converted into osteoblasts when the bones were cocultured with brain tissue. The potency of hypertrophic chondrocytes to convert into bone-forming cells was also demonstrated in chondrocyte cell cultures: Human chondrocytes changed their gene expression pattern from collagens II and X to collagen I within 4 weeks in the presence of ascorbate (Kirsch et al. 1992). Similarly, chondrocytes isolated from chick cartilage differentiated to hypertrophic cells in suspension culture in the presence of ascorbate and retinoic acid and changed rapidly after transfer to anchorage-dependent culture conditions into calcifying, osteoblast-like cells expressing collagen I and ovotransferrin, a peculiarity of chick chondrocytes (Gentili et al. 1993; Descalzi-Cancedda et al. 1992).

Strong morphological evidence for direct transdifferentiation of hypertrophic chondrocytes to osteoblasts was presented by Roach et al. (Roach 1992; Roach et al. 1995; Roach and Erenpreisa 1996) who demonstrated the appearance of osteoblast-like cells in intact lacunae of hypertrophic cartilage of 14-day chick femora several days after setting a cut through the hypertrophic zone. The cells within the lacunae stained positive for alkaline phosphatase, osteonectin, osteopontin, and type I collagen, and many hypertrophic chondrocytes were still mitotic as shown by tritiated thymidine incorporation. No osteogenic markers were observed in hypertrophic lacunae of uncut femurs; therefore, the authors suggested that the transdifferentiation to osteoblast-like cells was initiated by disruptions of the cell–cell associations (Roach and Erenpreisa 1996). Interestingly, the change from chondrogenic to osteogenic commitment was associated with an asymmetric cell division with diverging fates of the two daughter cells, with one daughter cell remaining viable and the other one dying (Roach et al. 1995).

Despite these and other experimental studies on the cell fate of hypertrophic chondrocytes, the notion of a continuous chondrocyte-to-osteoblast lineage was never fully accepted, either because of unsettled questions concerning the exclusion of contaminating osteoprogenitor or stem cells in the cell or organ culture systems or because of experimentally induced nonphysiological modulations of the microenvironment of hypertrophic chondrocytes. Furthermore, many of the relevant studies were performed with chick chondrocytes or chick organs which show a higher plasticity of the phenotype and different responses to growth factors and hormones. Therefore, the question remained open whether similar transitions of the chondrogenic to the osteogenic phenotype also occur on the mammalian growth plate in situ.

5.4.4.3 Lineage Tracing Experiments

A definite proof of a chondrocyte-to-osteoblast continuum during endochondral ossification requires labeling of chondrocytes with cell-specific and stable cellular markers which are transmitted to all progeny of the cell. Previous attempts to label the progeny of hypertrophic chondrocytes with *cherry*, the gene for a red fluorescent protein, expressed under the *Col10a1* promoter were, however, not entirely conclusive due to the limited half-life of the cherry protein (Maye et al. 2011). Another study using inducible and stable *Col2a1Cre*-induced reporter gene expression detected a chondrocyte-derived progeny associated with bone trabeculae in the spongiosa underneath the chondro-osseous junction, but these cells did not express bone-specific markers (Hilton et al. 2007). The use of *Col2a1Cre*-induced recombination is also hampered by the fact that C*ol2a1* is expressed very early in chondro-osteoprogenitor cells and in the perichondrium and thus will also label cells which follow a separate osteogenic pathway along the perichondrium (Maes et al. 2010; Ono et al. 2014).

Final affirmation of the concept of chondrocyte-to-osteoblast transdifferentiation was achieved independently in four laboratories in genetic lineage tracing studies based on Cre-induced reporter gene activation. To follow the cell fate of growth plate chondrocytes, hypertrophic chondrocytes were specifically labeled with tamoxifen-inducible or non-inducible Cre recombinases inserted into the *Col10a1* gene (Yang et al. 2014a, b) or driven by a *BAC Col10* transgene (Golovchenko et al. 2013; Zhou et al. 2014; Park et al. 2015). In addition, the chondrocyte lineage was followed using tamoxifen-inducible *Agc1*-CreERT2 mouse (Zhou et al. 2014) in which Cre was inserted in the 3′UTR of the aggrecan gene *Agc1* (Henry et al. 2009). *Col10Cre*- or *Agc1Cre*-induced genetic recombination was made visible by mating with reporter mice bearing LacZ, YFP, or tomato genes under the ROSA26 promoter (Soriano 1999). Using these tools, L. Yang et al. (2014b), G. Yang et al. (2014a), Zhou et al. (2014), and Park et al. (2015) detected reporter gene expression in transgenic mice in hypertrophic chondrocytes but also in trabecular osteoblasts of the primary spongiosa, in the endosteum, and in cortical bone (Figs. 5.1, 5.2, and 5.3). Importantly, no $LacZ^+$ or YFP^+ cells were found in calvaria, nor in the perichondrium/periosteum which is the other source for osteoprogenitor cells invading the spongiosa (Colnot et al. 2004; Maes et al. 2010).

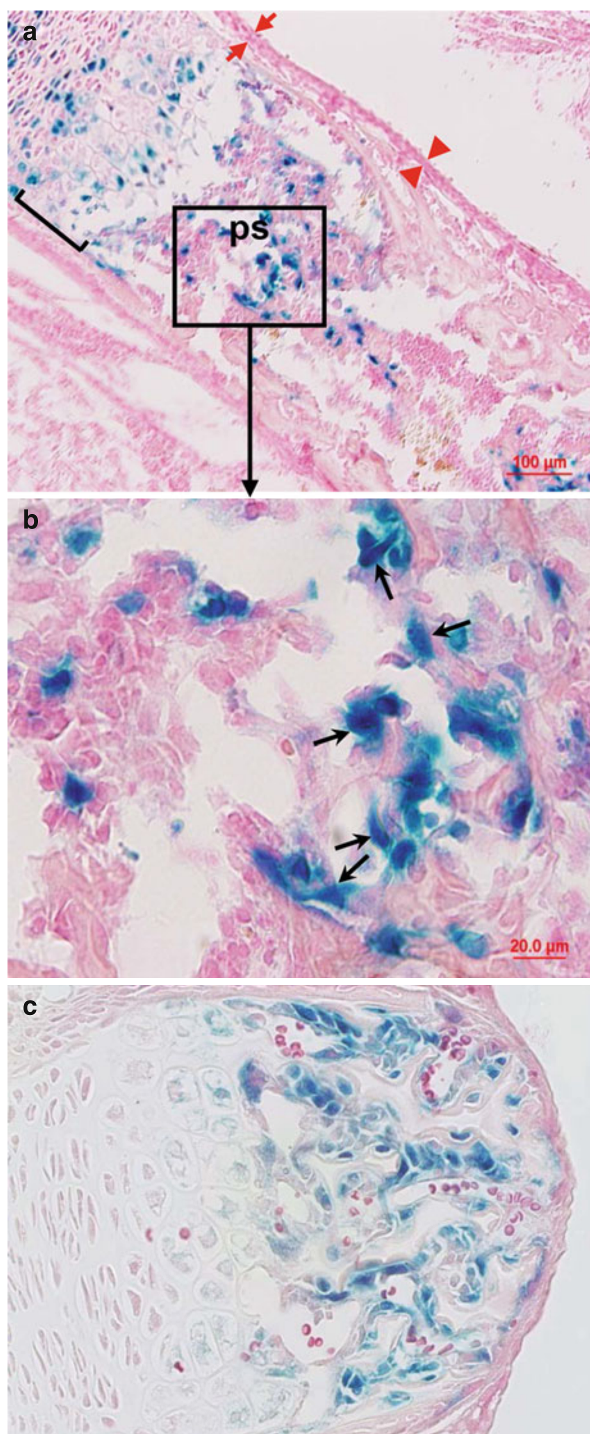

Fig. 5.1 Appearance of non-chondrocytic reporter + cells in the primary spongiosa of tamoxifen-treated Agc1-CreERT2 embryos that are derived from mature chondrocytes. (**a**) The LacZ-stained femur section of E16.5 Agc1-CreERT2;ROSA26R embryo treated with tamoxifen at E11.5. The *black arrows* indicate the non-chondrocytic LacZ+ cells in the primary spongiosa (*ps*). *Black brackets* hypertrophic zone. No LacZ+ cells were detected within the perichondrium (*red arrows*) and periosteum (*red arrowheads*). (**b**) Higher magnification of *black square* in (**a**). (From Zhou et al. (2014), with kind permission by PLOS genetics). (**c**) Similarly, in BACol10Cre;RosaLACZ transgenic mice in which the ROSA29 locus is activated under the Col10a promoter, LacZ-positive cells appear in the primary ossification center (here: ribs of a P5 mouse). The LacZ + cells have typical shape and location of osteoblasts adherent to bone trabeculae (From Park et al. (2015), with kind permission from The Company of Biologists)

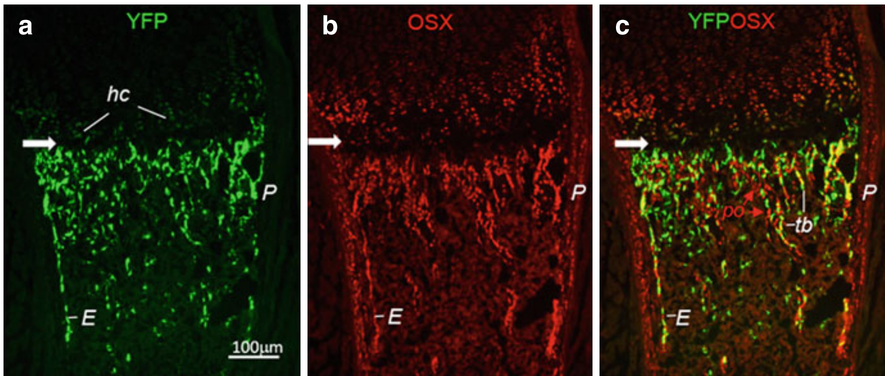

Fig. 5.2 Dual origin of trabecular osteoblasts. *BACCol10Cre*-induced recombination of the ROSA 26YFP locus in hypertrophic chondrocytes activates YFP expression in hypertrophic chondrocytes (*hc* in **a**) and a progeny of hc in the primary spongiosa and endosteum (*E*). Immunofluorescence double staining of a E18.5 tibia of a *BACCol10Cre*;*R26RYFP* mouse for YFP (**a**) and osterix (**b**) shows that about 30 % of the Osx + osteogenic cells are also positive for YFP (*yellow cells* in **c**), i.e., derived from hypertrophic chondrocytes. (**c**) The rest of the Osx^{+} cells in the spongiosa is derived from perichondrium (*red cells*, periosteal osteoblasts *po*). Note the complete absence of YFP+ cells from periosteum/perichondrium (*P*). *White arrow* chondro-osseous junction (Modified from Park et al. (2015))

These studies unanimously provided conclusive evidence that growth plate chondrocytes give rise to a progeny of osteoblasts which contribute substantially to endochondral bone formation. This conclusion relies in part on the premise that the *Col10Cre* activity used in the various transgenic mouse lines is restricted to hypertrophic chondrocytes. Lack of Cre expression in calvarial bone or any other tissue outside hypertrophic cartilage was demonstrated in all *Col10Cre* knock-in mice (Yang et al. 2014a, b) or BACCol10Cre transgenic mice by in situ hybridization (Zhou et al. 2014; Park et al. 2015; Golovchenko et al. 2013).

Comprehensive histological investigations of *Col10cre*- and *Agc1Cre*-induced reporter gene expression during skeletal development demonstrated specific expression of *LacZ*, *YFP*, or *Tomato* reporter genes coinciding with Cre expression in the zone of hypertrophic cartilage (Yang et al. 2014a, b; Park et al. 2015; Zhou et al. 2014) of long bones prior to capillary invasion and bone marrow formation, which begins at about E14–E15 in the mouse tibia anlage. With formation of the primary ossification center, strong expression of reporter genes appeared in the spongiosa in cells that were associated with bone trabeculae or lining the endosteal surface (Yang et al. 2014a, b; Zhou et al. 2014; Park et al. 2015). Many of the reporter gene-positive cells showed epithelioid, osteoblast-like morphology (Fig. 5.1) and co-expressed osteogenic markers including osterix and/or collagen I and osteocalcin, as shown by immunofluorescence double or triple staining, by immunohistochemistry or combinations with in situ hybridization (Fig. 5.2). Increasing numbers of reporter gene-positive cells, i.e., hypertrophic chondrocytes (HC)-derived osteogenic cells, were observed in the primary and secondary ossification centers of all long bones, ribs,

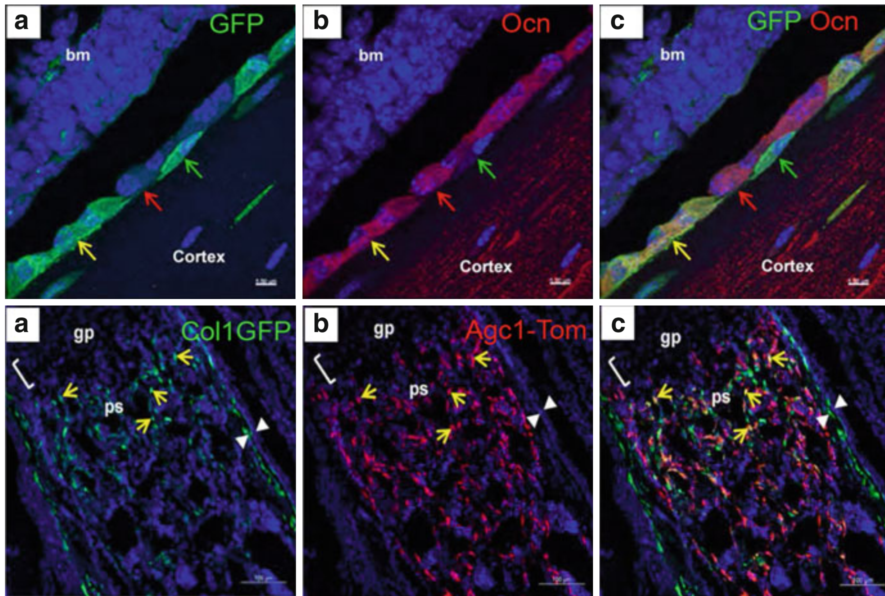

Fig. 5.3 *Upper panel* (**a**–**c**) Lineage tracing of hypertrophic chondrocytes using *Col10a1-Cre*;*Osx flox*/+ mice (Zhou et al. 2014). Double immunofluorescence (DIF) experiments revealed that the EGFP+ (Osx−/+) cells (*yellow arrows*) in the primary spongiosa and endosteum are mature chondrocyte-derived osteoblasts. DIF experiment with anti-Ocn and anti-GFP using femur frozen sections of 1-month-old *Col10a1-Cre*;*Osxflox*$^{/+}$ mice. *Green arrows* EGFP + Ocn- cells, *red arrows* Ocn + EGFP- cells. *Lower panel* (**a**–**c**) Lineage tracing using *Agc1-CreERT2*;*2.3Col1-GFP*;*ROSA-tdTomato* triple transgenic mice (Zhou et al. 2014). Cellular co-localization of the chondrocyte-derived tomato marker and the osteoblast-specific 2.3Col1-GFP marker shows collagen 1-expressing cells (*yellow arrows*) in the spongiosa of P1 femora that are derived from chondrocytes. Tamoxifen treatment was done at E14.5. Green cells are Tomato^{-} EGFP^{+} cells, *yellow arrows* Tomato^{+}EGFP^{+} cells. Only EGFP+ cells but no Tomato + cells were present in the perichondrium (between *white arrows*) and periosteum (between *white arrowheads*) (From Zhou et al. (2014), with kind permission from PLOS Genetics) *white brackets* growth plate (gp)

and vertebrae (Park et al. 2015) in fetal, postnatal, and even adult bones (Yang et al. 2014a, b), again confirming the notion that hypertrophic chondrocytes give rise to a non-chondrocytic, osteogenic progeny.

In light of remaining uncertainties with respect to antibody specificity, further efforts were made to ensure the osteogenic phenotype of HC-derived cells in the spongiosa. For example, Zhou et al. (2014) identified osteogenic cells in the spongiosa unequivocally by expressing EGFP+ under the *Col10Cre*-recombined *Osx* promoter (*Agc1-CreERT2 Osx*$^{+/}$*EGFP*) (Fig. 5.3). An elegant and specific way to mark *Col1a1*-expressing cells in an antibody-independent way was the insertion of the 2.3-*Col1*;*GFP* transgene which is expressed specifically in osteoblasts but not in fibroblasts (Kalajzic et al. 2002) into *Col10Cre*;*tomato* or *Agc1Cre*;*tomato* mice (Zhou et al. 2014) (Fig. 5.3).

The osteogenic character of YFP^{+}, i.e., HC-derived cells in the spongiosa of P5 *BACCol10Cre*;*ROSAYFP* bones, was further confirmed by qRT-PCR analysis of

FACS-sorted YFP+ trabecular osteoblasts which revealed expression of *Col1a1*, *bsp* (bone sialoprotein), and *Runx2* (Park et al. 2015). Similarly, cultured endosteal cells isolated from 3-week-old *BACCOL10Cre*; *ROSAYFP* bones and sorted for YFP fluorescence expressed Osx, *Runx2*, *osteocalcin*, and *Col1a1* as shown by RT-PCR (Park et al. 2015).

5.4.4.4 Tam-Inducible Cre Expression

Despite the results of in situ hybridization analysis showing the absence of Cre expression in cells other than hypertrophic chondrocytes or in calvarial bone (Yang et al. 2014a, b; Zhou et al. 2014; Park et al. 2015), questions remained whether minute levels of *Col10Cre* expression below the detection limit of in situ hybridization can be excluded in osteogenic cells of the primary spongiosa. Further strong evidence for the specificity of *Col10Cre* activity was, however, provided by L. Yang et al. (2014b) and Zhou et al. (2014) who confirmed the chondrocyte-to-osteoblast continuation in the growth plate by generating tamoxifen-inducible *Col10CreERt* (Yang et al. 2014b) or *Agc1CreERT2* (Zhou et al. 2014) reporter lines. CreERt and CreERT2 are Cre recombinases bearing mutated estrogen receptor binding domains (ERT) which do not respond to endogenous estradiols but are activated through nuclear localization by the estrogen analog 4-hydroxy-tamoxifen (Feil et al. 2014). The advantage of this system is that it allows time-controlled pulses of *cre* activation by administering tamoxifen (TAM) to the pregnant mothers at distinct stages of development. If the pulse is started in a defined window prior to onset of bone marrow formation, it will mark only hypertrophic chondrocytes, and the time course of reporter gene expression in hypertrophic chondrocytes and their progeny including osteogenic cells can be tracked by analyzing the offspring at various days after TAM injection. For example, when pregnant *Col10CreErt*;*LacZ*+ mothers were injected with tamoxifen at stage E 13.5, after a time lag of 2 days, many LacZ+ chondrocytes were detected in the hypertrophic zone of the humerus and some in the tibia at E15.5, whereas at E16.5, fewer LacZ+ cells were seen in the hypertrophic zone, but more in the primary ossification center (Yang et al. 2014b). This excluded unspecific *Col10Cre*-induced recombination of the ROSA26 locus in osteoblasts, because in that case, one would expect LacZ+ cells appearing in the primary spongiosa simultaneously with hypertrophic chondrocytes.

Col2a1Cre-driven reporter gene expression is activated already in early chondro-osteoprogenitor cells in the cartilage blastema as well as in the perichondrium and may therefore also mark the perichondrium-derived osteogenic lineage. In contrast, *Agc1* (aggrecan) is expressed in resting chondrocytes but not in chondro-osteoprogenitor cells nor in the perichondrium (Henry et al. 2009) and is therefore a suitable marker to follow the cell fate of mature (resting and proliferating) chondrocytes. *Agc1*;*CreERT2* activity was detected in limb chondrocytes already at embryonic stage E12.5 (Zhou et al. 2014). The activation window in *Agc1CreERT2*; *ROSALacZ* mice was 24–48 h after TAM injection. When TAM was injected at E11.5, LacZ+ chondrocytes were already detected at E13.5 (Zhou et al. 2014), and at E15.5, with onset of ossification in the primary spongiosa, cre-inducing activity of tamoxifen was no longer retained in the tissue, but at E16.5, LacZ+ cells were

seen in the primary spongiosa. The presence of osteogenic cells derived from mature chondrocytes was also confirmed by analyzing *Agc1-CreERT2*;*Osx*$^{+/}$*EGFP* mice which revealed chondrocyte-derived, osterix-positive (Osx + EGFP+) cells in the spongiosa at E15.5 2 days after tamoxifen treatment (Zhou et al. 2014).

5.4.4.5 Quantitative Contribution of Chondrocyte-Derived Osteoblasts to Endochondral Bone

These findings challenged not only the common view that late hypertrophic chondrocytes are eliminated at the chondro-osseous junction of the growth plate but also the concept that all trabecular and endosteal osteoblasts in the primary and secondary ossification center originate from osteoprogenitor cells invading from the periosteum along with bone marrow capillaries (Colnot et al. 2004; Maes et al. 2010; Ono et al. 2014). An important issue was therefore the question to what extent chondrocyte-derived osteoblasts contributed to endochondral bone formation in relation to periosteum-derived osteoblasts. Depending on the type of genetic lineage tracing, the markers to define osteoblasts (osteoprogenitors, osteoblasts, or mature osteocytes), and the stage and types of bones investigated, the estimated contributions of chondrocyte-derived osteoblasts to total endochondral bone cells varied between 8.5 and 30 % in the trabecular and endosteal zone of the spongiosa at embryonic stage E18.5 or newborns and 40–60 % in postnatal cortical bone (Yang et al. 2014a, b; Zhou et al. 2014; Park et al. 2015).

A quantitative analysis of the ratio of chondrocyte-derived osteogenic cells (defined by nuclear staining for osterix) to total osteogenic cells in the primary spongiosa of E16.5 to P7 *BACCol10cre*;*ROSAYFP*$^{+}$ tibiae indicated that chondrocyte-derived (YFP^{+}) Osx^{+} cells contributed 30 % to all Osx^{+} cells in the spongiosa at E18.5, leveling off at 18 % in the postnatal stages (Park et al. 2015). Similarly, L. Yang et al. (2014b) reported that 16 % of all Col1-positive cells in the primary ossification center of E18.5 tibiae of *BACCol10CreErt*;*ROSALacZ*$^{+}$ embryos were positive for LacZ. This corresponded to the 30 % ratio reported above (Park et al. 2015), considering that in *Col10Cre* knock-in mice, only 50 % of the hypertrophic chondrocytes were LacZ^{+} after TAM-induced reporter gene activation (Yang et al. 2014b), whereas 100 % of the hypertrophic chondrocytes in growth plates in *BACCol10Cre*;*ROSALAcZ* bone were LacZ positive (Gebhard et al. 2008; Park et al. 2015). Divergent results were published on postnatal stages: G. Yang et al. (2014a) reported that about 30 % of all Col1-positive cells in metaphyses of 20-day-old *Col10Cre*;*ROSALacZ* tibiae were LacZ^{+}, as compared to 21 % in the trabecular zone. Zhou et al. (2014), however, reported much higher rates with 60 % chondrocyte-derived Ocn^{+} Col1a1^{+} osteoblasts in the trabecular zone, 68 % in the endosteal zone of 3- to 4-week-old Col10a1Cre;Osx$^{flox/EGFP}$ or Col10a1-cre;2.3Col1-GFP; ROSA Tomato tibiae, and 69 % in the cortical zone. Similarly, a rate of 40–50 % LacZ-positive, matrix-embedded mature osteocytes expressed the LacZ marker in cortical bone of 4 week of *Col10Cre*;*ROSA LacZ* mice (Park et al. 2015). These studies also provided strong evidence for a dual origin of cortical bone in fetal and postnatal stages forming by appositional growth, with chondrocyte-derived osteoblasts accumulating matrix from the endosteal side and periosteum-derived osteoblasts providing the outer part of the cortical bone (Fig. 5.4).

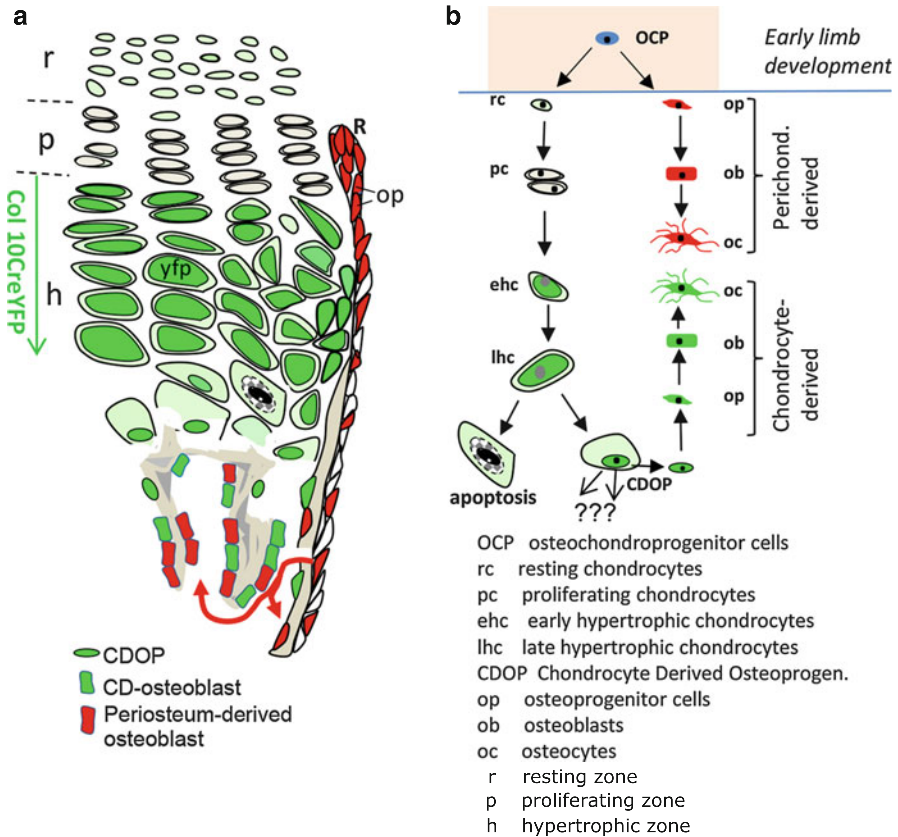

Fig. 5.4 (**a**) Cell fate analysis of hypertrophic chondrocytes reveals transdifferentiation to osteoblasts: Lineage tracing of hypertrophic chondrocytes with Col10Cre-activated reporter genes (here: YFP) revealed that in the fetal spongiosa, a substantial fraction of trabecular osteoblasts is derived from hypertrophic chondrocytes (*green cells*), whereas the majority of osteoblasts are derived from perichondrial osteoprogenitor cells (*red cells*) invading the spongiosa through capillaries (Modified after Park et al. (2015)). (**b**) Dual pathways but common origin of trabecular osteoblasts: Chondrogenic and osteogenic lineages start from a common Sox9^{+} osteochondroprogenitor cell in early limb development. In the growth plate, chondrocytes mature in several distinct differentiation steps to hypertrophic cells which either undergo programmed cell death (apoptosis or chondroptosis) or shrink to small osteoprogenitor cells (*CDOP*) which may be reprogrammed into the osteogenic lineage and other bone marrow cells

5.5 Transdifferentiation During Fracture Callus Formation and Bone Repair

Bone fracture healing involves in most cases a cartilaginous callus intermediate that is replaced by bone in a process analogous to endochondral ossification. To elucidate whether chondrocytes of the fracture callus also give rise to bone cells during

the repair process, L. Yang et al. (2014b) grafted pieces of hypertrophic cartilage isolated from *Col10Cre*;*RLacZ* pups into bone injury sites generated by drilling holes in the tibia of 3-month-old adult mice. Within 8 days post operation, LacZ-positive cartilage in the graft decreased and was replaced by bone containing LacZ+ osteoblast- and osteocyte-like cells.

In another study, healing of semi-stabilized tibia fractures was investigated in 2- to 3-month-old *Agc1-CreERT2*;*ROSA-tdTomato* mice (Zhou et al. 2014). Seven days post surgery, *Agc1*- mice were treated with tamoxifen, and at day 9, when chondrocyte differentiation occurred in the repair callus, Tomato$^+$ fluorescence was completely matching the areas of Safranin-O-stained chondrocytes in the callus, suggesting that the Tomato$^+$ cells in the cartilage callus were in fact chondrocytes. On day 14, the repair callus was partially ossified, showing a mixture of bone and cartilage, and almost all cells in the repair callus, both in the cartilage and in the bone regions, were positive for Tomato, suggesting the presence of non-chondrocytic Tomato$^+$ cells in the repair callus. In the repair callus of a post-surgery day 14 *Agc1-CreERT2*;*2.3Col1-GFP*;*ROSA-tdTomato* mouse treated with tamoxifen at post-surgery day 6, many of the Tomato$^+$ cells were also positive for GFP (Tomato$^+$ GFP$^+$), implying that the mature chondrocytes present in the repair callus have the ability to become *Col1a1*-expressing bone-forming osteoblasts. At day 29 post surgery, the number of Tomato$^+$ GFP$^+$ cells was substantially increased, and ossification was almost complete (Zhou et al. 2014). Thus, both studies provide convincing evidence that chondrocytes in the repair callus are a source of osteoblasts involved in bone fracture healing.

5.6 Mechanism of Chondrocyte-to-Osteoblast Conversion: Transdifferentiation or Redifferentiation and Reprogramming?

Despite the overwhelming evidence for a continuation of chondrocyte differentiation into osteogenic cells, many questions remain open concerning the mechanism and regulation of conversion of hypertrophic chondrocytes into osteoblasts. Do hypertrophic chondrocytes give rise to osteoblasts or osteoprogenitor cells by direct transdifferentiation, or does this process involve redifferentiation of hypertrophic chondrocytes to stemlike cells and reprogramming into osteoblasts? What is the sequence of gene expression changes during transdifferentiation? Are the osteogenic cells generated by asymmetric division of hypertrophic chondrocytes as proposed by Roach et al. (1995), with one daughter cell undergoing cell death and the other entering an osteogenic transdifferentiation program? Is the process of transdifferentiation a cell-autonomous process or regulated by the microenvironment of the hypertrophic chondrocytes in the calcifying lacunae adjacent to invading bone marrow capillaries? Which extracellular factors are involved in this case, and which factors are critical for the decision of transdifferentiation versus programmed cell death? How can small osteoprogenitor cells emerge from the substantially larger hypertrophic chondrocytes with diameters of 15–20 μm, do they shrink by autophagy or by other mechanisms?

5.6.1 Identification of Chondrocyte-Derived Osteoprogenitor Cells in the Growth Plate

In attempts to identify the origin of the *Col10Cre*-induced YFP-labeled osteoprogenitor cells at the chondro-osseous junction, growth plates isolated by microdissection of P5-P7 *Col10CreYFP*$^+$ tibiae, femora, and humeri were analyzed by confocal microscopy (Park et al. 2015). Detailed analysis of the chondro-osseous junction at serial levels along the longitudinal axis (*z*-axis) revealed the presence of small chondrocyte-derived (YFP$^+$) Col1$^+$ cells and YFP$^+$Osx$^+$ cells of 4–6 μm diameters in the lowest zone of hypertrophic chondrocytes adjacent to the cartilage–spongiosa interface. Importantly, these cells which were named chondrocyte-derived osteoprogenitor (CDOP) were mitotically active, as shown by BrdU injection into pregnant *Col10Cre*;*YFP*$^+$ mice 1 day before delivery (Park et al. 2015). Size and position of these cells resembled the "condensed hypertrophic chondrocytes" described ultrastructurally by Farnum and Wilsman (1987, 1989b) in the lowest row of closed lacunae of hypertrophic growth plate cartilage. These cells have a condensed nucleus and extensive cytoplasmic vacuolization and fill only a small part of the space of its lacuna. Initial attachments to the pericellular and territorial matrices eventually disappear, except at the last transverse septum (Farnum and Wilsman 1987, 1989b). Whether the CDOP cells identified by Park et al. (2015) actually represent these condensed cells, however, remains to be confirmed.

To confirm the ability of CDOP cells to differentiate into osteoblasts, CDOP cells were isolated from P5-P7 *Col10CreYFP*$^+$ growth plates by sequential enzymatic digestion of isolated growth plates with trypsin and collagenase, and three fractions were obtained which were cultured on fibronectin-coated culture dishes. After 7–12 days in monolayer culture, immunofluorescence analysis of fraction 2 cells with diameters of 4–5 μm revealed numerous small YFP$^+$ cells staining for the stem cell marker Sca1. When cells were expanded for 10 days and YFP$^+$ and YFP$^-$ cells were separated by FACS, almost all YFP$^+$ cells were positive for Osx. RT-PCR analysis of RNA isolated from sorted YFP$^+$ fraction 2 cells revealed expression of both stem cell-typical genes such as *CD34*, *sca1*, *sox2*, and *c-myc* and genes of the osteogenic lineage including *col1a1*, *osx*, and *Runx2*. After additional 14 days in culture, mRNA levels of *Col1a1*, *Ocn*, and *Runx2* in YFP$^+$CDOP cells were comparable to those of differentiating MC3T3 osteoblasts, confirming the ability of CDOP cells to differentiate into osteoblasts (Park et al. 2015). Altogether, these findings confirm that the population of small, 4–6 μm cells in fraction 2 isolated from growth plates contained chondrocyte-derived cells with osteoprogenitor character. They represent most likely the CDOP cells observed by confocal microscopy in the chondro-osseous junction of the growth plate.

5.6.2 Autophagy in Hypertrophic Chondrocytes: Preparation for Apoptosis or Cell Survival?

A major question arising from these observations relates to the dramatic changes in cell size during the transdifferentiation process: How do the large hypertrophic

chondrocytes shrink to osteoprogenitor cells with an approximately 20- to 50-fold lesser volume? In light of increasing evidence for autophagy occurring in growth plate and articular chondrocytes (Bohensky et al. 2009; Srinivas et al. 2009a, b; Carames et al. 2010), it is likely that cell shrinkage occurs by autophagy. Autophagy is primarily considered a cell-protective mechanism that permits cells to survive under stress conditions such as nutrient deficiency or hypoxia (Carames et al. 2010). This includes that "the cell cannibalizes itself to generate energy" (Srinivas et al. 2009b; Yang and Klionsky 2010). By sequestering dysfunctional organelles, proteins, and membranes into double-membrane vesicles called autophagosomes which later fuse with lysosomes, cells generate energy and remain viable and complete their life cycle (Kimura et al. 2007; Srinivas et al. 2009b; Yang and Klionsky 2009). In fact, most YFP-positive hypertrophic chondrocytes in the prehypertrophic and hypertrophic zone of *BACCol10CreYFP* mice showed a punctate fluorescence after both anti-beclin-1 and anti-LC3B staining (Park et al. 2015), characteristic of autophagic vacuoles (Srinivas et al. 2009b; Carames et al. 2010). Beclin-1 is a protein that participates in the nucleation of autophagic vesicles (Thorburn 2008; Zhang et al. 2013) and LC3B is required for the expansion of autophagosomes (Weidberg et al. 2011).

On the other hand, autophagy is also closely related to apoptosis caused by stress or hypoxia (Wang 2015; Li et al. 2015). Generally, autophagy blocks the induction of apoptosis and inhibits the activation of apoptosis-associated caspases which could reduce cellular injury (Wu et al. 2014). Thus, autophagy is very likely the first step in the condensation process of hypertrophic chondrocytes and either may lead to cell death or permit cell survival and reprogramming to osteogenic cells depending on the microenvironmental conditions of the cells (Carames et al. 2010). Identification of these conditions which may include hypoxic conditions, reactive oxygen species (ROS) (Poillet-Perez et al. 2015), or pH changes appears an important question to be solved.

5.6.3 Programmed Cell Death or Transdifferentiation?

As the current data strongly suggest that hypertrophic chondrocytes have the option to undergo programmed cell death, or to reenter the cell cycle and activate an osteogenic fate (Fig. 5.4), the question arises at which stage of the chondrocyte differentiation program this decision is made and which factors of the microenvironment of the growth plate are involved in the control of this decision. As hypertrophic chondrocytes are arranged in vertical columns, each representing most likely one clone derived from a single prehypertrophic chondrocyte at the top of the column, the decision for cell death versus reprogramming after onset of autophagy may occur at any stage before reaching the lowest lacuna in the growth plate. Using the multicolor Confetti reporter system (Muzumdar et al. 2007) activated by *Col2a1CreERT*-mediated recombination, G. Yang et al. (2014a) observed few chondrocyte columns 5 days after tamoxifen activation, each labeled uniformly with different colors, confirming the clonal character of the chondrocyte columns.

Several days later, the colored columns extended into the spongiosa underneath the growth plate, indicating direct conversion of some chondrocytes into osteogenic cells and other bone marrow cells. This powerful lineage tracing system could be extended to investigate whether different types of clonal chondrocyte columns could be distinguished, perhaps on the basis of differentially expressed genes, which would undergo cell death rather than reprogramming into osteogenic cells. The clonal character of hypertrophic chondrocytes within a column and the heterogeneity of the cell fate in individual columns are consistent with the report by Farnum and Wilsman (1987, 1989b) who described the appearance of small "condensed" cells in some, but not all lowest intact lacunae of hypertrophic chondrocytes.

For the decision of cell death by apoptosis versus survival, the expression levels of Bcl2 and BAX have been shown to be instrumental. Bcl-2 and BAX are members of an emerging family (Bcl family) of proteins which are involved in the regulation of programmed cell death (Vaux et al. 1988; Korsmeyer 1992). Bcl-2, an inhibitor of apoptosis, and BAX, an apoptosis inducer, form heterodimers which neutralize the anti-apoptotic effect of Bcl-2, causing accelerated cell death. Within a cell, it is the ratio of Bcl-2 to Bax that determines whether a cell dies or not (Amling et al. 1998; Sedlak et al. 1995; Oshima et al. 2008; Yin et al. 1994).

Very little is known, however, on the heterogeneity of the microenvironment in different zones or different columns of hypertrophic chondrocytes in the growth plate. Gibson et al. (1995) have reported enhanced apoptotic activity in hypertrophic chick sternal chondrocytes located close to invading bone marrow vessels, and also in the mammalian growth plate, the bone marrow invasion front is rather irregular, suggesting that different hypertrophic chondrocytes along the chondro-osseous junction may be exposed to different levels of invading cytokines or growth factors and to different pH or oxygen levels. Elucidation of these parameters and their influence on autophagy, cell death, or cell survival and transdifferentiation will be one of the demanding challenges in the future to clarify this issue.

Conclusion

The recognition of a continued chondrocyte-to-osteoblast lineage sheds new light on the role of hypertrophic chondrocytes in endochondral ossification. We now understand why such seemingly intricate and complex mechanisms are necessary to regulate chondrocyte differentiation in the growth plate, leading at the end to a hypertrophic chondrocyte which not only produces a specialized calcified cartilage matrix but also induces invasion of vascular sprouts, expresses bone-specific genes, and finally gives rise to a progeny of osteoblasts. Numerous previous studies reporting complex alterations of endochondral bone formation after genetic deletion or overexpression of osteogenic or chondrogenic regulatory genes in chondrocytes may have to be reconsidered in light of the concept of a continued chondrocyte-to-osteoblast lineage, not only in transgenic animal studies but also in human chondrodysplasias.

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Hypoxia-Driven Pathways in Endochondral Bone Development

6

Naomi Dirckx and Christa Maes

Abstract

Endochondral ossification, the mechanism responsible for the development of the long bones, is dependent on the vigorous and tightly regulated proliferation, differentiation, and matrix synthesis by chondrocytes and osteoblasts. Chondrocytes first generate cartilaginous templates of the forming bones from mesenchymal condensations and subsequently create the growth plates that provide the prime engine for bone growth. These cartilaginous structures are inherently avascular and represent physiologically hypoxic tissues. Hypoxia-driven pathways, governed by the hypoxia-inducible factors (HIFs), are absolutely essential for the survival and functioning of chondrocytes in these challenging conditions. Following chondrogenesis, further bone development and growth is driven by the progressive conversion of the prefiguring cartilage into bone tissue. This process depends on cartilage neovascularization and the concomitant infiltration of the future ossified region by osteoprogenitors, and is molecularly steered by the potent angiogenic stimulator vascular endothelial growth factor (VEGF). Later in life, HIFs and VEGF remain vital players in the regulation of bone remodeling, in line with the hypoxic status of the postnatal bone and marrow environment. HIF-mediated signaling has also been implicated in joint formation and the integrity of the adult articular cartilage. Thus, the oxygen-regulated genetic program mediated by HIFs is a key to the controlled development, growth, health, and disease of endochondral bones. Much of the current knowledge is based on the investigation of a growing number of genetically modified mouse models, dissecting the roles of the HIFs as well as their key downstream effectors and upstream regulators in mesenchymal progenitors, chondrocytes, and osteoblast lineage cells, as will be reviewed in this chapter.

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6.1 The Hypoxia-Inducible Factor (HIF) Pathway

Hypoxia-driven pathways play major roles in pathological conditions such as cancer and ischemia, but are also vitally important in normal development and tissue homeostasis (Semenza 2012, 2014; Ratcliffe 2013). Particularly cartilage, an inherently avascular tissue, is highly dependent on cellular hypoxia-adaptation mechanisms, as will be discussed in this chapter.

The main orchestrator of the cellular responses to hypoxia is the transcription factor hypoxia-inducible factor (HIF) (Semenza 2012, 2014; Ratcliffe 2013). HIFs are heterodimers consisting of an α subunit which is regulated by oxygen (HIF-1α, HIF-2α/EPAS1, or the less characterized HIF-3α) and a β subunit that is constitutively expressed in an oxygen-independent manner. The best characterized member, HIF-1, is formed in hypoxic conditions by the subunits HIF-1α and HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator, ARNT), which both contain basic helix-loop-helix-PAS domains that mediate heterodimerization and DNA binding (Fig. 6.1). In well-oxygenated conditions, the HIF-1α protein is hydroxylated on specific residues within its amino acid sequence. Specifically, members of the superfamily of 2-oxoglutarate (2-OG)-dependent dioxygenases termed the HIF prolyl-4-hydroxylases (PHDs, the three prime members being PHD1-3, also known as Egln2, Egln1, and Egln3) mediate the hydroxylation of prolines P402 and P564 in the oxygen-dependent degradation domain (ODD) using O_2 as a substrate (Myllyharju and Koivunen 2013). This hydroxylation reaction leads to the recognition of HIF-1α by the von Hippel-Lindau protein (pVHL), part of an E3 ubiquitin ligase complex, associated with the ubiquitination and instant proteosomal degradation of HIF-1α in non-hypoxic conditions (Fig. 6.1) (Semenza 2012, 2014; Ratcliffe 2013). PHD2, which is ubiquitously expressed, represents the most important PHD isoform with regard to the normoxic degradation of HIF-1α and as based on knockout mouse models. Indeed, PHD2 null mice are embryonic lethal due to severe placental and heart problems, and conditional inactivation of the PHD2 gene leads to defects in several organs, while inactivation of the more tissue-restricted isoforms PHD1 and PHD3 is not associated with developmental defects (for references and details, see review (Myllyharju and Koivunen 2013)).

In hypoxic conditions, estimated at oxygen tension levels dropping below a threshold of 5 %, this oxygen-dependent hydroxylation reaction and rapid degradation of HIF-1α is inhibited. As a result, the HIF-1α protein is stabilized and can translocate to the nucleus, where it dimerizes with HIF-1β to form the transcriptional complex HIF-1 regulating the hypoxia-response program of the cell (Fig. 6.1) (Semenza 2012; Maes et al. 2012b). Besides the PHDs regulating the protein stability of HIF-1α (and HIF-2α), another enzyme termed factor-inhibiting HIF-1 (FIH-1) negatively regulates the transcriptional activity of HIF-1α (and HIF-2α) in normoxia. This reaction is mediated by FIH-1-dependent hydroxylation of a specific asparaginyl residue, which blocks the binding of HIF to the coactivator proteins p300 and CREB-binding protein (CBP) (Fig. 6.1).

HIF-1 executes its transcriptional program by binding to hypoxia-response elements (HRE) within the promoters of its target genes. More than a hundred putative

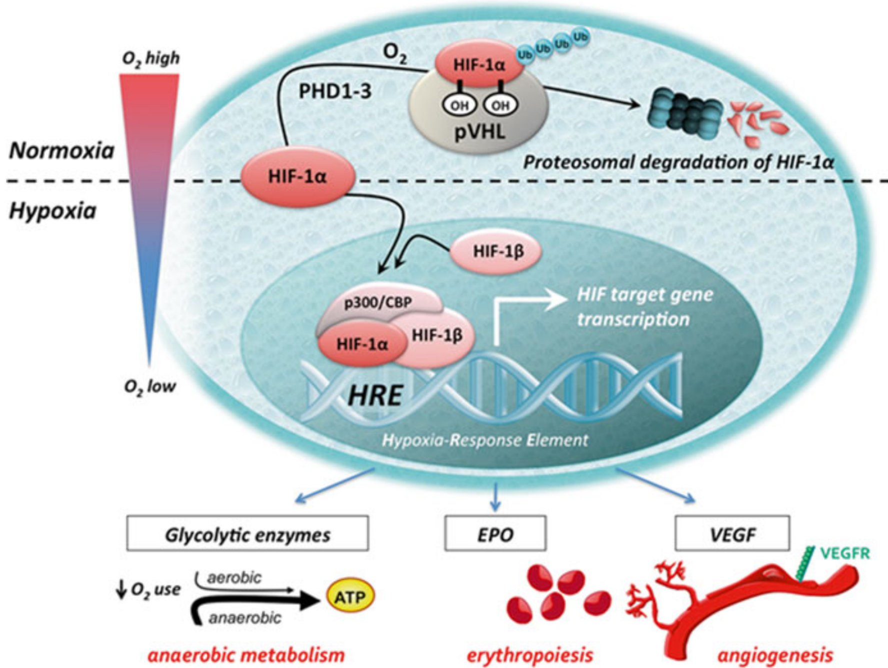

Fig. 6.1 The HIF pathway. In normoxia, hypoxia-inducible factor (*HIF*)-1α is targeted for proteosomal degradation by interaction with the von Hippel-Lindau protein (*pVHL*) that is part of an ubiquitin ligase complex. The recognition of HIF-1α by pVHL is regulated through an oxygen-dependent hydroxylation of specific proline residues (402 and 564) in HIF-1α; this reaction is executed by HIF prolyl-hydroxylases (*PHD1-3*). In hypoxia, HIF-1α is stabilized, allowing its interaction with the constitutively expressed HIF-1β subunit to form the HIF transcriptional complex. The HIF complex binds to the hypoxia-responsive element (HRE) in a plethora of target genes and induces transcription. HIF target genes include glycolytic enzymes involved in anaerobic metabolism, erythropoietin (*EPO*), and vascular endothelial growth factor (*VEGF*). Other abbreviations: *CBP* CREB-binding protein, *ATP* adenosine triphosphate, *VEGFR* VEGF receptor

HIF-1 target genes have been identified, several of which function in cell proliferation, differentiation, survival, resistance to oxidative stress, apoptosis, and extracellular matrix homeostasis, but most typically and abundantly involving genes regulating energy metabolism and angiogenesis (Semenza 2012, 2014; Ratcliffe 2013). Indeed, key hypoxia-responsive genes and pathways include regulators of glucose utilization, in particular those stimulating anaerobic metabolism or glycolysis, which mediate the cell's adaptation to hypoxia by reducing its oxygen consumption and its dependence on oxygen to generate ATP. The shift toward glycolysis at the same time prevents the generation of excess reactive oxygen species (ROS) in the mitochondria due to inefficient electron transport under hypoxic conditions. Secondly, cells respond to low oxygen by stimulating the delivery of oxygen, which is achieved by activating erythropoiesis via HIF-induced erythropoietin (EPO) expression and by stimulating angiogenesis through HIF-driven expression of

vascular endothelial growth factor (VEGF) (Fig. 6.1). Through these combined transcriptional actions, HIFs thus function as master regulators of oxygen homeostasis (Semenza 2012, 2014; Ratcliffe 2013).

Although HIF-1α and HIF-2α both dimerize with HIF-1β and share extensive structural homology (48 % homology in overall amino acid sequence, including a few highly conserved key domains within the proteins), they do not exhibit the same distribution, target genes, and functions, and they are consequently nonredundant. For instance, while HIF-1α is primarily active in insufficiently vascularized and hypoxic tissues, HIF-2α can be expressed in well-vascularized tissues independently of hypoxia and in a more cell-restricted manner than HIF-1α. HIF-2α plays important roles in erythropoiesis, vascularization, and pulmonary development (Semenza 2012, 2014; Ratcliffe 2013).

6.2 HIF-Mediated Regulation of Chondrogenesis and Chondrocyte Survival in Mesenchymal Condensations and Developing Growth Plates

A growing number of studies using genetically modified mice support the essential roles of HIF-1 and hypoxia-regulated molecular pathways, including VEGF signaling, in chondrocyte biology and the regulation of the early development and growth of the long bones. Given the early fetal lethality of ubiquitous knockout mice for most of these important molecules, insights in skeletal biology have been gained particularly by employing Cre-loxP strategies to generate conditional knockout (cKO) models. During embryogenesis some bones form directly through intramembranous ossification, but most of the bones of the skeleton develop via temporary cartilage anlagen through the complex, multistep process of endochondral bone formation (Fig. 6.2 and Chaps. 4, 8, and 10). Both processes start with mesenchymal condensations outlining the future bones and consisting of progenitors that bear the potential to differentiate into osteoblasts. In endochondral bones, however, the mesenchymal cells in the condensations initially differentiate into chondrocytes, generating cartilaginous templates of the forming long bones and subsequently making up the growth plates that provide the prime engine for the longitudinal growth of the bones (Fig. 6.2). These cartilaginous structures are rather unique tissues in that they are inherently avascular and hypoxic (Maes et al. 2012a; Provot et al. 2007; Schipani et al. 2001), and the chondrocytes absolutely require hypoxia-driven pathways and HIF-1α-mediated signaling in order to survive these challenging conditions (Schipani et al. 2001). In fact, the genetic program controlled by hypoxia is a key to the controlled development and growth of endochondral bones by modulating the process of chondrogenesis at multiple steps (Fig. 6.3). Defining these roles of the pathway and of specific molecular components thereof has been advanced greatly by the use of Prx1-Cre- and type II collagen (Col2)-Cre-driver strains to generate conditional mouse mutants, thereby targeting the fetal limb bud mesenchyme or early osteochondroprogenitors and chondrocytes, respectively (Table 6.1, Part A).

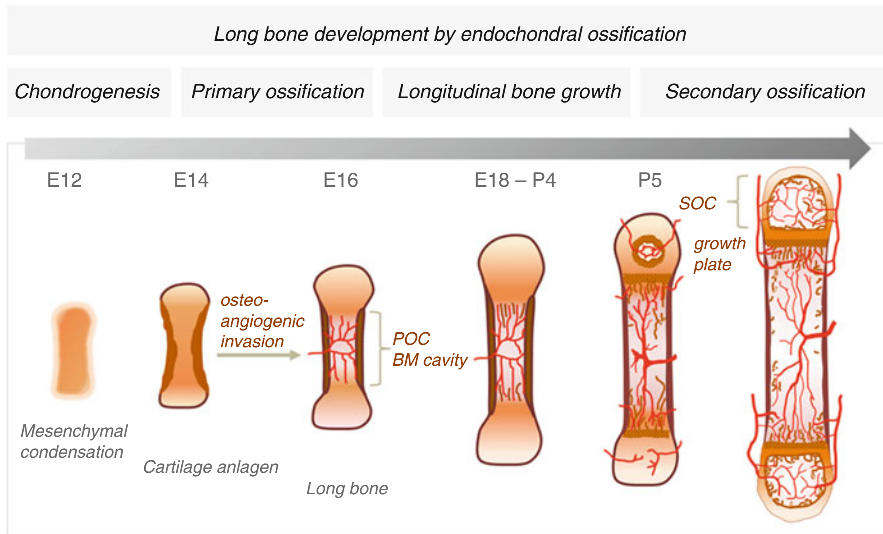

Fig. 6.2 Endochondral ossification. The sequence of events that take place during development to generate the long bones imply the transformation of avascular, hypoxic cartilage into highly vascularized bone tissue, through consecutive stages of vascularization. Figures 6.3, 6.4, and 6.5 detail the regulation by hypoxia-driven pathways and VEGF family members of specific aspects and stages shown in this overview. *POC* primary ossification center, *SOC* secondary ossification center

The first study that revealed the essential physiological role of hypoxia-driven pathways in cartilage used a Col2-Cre-driven conditional deletion model of HIF-1α, showing that loss of HIF-1α in chondrocytes led to massive cell death in the developing growth plate (Schipani et al. 2001). Specifically, the chondrocytes located in the center of the growth cartilage, farthest from the perichondrial blood vessels, died in the absence of HIF-1α. These regions co-localized with the presence of hypoxia, as detected by a marker for bioreductive activity (EF5) (Schipani et al. 2001). Since this pioneering work, several studies have increasingly shed light on the mechanisms by which HIF-1α ensures the survival of hypoxic chondrocytes, including the direct activation of genes that enable chondrocytes to switch to oxygen-sparing metabolic pathways and the indirect consequences of induction of VEGF (Maes et al. 2012a; Schipani et al. 2015) (Fig. 6.3). Indeed, mutant mice in which VEGF was deleted in cartilage, or which lacked specifically the diffusible splice isoforms $VEGF_{120}$ and $VEGF_{164}$, also showed massive apoptosis of non-hypertrophic, hypoxic chondrocytes in the interior of the growth plate (Maes et al. 2004, 2012a; Zelzer et al. 2004). Although an additional cell-autonomous protective effect of VEGF on chondrocyte survival in the avascular cartilage cannot be excluded, the prime function of VEGF appears to be stimulating angiogenesis in the immediate surrounding perichondrial tissues, thereby increasing the oxygen supply into the growing cartilage and lessening the degree of hypoxia to prevent cell death (Maes et al. 2004, 2012a). This model reconciles the published mouse studies including the crucial role of the diffusible VEGF isoforms,

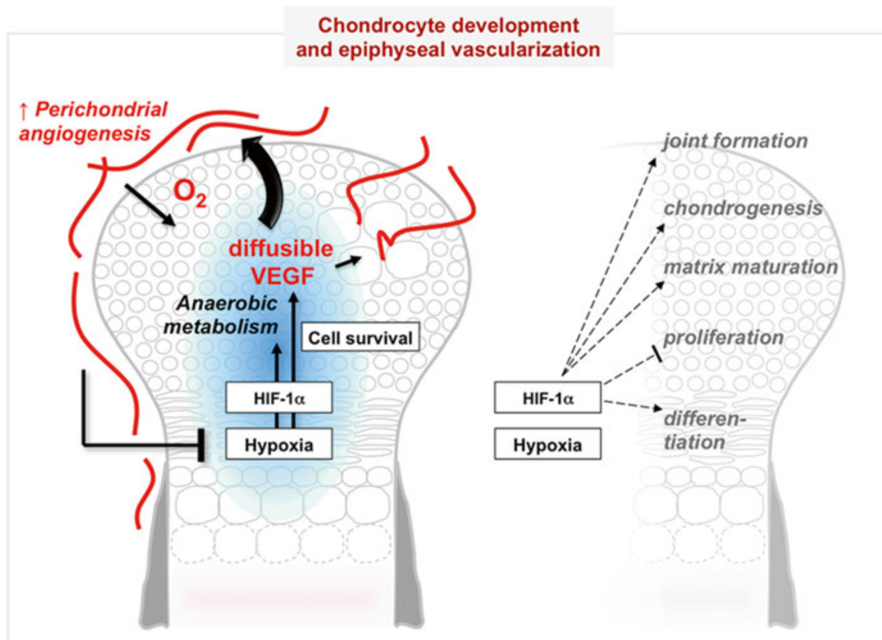

Fig. 6.3 The role of the HIF-VEGF network in the survival of hypoxic chondrocytes and the development of the growth cartilage of endochondral bones. (*Left*) Being an avascular tissue, a gradient of reducing oxygenation exists from the peripheral zones toward the central portion of the fetal growth plate, which inherently becomes hypoxic (*blue in diagram*) during development as the cartilage structure grows. Hypoxia induces a highly orchestrated homeostatic response to maintain cell viability and chondrocyte development, which includes several adaptations to overall balance oxygen availability and consumption. Hypoxia, at least in part via HIF-1α, induces expression of VEGF by the chondrocytes. The soluble VEGF isoforms ($VEGF_{120}$ and $VEGF_{164}$, but not the matrix-bound $VEGF_{188}$ isoform) can diffuse to the perichondrial soft tissues surrounding the cartilage and stimulate angiogenesis. As a result, the O_2 supply into the cartilage increases, limiting the degree of hypoxia. Later on, VEGF signaling is involved in triggering the blood vessels to invade the epiphysis, thereby starting the formation of the secondary ossification center (also see Fig. 6.2). HIF-1 additionally exerts VEGF-independent functions promoting the survival of hypoxic chondrocytes, including by adapting their cellular energy metabolism to the scarcity of oxygen by inducing the expression of enzymes regulating anaerobic glycolysis. (*Right*) HIF-1α also limits chondrocyte proliferation, an effect that further contributes to the lowering of the oxygen consumption in the tissue. Furthermore, hypoxia-driven pathways and HIF-1α affect chondrocyte development and cartilage matrix maturation at different steps during endochondral bone development. The molecular mechanisms responsible for these pleiotropic actions in mesenchymal condensations and developing growth plates are still incompletely understood

as they could induce angiogenesis at the surface of the cartilage following their hypoxia-induced production in the interior of the growth plate (Maes et al. 2004). In addition to regulating blood vessel formation in the perichondrium, VEGF also induces blood vessel invasion and growth within the endochondral bones at various stages during skeletal development, as discussed in the next section of this chapter (see further).

Table 6.1 Mouse mutants generated to study the hypoxia-HIF-VEGF molecular axis in cartilage and bone

Part A: Cartilage development			
Cre-driver	Mutation	Phenotype	Refs.
Prx1-Cre	HIF-1α cKO	Limb shortening and malformation caused by impaired chondrogenic differentiation and formation of the cartilage anlagen and joints starting at E13.5	Provot et al. (2007), Amarilio et al. (2007)
Col2a1-Cre	HIF-1α cKO	Massive cell death in central cartilaginous regions, skeletal malformations	Schipani et al. (2001)
Col2a1-Cre-ERt	HIF-1α i-cKO (tam at E15.5)	Hypocellularity in the growth plate at E17.5; impaired collagen hydroxylation and folding; ER stress and unfolded protein response activation; impaired ECM secretion	Bentovim et al. (2012)
Ubiquitous knock-in mutant	Lack of $VEGF_{120}$ and $VEGF_{164}$ (exclusive expression of the $VEGF_{188}$ isoform)	Chondrocyte cell death, disturbed development of the growth plate and secondary ossification center, dwarfism, knee joint dysplasia	Maes et al. (2004)
Col2a1-Cre	VEGF cKO	Chondrocyte cell death, delayed invasion of blood vessels into developing bones	Zelzer et al. (2004), Maes et al. (2012a)
Col2a1-Cre	HIF-1α cKO; R26-$Vegf_{164}$ cTg	Partial rescue of cell death in HIF-1α deficient cartilage by $VEGF_{164}$ overexpression	Maes et al. (2012a)
Ubiquitous knockout mutant	HIF-2α$^{+/-}$ (het-KO)	Mild and transient dwarfism that resolves within 2 weeks following birth	Saito et al. (2010)
Prx1-Cre	HIF-2α cKO	Slightly but transiently impaired differentiation to late hypertrophic chondrocytes causing shorter limbs at E17.5	Araldi et al. (2011)
Col2a1-Cre	VHL cKO	Dwarfism, reduced chondrocyte proliferation, increased ECM production, hypocellularity, and atypical large cells in resting zone	Pfander et al. (2004)
Prx1-Cre	VHL cKO	Impaired proliferation and delayed terminal differentiation of chondrocytes; lack of secondary ossification center formation. Aberrant bone formation, fibrosis, and soft tissue tumors in postnatal mice	Mangiavini et al. (2014, 2015)

(continued)

Table 6.1 (continued)

Part B: Bone development and homeostasis			
Cre-driver	Mutation	Phenotype	Refs
Ubiquitous knock-in mutant	Lack of $VEGF_{164}$ and $VEGF_{188}$ (exclusive expression of the $VEGF_{120}$ isoform)	Delayed formation of the primary ossification center; reduced angiogenesis, impaired hypertrophic cartilage resorption, and reduced bone formation; decreased bone growth	Maes et al. (2002)
Col2a1-Cre	R26-$VEGF_{164}$ cTg	Hypervascularization and aberrant bone formation during development, leading to skeletal malformations	Maes et al. (2010a)
Col2a1-Cre + induction by dox in adult	tet(O)-$VEGF_{164}$; R26-rtTA i-cTg (induced VEGF overexpression)	Increased bone mass with excessive vascularization, bone marrow fibrosis, and hematological abnormalities	Maes et al. (2010a)
Col2a1-Cre-ERt + tam at 1 month of age	VHL i-cKO	Striking and progressive increase in trabecular bone volume with increased bone formation and vascularization	Weng et al. (2014)
Osx-tTA; dox regulated	tet(O)-HIF-1α PPN (oxygen-stable HIF-1α)	Upregulation of glycolytic metabolism, expansion of the osteoblast population, and increased trabecular bone formation	Regan et al. (2014)
Osx-Cre:GFP	VEGF cKO	Delayed primary ossification center formation and reduced osteoblast differentiation during bone development; reduced bone mass and increased marrow fat due to altered progenitor cell differentiation and fate in postnatal mice	Liu et al. (2012), Duan et al. (2015)
Osx-Cre:GFP	HIF-1α/HIF-2α double-cKO	Decreased trabecular bone volume; reduced erythroid progenitors in bone marrow but normal hematocrit	Rankin et al. (2012)
Osx-Cre:GFP	VHL cKO	Increased EPO production in the bone and expansion of erythroid lineage cells; excessive trabecular bone and increased bone vascularization	Rankin et al. (2012)
Osx-Cre:GFP	PHD1/2/3 triple-cKO	Increased EPO levels in the bone and elevated hematocrit value; excessive bone accumulation and hypervascularization	Rankin et al. (2012), Wu et al. (2015)
Osx-Cre:GFP	PHD2/3 double-cKO	Increased trabecular bone volume, reduced number of osteoclasts associated with HIF-induced OPG upregulation	Wu et al. (2015)
Col1a2-Cre	PHD2	Short stature, smaller bones, reduced bone volume, and premature death at 12–14 weeks of age due to erythrocytosis	Cheng et al. (2014)

(continued)

Table 6.1 (continued)

Part B: Bone development and homeostasis			
Cre-driver	Mutation	Phenotype	Refs
Osteocalcin-Cre	HIF-1α cKO	Narrower bones with impaired bone formation, reduced trabecular bone volume, and decreased vascularization	Shomento et al. (2010), Wang et al. (2007)
Osteocalcin-Cre	HIF-2α cKO	Decreased bone vascularization but only modestly reduced bone volume	Shomento et al. (2010)
Osteocalcin-Cre	VHL cKO	Extremely dense and heavy vascularized bones; protected against ovariectomy-induced bone loss and vascular defects	Wang et al. (2007), Zhao et al. (2012)

Abbreviations: *KO* knockout (loss-of-function or null mutation); *cKO* conditional knockout, using Cre-LoxP recombination strategy; *i* inducible; *cTg* conditional transgenic line; *het* heterozygous; *R26* Rosa26 genomic locus; *tTA* tetracycline-transactivator; *tet* tetracycline; *tam* tamoxifen; *dox* doxycycline

Interestingly, forced expression of $VEGF_{164}$ in cartilage lacking HIF-1α could only partially rescue the cell death phenotype in a combined mouse mutant. Further investigation revealed metabolic adaptation as a second vital mechanism downstream of HIF-1α, independent of VEGF, contributing to the survival of hypoxic chondrocytes (Maes et al. 2012a). Although much remains to be learned about energy metabolism in chondrocytes, the evidence indicates that HIF-1α reprograms their metabolism toward the use of oxygen-sparing anaerobic metabolic pathways (Maes et al. 2012a). This is achieved through induction of the expression of classical HIF target genes that mediate glycolysis, such as the enzyme phosphoglycerate-kinase 1 (PGK1), or that impair mitochondrial respiration, such as pyruvate dehydrogenase kinase 1 (PDK1), which shunts pyruvate away from the mitochondrial tricarboxylic acid cycle (Maes et al. 2012a; Schipani et al. 2001; Bentovim et al. 2012). These adaptations serve to limit the oxygen consumption within the cartilage. Thus, HIF-1α regulates a fine balance of oxygen delivery and utilization in this challenged avascular tissue, to maintain the level of hypoxia within the delicate optimal range for chondrocytes to survive and develop and function normally (Maes et al. 2012a) (Fig. 6.3).

Besides its prime function in mediating the chondrocytes' adaptation to and survival in their hypoxic environment, HIF-1α indeed also regulates chondrogenesis and chondrocyte development at multiple steps (Fig. 6.3, right panel). The net result of all these effects is that conditional inactivation of HIF-1α in chondrocytes (Col2-Cre-driven) or in the mesenchymal cells of the limb buds (mediated via the use of Prx1-Cre mice) is associated with dwarfism with marked shortening of the limbs (Schipani et al. 2001; Provot et al. 2007; Amarilio et al. 2007) (Table 6.1, Part A). Specifically, the early loss of HIF-1α in limb bud mesenchyme did not seem to affect the initial formation of the mesenchymal condensations, but delayed the specification of the joints, the differentiation of mesenchymal cells into chondrocytes,

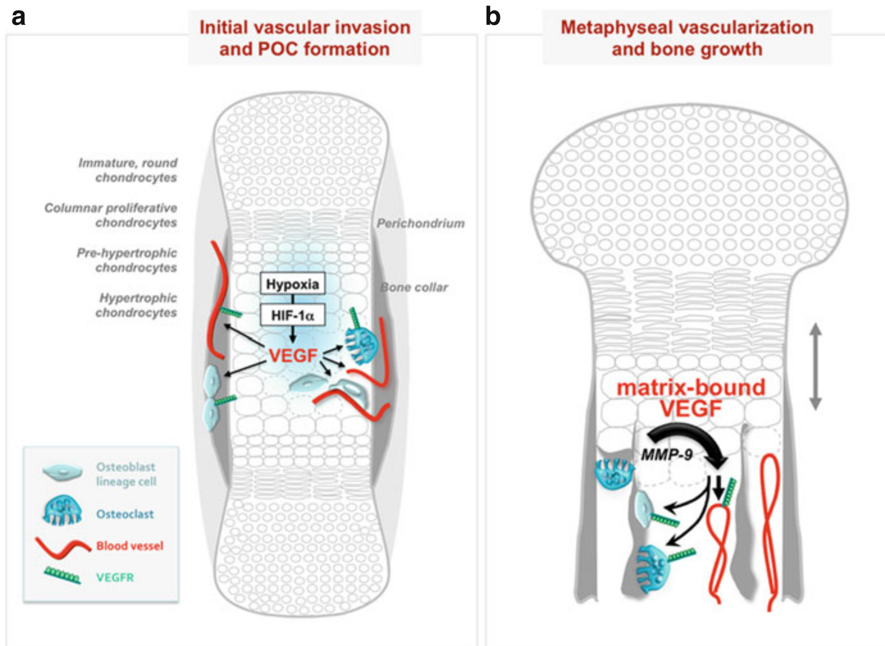

Fig. 6.4 The role of HIF and VEGF in the neovascularization of cartilage and its conversion to bone. (**a**) VEGF controls the initial osteo-angiogenic invasion of the endochondral bone template. During long bone development, hypertrophic chondrocytes in the middle diaphyseal region of the avascular cartilage template become hypoxic (*blue shading*) and express high levels of VEGF. Both HIF-1α and VEGF are required for the timely invasion of the template by blood vessels from the perichondrium. Along with the endothelium, osteoprogenitors move into the tissue and will start to deposit bone and establish the primary ossification center. Osteoclasts, cells of hematopoietic origin, also appear coinciding with vascular accumulation in the perichondrium and co-invade the cartilage. All the cell types involved express VEGF receptors (*VEGFR*) and can respond directly to VEGF signaling by enhanced migration, recruitment, proliferation, and/or differentiation. (**b**) VEGF actions at the chondro-osseous junction and the metaphysis of growing long bones. The matrix-binding isoforms of VEGF ($VEGF_{164}$, and $VEGF_{188}$) are stored in the cartilage matrix after their secretion by hypertrophic chondrocytes. Upon cartilage resorption, mediated by osteoclasts and matrix metalloproteinase (*MMP*)-9, the released VEGF attracts blood vessels toward the growth plate and stimulates angiogenesis. Indirectly (via the vascular growth) and directly (via VEGFR signaling), VEGF stimulates bone formation by osteoblasts and cartilage resorption and bone remodeling by osteoclasts, thereby coordinating the conversion of cartilage into bone at the chondro-osseous junctions and stimulating the growth of the long bones

and their terminal transition to hypertrophic chondrocytes (Provot et al. 2007; Amarilio et al. 2007). The function of HIF-1α as an early differentiation factor in limb bud mesenchyme may be mediated by Sox9, the master transcriptional regulator of chondrogenesis, as HIF-1α has been noted to upregulate Sox9 expression (Amarilio et al. 2007; Robins et al. 2005). Such a mechanism, albeit not fully elucidated at present, could well be in line with a myriad of reports substantiating that low oxygen tension promotes differentiation of mesenchymal stem cells into

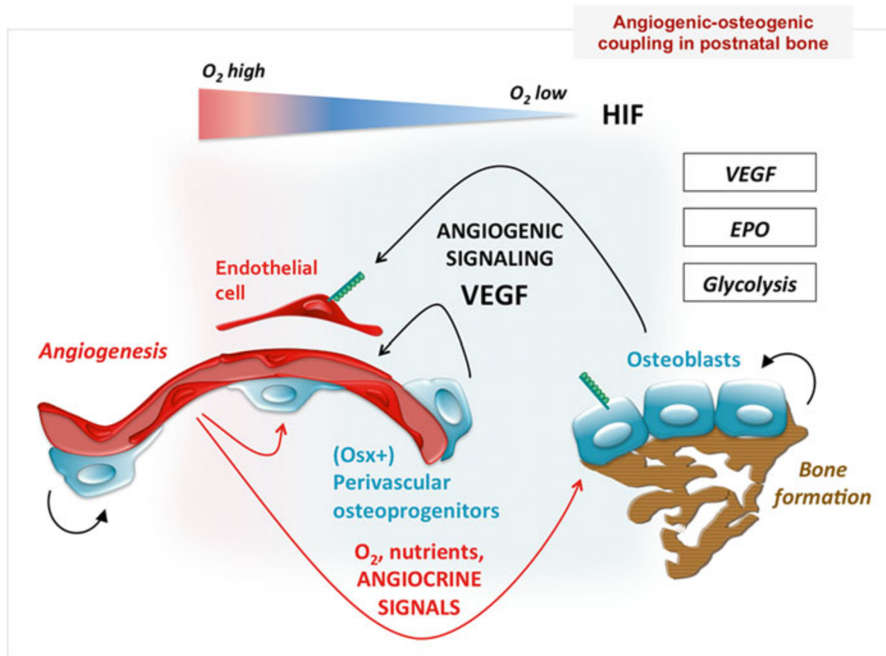

Fig. 6.5 Regulation of angiogenic-osteogenic coupling by HIF and VEGF signaling in the postnatal bone environment. Osteoblast lineage cells respond to low oxygen tension through several actions orchestrated by HIFs. First, a major consequence of hypoxia is HIF-1α- and HIF-2α-induced upregulation of VEGF expression, which will act upon endothelial cells and thereby stimulate angiogenesis. This angiogenic signaling indirectly stimulates bone formation, through the enhanced delivery of oxygen, nutrients, and angiocrine signals such as Noggin by the vasculature and possibly also by increased recruitment of perivascular-residing osteoprogenitors to sites of bone formation. Both HIF-1α and VEGF can also directly affect the proliferation, differentiation, and/or migration of osteoblast lineage cells, which express all the major VEGF receptors. Second, HIF-2α transcriptional activity can induce EPO expression in osteoblast lineage cells and thereby modulate erythropoiesis. Finally, HIF-mediated responses in osteoblast lineage cells in conditions of low oxygen availability include direct effects on cellular metabolism; in particular, HIF-1α signaling can stimulate glycolysis in osteoprogenitors, a mechanism significantly contributing to the bone-forming activity

chondrocytes in vitro (Adesida et al. 2012; Duval et al. 2012; Munir et al. 2014; Portron et al. 2013; Xu et al. 2007; Lafont et al. 2007; Khan et al. 2007). The delayed terminal hypertrophic differentiation of chondrocytes in the absence of HIF-1α has been suggested to rather be a downstream effect of delays in the initiation of chondrogenesis (Provot et al. 2007; Amarilio et al. 2007).

Some of the mutant mouse models also indicated that HIF-1α negatively regulates the proliferation of chondrocytes in the fetal growth cartilage (Fig. 6.3), which could be consistent with the notion that hypoxia leads to growth arrest (Goda et al. 2003) as well as with the HIF-1α-mediated control of oxygen consumption in the hypoxic tissue (by reducing the number of

metabolically active cells). In cartilage lacking HIF-1α, the proliferation rate of the viable chondrocytes in the peripheral regions of the fetal growth plate was strikingly increased (Schipani et al. 2001). Conversely, the chondrocyte proliferation rate was markedly reduced in mice with conditional inactivation of VHL (the E3 ubiquitin ligase that targets HIF-1α and HIF-2α to the proteasome for degradation and thus serves as a negative regulator of HIF activity) in cartilage or limb buds, concomitant with an increased mRNA expression of the cyclin-dependent kinase inhibitor p57. The loss of VHL in growth plate chondrocytes in vivo thereby resulted in a pronounced hypocellularity in the growth plates and the mutant mice displayed severe dwarfism (Pfander et al. 2004; Mangiavini et al. 2014).

Finally, loss of VHL in cartilage or in the limb bud mesenchyme appeared to also be associated with an increased accumulation of matrix in between the chondrocytes (Pfander et al. 2004; Mangiavini et al. 2014), pointing at one more function of HIF signaling in cartilage, in the control of matrix maturation (Fig. 6.3). Indeed, the relationship between hypoxia sensing and extracellular matrix (ECM) accumulation in cartilage is increasingly being elucidated at the molecular and cellular levels. In providing the template for progressive long bone growth, chondrocytes produce large amounts of matrix, the main constituent of which is type II collagen. The acquisition of the mature triple helix structure of collagens requires specific posttranslational modifications, including critical hydroxylation reactions of proline residues in the collagen proteins. These reactions are catalyzed by a family of collagen prolyl-4-hydroxylases (cP4Hs, in particular cP4HaI and cP4HaII), which are distinct from the family of prolyl-hydroxylases that hydroxylate HIFs (the PHDs, introduced above) but also function in an oxygen-dependent manner by using oxygen as a substrate for proline hydroxylation (Myllyharju and Schipani 2010). It may therefore seem somewhat paradoxal, but all the evidence indicates that hypoxia induces the expression of cP4Hs in chondrocytes (as in other cell types (Takahashi et al. 2000; Hofbauer et al. 2003; Myllyharju and Schipani 2010)), through HIF-1α-dependent transcriptional stimulation (Bentovim et al. 2012; Aro et al. 2012; Grimmer et al. 2006). As such, HIF-1α improves the efficiency of essential posttranslational modifications of type II collagen, which can explain its documented role in vivo in facilitating the correct folding of the collagen proteins in the endoplasmic reticulum (ER) and preventing ER stress and in increasing the secretion and accumulation of mature type II collagen in the cartilage ECM (Pfander et al. 2003, 2004; Bentovim et al. 2012). The positive effect of HIF-1α on ECM maturation and accumulation may present another contribution to the functioning of hypoxia-driven pathways in ensuring chondrocyte survival, since accumulation of inappropriately processed collagen could trigger the unfolded protein response, lead to ER stress, and cause cell death (Bentovim et al. 2012). In support, growth plates from mice with an almost 70 % decrease of cP4Hs enzymatic activity, by heterozygous inactivation of cP4HaI combined with homozygous inactivation of cP4HaII ($cP4HaI^{fl/-}$;$cP4HaII^{-/-}$ mice), displayed an inner cell death phenotype reminiscent of that observed in growth plates lacking HIF-1α, albeit far more mild and transient (Aro et al. 2015). The chondrodysplasia observed in the

$cP4HaI^{fl/-}$;$cP4HaII^{-/-}$ double mutant mice appeared to be due to severe matrix abnormalities rather than being secondary to uncompensated ER stress (Aro et al. 2015). Although the initial differentiation of mesenchymal cells into chondrocytes in the early limb buds was unaffected in these cP4Hs mutants, it is still conceivable that the role of HIF-1α in promoting the formation of an appropriately structured ECM may also contribute to its role in regulating chondrocyte differentiation, for instance, by supporting key cell-matrix interactions (Maes et al. 2012b; Schipani et al. 2015).

Distinct from HIF-1α, HIF-2α is not critical for growth plate development, as lack of HIF-2α was found to cause only a modest and transient delay of endochondral bone development in several mouse models. Mouse embryos ubiquitously lacking one allele of HIF-2α (heterozygous HIF-$2\alpha^{+/-}$ fetuses) develop mild and transient dwarfism that resolves within 2 weeks following birth (Saito et al. 2010). Similarly, conditional inactivation of HIF-2α in the limb bud mesenchyme results in a mild and transient delay in endochondral bone development at E17.5 (Araldi et al. 2011). The molecular mechanisms responsible for this phenotype are still largely unknown, but may involve an impairment of the last stage of chondrocyte hypertrophy in the fetal growth plate (Araldi et al. 2011; Saito et al. 2010). In contrast to its limited importance in cartilage during development, HIF-2α does appear to play a significant role in the postnatal joint and the pathogenesis of osteoarthritis, as discussed further in this chapter.

Thus, taking together the gained knowledge from a substantial number of studies performed over the last 15 years, it is clear that the roles of hypoxia-driven pathways in cartilage during endochondral bone development are multifactorial and absolutely essential for the survival and controlled proliferation and differentiation of hypoxic chondrocytes, even though the underlying molecular mechanisms are at present incompletely understood.

6.3 Cartilage Neovascularization and Conversion into Bone: A Central, Coordinating Role for Angiogenic Signaling by VEGF

While the fetal growth plate is a unique mesenchymal tissue in that it is itself avascular, the completion of the chondrocytes' developmental program is followed by vascular invasion of the terminally differentiated, hypertrophic cartilage. This angiogenic invasion of the terminal hypertrophic cartilage is associated with its decay and replacement by bone, as osteoclast and osteoblast precursors co-invade the region along with its neovascularization, degrade the cartilage matrix, and populate the newly forming bone center (see Chaps. 4 and 5). In fact, the process of endochondral bone development involves a series of consecutive vascularization events that drive the gradual replacement of the avascular cartilage template, and subsequently the growth plate, by highly vascularized bone and bone marrow tissue forming the mature long bone (Fig. 6.2) (Maes 2013).

In the first step, the embryonic cartilage anlagen becomes invaded by blood vessels that accumulated in the surrounding perichondrium, more particularly in the mid-diaphyseal region adjacent to the differentiated hypertrophic chondrocytes that, around E14.5 in the mouse, constitute the center of the cartilaginous template (Fig. 6.4a). This initial vascular invasion event launches the formation of the primary ossification center or primitive bone marrow cavity of the long bone. It was shown, by using lineage-tracing approaches in mice, that the blood vessel invasion is closely associated with the invasion of the nascent bone center by osterix (Osx)-expressing osteoprogenitors from the perichondrial tissue, a subset of which co-migrates with the endothelial cells while positioning as pericytes around the blood vessels (Maes et al. 2010b). This may provide a partial explanation for the fact that cartilage neovascularization is an absolute requirement for the endochondral replacement of cartilage by bone, as supported by experiments blocking the process by physical, chemical, or genetic manipulations (Gerber et al. 1999; Maes et al. 2002; Colnot et al. 2004) (see below). Second, as the growth plates become established at both sides of the developing bone and marrow cavity, the further lengthening of the bone is driven by the continual capillary invasion of the last row of hypertrophic chondrocytes at the metaphyseal border of the growth cartilage, which mediates the replacement of the cartilage by trabecular bone (Fig. 6.4b). Finally, neovascularization of the cartilage at the ends of the bone (epiphyses) initiates the formation of secondary ossification centers, leaving mere “plates” of chondrocytes in between the diaphysis and the epiphyses (Maes 2013).

Molecularly, the avascular status of developing cartilage is believed to be maintained through the production of angiogenic inhibitors such as chondromodulin-I, troponin-I, and thrombospondins by immature chondrocytes (Moses et al. 1990, 1999; Shukunami and Hiraki 2007). In contrast, when chondrocytes become hypertrophic, they switch to production of angiogenic stimulators, including high levels of VEGF, steering the angiogenic growth of blood vessels toward the terminal hypertrophic chondrocytes and the replacement of cartilage by bone (Gerber et al. 1999; Maes et al. 2002, 2010a). This molecular switch to proangiogenic signals that is embedded in the chondrocyte differentiation program explains why capillary invasion and cartilage neovascularization events are at all times preceded by chondrocyte hypertrophy. The regulatory mechanisms responsible for the high-level expression of VEGF in hypertrophic chondrocytes are not completely resolved at present, although several factors have been implicated and may cooperatively regulate the VEGF induction. For instance, VEGF transcription may be controlled by Runx2 and Osx, transcription factors that are expressed in hypertrophic and prehypertrophic chondrocytes, respectively, in addition to their prime role in osteoblastogenesis ((Zelzer et al. 2001; Chen et al. 2012; Tang et al. 2012) and Chap. 5)). Hypoxia too is most certainly able to induce VEGF expression in chondrocytes, through mechanisms that involve direct HIF-1α (but not HIF-2α-)-mediated transcriptional regulation, at least in vitro (Pfander et al. 2003; Cramer et al. 2004; Lin et al. 2004; Aro et al. 2012). The in vivo significance and subtleties of these hypoxia-driven control mechanisms for the fine regulation of the VEGF levels are, however, more challenging to decipher. The relatively low level of VEGF expression in

non-hypertrophic chondrocytes located centrally in the developing growth cartilage co-localizes with hypoxia and detectable HIF-1α proteins, which are highly likely to contribute to modulating the expression of VEGF in this inner area (Fig. 6.3) (Schipani et al. 2001; Maes et al. 2004, 2012a; Pfander et al. 2004). In vivo observations similarly underscore a potential hypoxia- and HIF-1α-mediated control mechanism of the VEGF expression in the preinvasion cartilaginous template (Fig. 6.4a) (Maes et al. 2012a; Provot et al. 2007; Amarilio et al. 2007). In contrast, it remains enigmatic whether hypoxia and/or HIF-1α significantly contribute to the high levels of VEGF production in the late hypertrophic chondrocytes of the growth plate during longitudinal bone growth. A recent study suggested that Runx2 might be involved in the protein stabilization of HIF-1α and induction of VEGF in hypertrophic chondrocytes (Lee et al. 2012).

Both HIF-1α and VEGF play critical roles in the initial angiogenic invasion and the early development of the long bones in vivo. At this time in development, endothelial cells, osteoblasts, and osteoclasts – cell types that all express VEGF receptors (VEGFRs) and can respond to VEGF signaling (reviewed in (Maes 2013)) – coordinately invade the cartilaginous template (Maes et al. 2010b) (Fig. 6.4a). In mice lacking HIF-1α or VEGF in cartilage, the invasion process and primary ossification center formation were delayed, which could be rescued by forced $VEGF_{164}$ overexpression in the combined mutant (Maes et al. 2012a). The $VEGF_{164}$ isoform is of particular importance, as embryos expressing only the $VEGF_{120}$ isoform or only the $VEGF_{188}$ isoform showed a delay in the early vascular invasion and development of the long bones. Conversely, in mice overexpressing $VEGF_{164}$ in the endochondral skeleton, achieved by a Col2-Cre-mediated conditional overexpression, the osteo-angiogenic invasion of developing bone centers occurred prematurely and excessively, with aberrant bone deposition in this vascularized area leading to misshapen limbs (Maes et al. 2010a). These findings strongly suggest that hypoxia-induced, HIF-mediated VEGF expression in cartilage is required for the concomitant recruitment of angiogenic blood vessels and osteoprogenitors into developing endochondral bone centers (Fig. 6.4a).

During longitudinal bone growth, VEGF is well established to provide a major engine to the coordinated decay and resorption of the hypertrophic cartilage matrix, vascular expansion, and formation of trabecular bone (Fig. 6.4b). Inhibition of VEGF action in juvenile mice, through administration of a soluble VEGF receptor chimeric protein (sFlt-1), impaired vascular invasion of the growth plate, and concomitantly, trabecular bone formation and bone growth were reduced and the hypertrophic cartilage zone became enlarged, likely as the result of reduced osteoclast-mediated resorption (Gerber et al. 1999). Additional mouse genetic studies exposed the specific roles of VEGF and of its major splice isoforms ($VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$) in endochondral ossification (Table 6.1, Part B) (Maes et al. 2002, 2004, 2010a, 2012a; Liu et al. 2012; Duan et al. 2015). Altogether, these studies support the model that VEGF, secreted at high levels by hypertrophic chondrocytes, becomes partially sequestered in the cartilage matrix, particularly the longer VEGF isoforms ($VEGF_{164}$ and $VEGF_{188}$) that have strong matrix-binding affinity. The shorter VEGF isoforms ($VEGF_{120}$ and $VEGF_{164}$) are largely soluble and diffuse

from the cartilage, attracting blood vessels toward the chondro-osseous junction and stimulating endothelial cells to form new blood vessels (angiogenesis). This is indirectly associated with increased delivery of osteoclast and osteoblast progenitors. The osteoclasts and osteoclast-derived matrix metalloproteinase (MMP)-9 (Vu et al. 1998; Engsig et al. 2000) can release more matrix-bound VEGF from the cartilage that is being resorbed, creating a positive-feedback system. Moreover, VEGF also has chemo-attractive activity in stimulating osteoclast invasion of cartilage and enhances osteoclast differentiation, survival, and resorptive activity (Engsig et al. 2000; Yang et al. 2008). The osteoblasts on their turn deposit bone on the remnants of the cartilage matrix, in part stimulated directly by VEGF signaling, which enhances the recruitment and differentiation of osteoblasts. This model reconciles the data of a large set of mutant mouse models and in vitro studies, which altogether identified VEGF as a crucial physiological driver of the endochondral turnover process that progressively transforms cartilage into bone at the metaphyseal growth plate during skeletal growth (for more extensive reviews, see (Maes 2013; Dirckx et al. 2013)).

VEGF-mediated vascular invasion of cartilage has also been implicated in the formation of the secondary ossification center, as $VEGF^{188/188}$ mice (a knock-in model replacing the endogenous VEGF gene by $VEGF_{188}$ cDNA, such that the other two major VEGF isoforms $VEGF_{120}$ and $VEGF_{164}$ are no longer expressed) showed drastically impaired formation of the epiphyseal bone center, associated with knee joint dysplasia and dwarfism (Maes et al. 2004) (Fig. 6.3).

6.4 Angiogenic-Osteogenic Coupling in Mature Bone by HIF and VEGF

The vascular system of the skeleton remains a vital player in the regulation of bone formation also beyond development. Processes of adult bone remodeling, repair, and regeneration all rely on optimal tissue vascularization and characteristically involve a close spatial and temporal association between bone formation and vascularization of the ossified tissue. This principle of angiogenic-osteogenic coupling is crucial for bone homeostasis and tightly regulated by hypoxia-driven pathways, in line with the highly hypoxic status of the bone and bone marrow microenvironment.

Hypoxia, HIF, and VEGF indeed represent major regulators of the tight coupling between angiogenesis and osteogenesis in postnatal bone formation and bone remodeling. Again, these functions in the complex bone environment have been exposed by studies using a growing collection of mutant mouse models, primarily Cre-loxP-mediated conditional knockouts employing Osx-Cre, type I collagen (Col1)-Cre, and osteocalcin (OC)-Cre-driver strains to target osteoprogenitors, maturing osteoblasts, and differentiated osteoblasts, respectively (Table 6.1, Part B). The role of HIF pathway components in the adult skeleton was first revealed by the genetic disruption of the genes encoding HIF-1α and VHL in differentiated osteoblasts (Wang et al. 2007). Mice lacking HIF-1α in OC-expressing osteoblasts

exhibited narrower bones and a reduced trabecular bone volume, associated with a reduced vascular density and a decreased bone formation rate (Wang et al. 2007; Shomento et al. 2010). Conversely, the phenotype of the VHL mutants was characterized by a massive increase in trabecular bone and in the number of osteoblasts, along with a dramatic increase in vascular density. In vitro, osteoblast proliferation, differentiation, and apoptosis were not noticeably altered upon VHL inactivation, suggesting that primarily non-cell-autonomous mechanisms explained the in vivo phenotype. In line with the role of VHL as upstream negative regulator of HIFs, VHL inactivation led to constitutive stabilization of HIFs and upregulated expression of VEGF, correlating with the observed vascular expansion (Wang et al. 2007). Accordingly, the proposed mechanism postulated that HIF signaling in osteoblasts regulates angiogenesis in the bone microenvironment in response to hypoxia and thereby indirectly stimulates osteogenesis through the principle of angiogenic-osteogenic coupling (Fig. 6.5). This coupling can result from the intrinsic function of the vascular system to supply oxygen, nutrients, hormones, and growth factors to osteoblast lineage cells, as required for their bone-forming activity. Locally produced angiocrine signals, secreted by vascular endothelial cells in the bone microenvironment, likely influence osteoblast lineage cells and bone formation significantly, as suggested by recent work implicating Notch signaling-regulated endothelial production of Noggin, a major modulator of the bone morphogenetic protein (BMP) pathway, in angiogenic-osteogenic coupling (Ramasamy et al. 2014). In addition, the vascular system may deliver osteoprogenitors, such as those marked by the expression of Osx found residing in the blood vessel walls, to the bone formation sites (Maes et al. 2010b; Dirckx et al. 2013) (Fig. 6.5). Interestingly, a recent study revealed that HIF signaling in specific subsets of endothelial cells in the long bones (in particular those defining the blood vessels of the metaphysis and the endosteum, recently termed the type H vasculature) contributes considerably to the coupling, most likely by affecting the pools of Runx2- or Osx-expressing osteoprogenitors (Kusumbe et al. 2014). Specifically, endothelium-specific and inducible inactivation of VHL, achieved by using *Cdh5*(*PAC*)-*CreERt2* mice, led to a striking increase in (type H) blood vessels in the bones of juvenile mice, associated with a dramatic expansion of the pool of Runx2+ or Osx + cells and with an increased bone volume. Conversely, endothelial cell-specific genetic deletion of HIF-1α led to severe vascular defects and a reduced number of osteoprogenitors (Kusumbe et al. 2014). Thus, endothelial cells and osteoblast lineage cells both contribute to angiogenic-osteogenic coupling, by sensing changes in the oxygen tension and directing adjustments in vascularization and ossification through HIF signaling and an intense molecular cross-talk (Fig. 6.5) (Maes and Clemens 2014).

In addition to these bidirectional paracrine signaling events between osteoblast lineage cells and the skeletal vasculature that are sensitive to the local oxygenation of the bone, recent studies in this active field of research have added to the model a number of emerging direct roles of hypoxia-driven pathways in osteoblast lineage cells (Fig. 6.5). In fact, each of the typical downstream consequences of HIF activity, namely, modulation of cellular metabolism, EPO expression, and VEGF regulation as indicated in Fig. 6.1, appears to play important roles in skeletal homeostasis

and health, as discovered by studies of Osx + osteoprogenitor-directed mouse models (Table 6.1, Part B). Many of these roles appear to be primarily driven by HIF-1α, while a few specific functions have been assigned to HIF-2α.

Firstly, in line with the established function of HIF to regulate key enzymatic components of the glycolytic pathway, HIF signaling emerged as a crucial regulator of cellular metabolism in osteoblasts, thereby stimulating bone formation (Regan et al. 2014). In a model of activated HIF-1 signaling in osteoprogenitors, obtained through temporal expression of a stabilized form of HIF-1α directed by the promoter of the Osx gene (*Sp7*) in postnatal mice, osteoblast lineage cells were found to upregulate glycolytic metabolism. This was associated with a marked expansion of the osteoblast population and increased trabecular bone mass. Of note, these effects were independent of HIF-1-driven increased VEGF expression and angiogenesis, as the phenotype of the mice was not blunted by concomitant loss of VEGF. Rather, the high bone mass was reversed by administration of an inhibitor of PDK1, triggering mitochondrial respiration at the expense of glycolysis. The authors thus concluded that glycolytic metabolism is required for HIF-1α-driven bone formation (Regan et al. 2014).

Secondly, the actions of VEGF, either or not downstream of HIF, include cell-autonomous effects in osteoblast lineage cells independent of its functioning as angiogenic stimulator by acting upon endothelial cells. Hypoxia, through both HIF-1α and HIF-2α, does provide a strong trigger of VEGF expression in osteoblasts (Akeno et al. 2001; Kim et al. 2002; Shomento et al. 2010), but several other mechanisms can additionally regulate the levels of VEGF. Osteoblasts express all the major VEGF receptors and can respond to VEGF signaling with chemotactic migration, differentiation, and bone-forming activity (reviewed in (Maes 2013)). Direct actions of VEGF signaling in osteoblast lineage cells in vivo were suggested in a model of induced conditional overexpression of VEGF in the adult bone environment, with increased VEGF leading to excessive β-catenin signaling, expansion of the osteoprogenitor pool, bone marrow fibrosis and hematopoietic defects, and a high bone mass phenotype (Maes et al. 2010a). Interestingly, conditional inactivation of the *VEGF* gene in Osx-expressing osteoprogenitors revealed that in addition to its typical paracrine actions, VEGF can also act in an intracrine mode to determine cell fate decisions; in the absence of intracellular VEGF signaling, bipotent progenitors in the target population differentiated into adipocytes rather than osteoblasts (Liu et al. 2012).

Thirdly, in addition to stimulating bone vascularization and ossification, HIF stabilization in osteoprogenitors was found to induce EPO expression in bone, associated with a selective expansion of the erythroid lineage and elevated hematocrit values. These findings were made by using mice with conditional inactivation of VHL in Osx-expressing cells and recapitulated through pharmacologic or genetic inhibition of the PHDs (PHD1/2/3 triple-cKO mice) (Rankin et al. 2012). Inactivation of HIF-2α, but not HIF-1α, prevented the excessive EPO expression in the VHL mutant bones, indicating that HIF-2 signaling in osteoprogenitors plays a role in controlling hematopoiesis, particularly red blood cell production, in an EPO-dependent manner (Rankin et al. 2012).

Thus, a growing number of typical HIF target genes are being implicated in the responses to low oxygen in osteoblasts. The substantial repercussions of these molecular changes on ossification, vascularization, and hematopoiesis mandate a tight

regulation of HIF activity in the osteoblast lineage. Very recent work started to dissect the roles of the PHD enzymes (PHD1-3), the critical cellular oxygen-sensing components upstream of HIF. Combined inactivation of PHD1, PHD2, and PHD3 in osteoprogenitors resulted in extreme HIF signaling, polycythemia, and excessive bone accumulation associated with overstimulation of angiogenic-osteogenic coupling (Wu et al. 2015). Mice with combined genetic inactivation of two of the PHD enzymes revealed that HIF-2α activation associated with PHD2 and PHD3 inactivation drove bone accumulation by modulating osteoblast-osteoclast cross-talk through the direct regulation of osteoprotegerin (OPG), one of the key osteoclast-regulatory molecules produced by osteoblast lineage cells. Thus, in addition to regulating angiogenic-osteogenic coupling, the PHD isoforms are required to fine-tune hypoxia signaling and HIF activity in the control of the coupling between bone formation and resorption, a crucial aspect of bone homeostasis that is coordinated by the cross-talk between osteoblasts and osteoclasts (Wu et al. 2015). Of note, inactivation of PHD1 and PHD3 did not modulate the expression of the HIF target genes VEGF, EPO, and PGK1 and did not cause alterations in the bone volume, suggesting that PHD2 is the most important PHD in osteoblast lineage cells. Surprisingly, conditional inactivation of PHD2 in maturing osteoblasts (Col1α2-Cre-driven) led to reduced bone volume as a consequence of impaired osteoblast function and bone formation; these mice developed smaller bones and died between 12 and 14 weeks of age, probably because of erythrocytosis caused by Cre-expression and PHD2 inactivation in the kidney, which led to dramatically increased circulating EPO levels and elevated hematocrit (Cheng et al. 2014). Further studies using skeletal-specific mutants will be necessary to fully understand the roles of the critical oxygen-sensing PHD enzymes in the cell types of bone.

Altogether, studies performed in this field are progressively uncovering the modes of action of the HIF-VEGF network in bone formation and remodeling, with increasing insights emerging on the roles of a variety of regulatory components of the hypoxia-regulated pathways, both upstream and downstream of HIF signaling. Understanding the roles of the hypoxia-driven pathways in osteogenesis may bear important therapeutic implications in widespread low bone mass disorders such as osteoporosis and in compromised fracture healing. For instance, age-related and osteoporosis-linked declines in both bone mass and bone vascular density have been documented; genetic inactivation of VHL or PHDs or administration of PHD inhibitors in mice can revert these effects and protect mice against age- or ovariectomy-induced bone loss (Kusumbe et al. 2014; Zhao et al. 2012; Weng et al. 2014; Liu et al. 2014; Wu et al. 2015). As well, bone regeneration and fracture healing in experimental animal models can be improved by activation of HIFs (Wan et al. 2008; Shen et al. 2009).

6.5 Hypoxia and HIF in Articular Cartilage and Joint Pathophysiology

A role for hypoxia and HIF-1α in joint specification during embryonic development has been reported, as mentioned earlier in this chapter (Provot et al. 2007; Amarilio et al. 2007). Later in life, hypoxia-driven pathways remain crucial in articular cartilage and have been implicated in joint disease. In particular, HIFs appear to be

involved in the pathogenesis of osteoarthritis (OA), as explained below. The role of hypoxia and HIFs, acting in conjunction with inflammatory pathways, in rheumatoid arthritis is complex and beyond the scope of this chapter (for review of this subject, see (Konisti et al. 2012)).

Articular cartilage represents a unique structural organization of chondrocytes, collagens (mostly type II collagen), and proteoglycans such as aggrecan. It is an avascular, aneural, alymphatic, and viscoelastic connective tissue, which relies for its nutrient supply and oxygenation on diffusion from the synovial fluid and the subchondral bone. The limited diffusion capacity of oxygen has been reported to create a gradient of 6 % oxygen at the joint surface to only 1 % in the deeper layers (reviewed in Zhang et al. (2015)). Articular chondrocytes are consequentially maintained in a hypoxic environment throughout life and are very well adapted to these challenging conditions by the relatively constitutive expression of HIFs (Zhang et al. 2015). Both HIF-1α and HIF-2α are expressed in articular surface chondrocytes and can induce expression of the master chondrogenic transcription factor Sox9 (Amarilio et al. 2007; Robins et al. 2005; Thoms et al. 2013; Lafont et al. 2007), but particularly HIF-1α is thought to be crucial for maintaining articular chondrocyte survival and function. Much of the functioning of HIF-1α in the processes of chondrocyte differentiation, ECM synthesis, proliferation, metabolic adaptation, and protection against apoptosis as occurring in the fetal cartilaginous anlagen and growth plates of the endochondral bones, as outlined earlier in the chapter (Fig. 6.3), may be sustained in the articular cartilage and contribute to the maintenance of the stable articular chondrocyte phenotype.

Articular chondrocytes need to keep a delicate balance between anabolic and catabolic processes regulating the integrity of their ECM, to enable the friction-free movement of the joint and the functioning of the cartilage as a load-bearing tissue and shock absorber. Disturbances in this homeostatic balance, instigated by, for instance, mechanical stress or inflammatory cytokines, can lead to the synthesis of excessive ECM-degrading enzymes such as aggrecanases and MMPs, resulting in degenerative pathologies. OA is the most common degenerative joint disorder, particularly among the elderly, and typified by articular cartilage destruction along with fibrosis of the bone marrow, thickening of the subchondral bone, and formation of osteophytes. The disease leads to loss of joint mobility and function and is associated with chronic pain. Treatment of OA is challenging, especially considering the avascular status of the articular cartilage, and largely symptomatic, alleviating pain and interfering with the cartilage degenerative processes and ultimately entailing total joint replacement.

In human OA cartilage, the mRNA expression of HIF-1α and its target genes increase, positively correlating with disease progression and the severity of cartilage degradation (Yudoh et al. 2005). Inhibition of HIF-1α in the knee joint of normal healthy mice (by intra-articular injection of 2-methoxyestradiol) led to chondrocyte apoptosis and osteoarthritic changes, including articular cartilage degeneration and osteophyte formation (Gelse et al. 2008). Therefore, HIF-1α has been postulated to be protective against OA and/or drive compensatory mechanisms to conserve articular cartilage integrity during OA development (Pfander and Gelse 2007).

Nevertheless, HIF stabilization with the nonselective PHD inhibitor dimethyloxaloylglycine (DMOG) could not prevent cartilage destruction in the knee joints of STR/ORT mice, a model for spontaneous OA (Gelse et al. 2008).

In contrast to HIF-1α, HIF-2α appears to be a catabolic regulator, mediating osteoarthritic cartilage matrix breakdown in OA, at least in mice. HIF-2α was identified as a potent transcriptional activator of a number of key genes typically expressed by hypertrophic chondrocytes and involved in endochondral ossification, namely, *COL10A1* (encoding the alpha chain of type X collagen), *VEGFA*, and genes encoding matrix-degrading enzymes including MMP-13 (Saito et al. 2010). Chondrocyte hypertrophy followed by vascular invasion and cartilage matrix degradation represents the central driving force of endochondral ossification during skeletal growth and is largely recapitulated during OA development. In OA, blood vessels infiltrate the cartilage via the synovium and the tendons at the periphery, causing the typical formation of osteophytes. The HIF-2α-mediated activation of these key cartilage-catabolic genes may thus reconcile the analogy between endochondral ossification and OA progression. HIF-2α levels were higher in surgical specimens of OA patients and osteoarthritic mice than in non-diseased cartilage (Saito et al. 2010; Yang et al. 2010). Moreover, several animal models implied a pathophysiological role for HIF-2α in OA. HIF-2α haploinsufficiency in *Epas1*$^{+/-}$ mice prevented cartilage degradation and osteophyte formation after surgically induced instability of the knee joint, thus protecting the mice against OA (Saito et al. 2010; Yang et al. 2010). Another loss-of-function study developed cartilage-targeting nanoparticles to deliver small-interfering RNA (siRNA) silencing HIF-2α expression to chondrocytes, which was associated with a decrease in HIF-2α, catabolic factors, and VEGF in vitro. Upon intra-articular injection of the nanoparticles into the knee joints of OA-affected mice, cartilage integrity was maintained and synovial inflammation alleviated (Pi et al. 2015). Conversely, gain-of-function of HIF-2α, obtained genetically or via adenovirus-mediated overexpression of the *Epas1* gene following local injection in the mouse knees, induced expression of the catabolic HIF-2α target genes and triggered severe cartilage destruction (Yang et al. 2010). The upstream mechanisms leading to aberrant HIF-2α activity in OA appear to involve nuclear factor (NF)-κB signaling, itself activated by mechanical stress and/or proinflammatory cytokines, rather than HIF-2α regulation through PHD activity (Saito et al. 2010; Yang et al. 2010; Li et al. 2015).

Given the considerable differences between mice and humans both in terms of articular cartilage thickness (and hence, oxygenation) and mechanical forces experienced by the joints, not all these findings in mice may easily be extrapolated to the human OA condition. Interestingly, however, a role for HIF-2α in human OA was suggested as well, as a functional single nucleotide polymorphism (SNP) in the human *EPAS1* gene encoding HIF-2α was found to associate with knee OA in a Japanese cohort (Saito et al. 2010). Although to date the clinical significance of HIF-2α in human idiopathic OA is still questioned and the genetic links could not be recapitulated in all patient cohorts (Nakajima et al. 2011), these findings altogether suggest that targeting HIF-2α could have therapeutic value for this common form of arthritis.

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Joint Development

7

Xue Wang, Wilson CW Chan, and Danny Chan

Abstract

Synovial joints between different skeletal elements are essential for mobility. The joint is encased in a capsule, lined by a synovial membrane inside, and enforced by ligaments. Within the synovial joints, articular cartilage lining bone surfaces provide a smooth, wear-resistant structure that reduces friction and absorbs impact forces. Joint formation is complex with structures of different shapes and sizes that are fit for purpose. In adult life, these structures need to be maintained, as loss or damage to articular cartilage is the hallmark of arthritic diseases. The joint when damaged does not repair well and the reason is not clear. However, understanding the developmental process will provide critical insights into how early limb patterning is linked to later skeletal morphogenesis. This chapter focuses on our current understanding at the cellular and molecular levels, from creation to maturation of a synovial joint. Morphologically, we know there is the formation of interzone regions at the presumptive sites of the future joint. Molecularly, we have some insights into signals that direct the initiation and progression of interzone regions toward a joint. And through innovative technologies in mouse genetics and genomics, we are beginning to understand the developmental processes, with the identification of progenitor cell pools, and to trace the origin of cells and track the fate of descendent cells from initiation to formation of the complete joint. The information gained from development will enable potential therapeutic strategies, from activation of endogenous repair mechanisms to the use of appropriate progenitors for cell therapy.

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7.1 Skeletal Joints

A joint is where two or more bones meet, allowing motion of the skeletal elements. Diarthrosis (synovial) joints are highly mobile, amphiarthrosis (intervertebral disk) joints are less mobile, and synarthrosis (suture and gomphosis) joints are relatively immobile. Most common in the human body are synovial joints and their formation, maintenance, and degeneration are best studied (Li et al. 2013). This chapter will focus on synovial joints that are complex structures as a unit, consisted of articular cartilage at the surface of opposing bones, ligaments, synovium, and the joint capsule (Ward et al. 1999; Archer et al. 1999). The synovial fluid provides lubrication for the joint during movement, whereas the synovial membrane, a saclike structure, encloses the joint cavity and synovial fluid. These joints differ in shape with distinct architecture for the required movements and loading, exemplified by the different joints of the limbs and digits.

Synovial joints when damaged through trauma do not repair well, and the reasons are not well understood. The repair processes that do occur make fibrous cartilages that cannot fulfill compressive and articulation functions. Repair of many tissues rely on reactivation of developmental cues and availability of progenitor cells, in which a damaged articular cartilage appears to be unable to activate and/or due to limited/absence of progenitor cells. With aging, as with other tissues, the articular cartilage will degenerate leading to conditions such as osteoarthritis (OA). While aging is a natural process, there are strong evidences for genetic contributions that could influence maintenance and repair potentials or abnormal developmental processes that lead to enhanced abnormal responses to daily wear and tear. Thus, a clear understanding of the developmental processes in joint formation would facilitate the identification of related processes or progenitor cells that need to be reactivated for better repair of articular cartilage and the associated joint tissues.

7.2 Structure of Synovial Joints

Synovial joints are composed of articular cartilage, synovial membrane, ligaments, and a fibrous capsule but diverse in shape, construction, and biomechanical function. For example, the shoulder and hip have universal "ball and socket" joints allowing multidirectional movements, the elbow has hinge joint for flexion and extension in one plane, while the knee has a modified hinge joint allowing flexion, extension, abduction, and adduction movements. Some joints have additional structures such as the meniscus and intra-joint ligaments in the knee, phalangeal joints have externally positioned collateral ligaments, and the hip displaced a centrally located ligamentum teres of the femur head. The anatomical functions of the different joints and structures are well understood; however, how these structures come about in development with the appropriate shape is not clear at all. Recent developmental studies in mice suggest that structures of joints are derived from a pool of cells with progenitor properties within the developing interzones, sites of the future joints.

The cellular and structural organization of the articular cartilage is similar between various types of joints with minor difference due to loading requirement.

Articular cartilage at the surface of joints has been extensively studied in knee joints because of pathological relationship with osteoarthritis. With articular cartilage of the knee as an example, the superficial zone contains elongated and flattened cells oriented parallel to the articular surface that produces lubricin and hyaluronic acid (HA) to lubricate joint movement (Jay et al. 2001). Chondrocytes in the middle zone are round in shape, usually organized in vertical rows of cells, and produce and maintain extracellular matrix components such as type II collagen and aggrecan for biomechanical function. Chondrocytes in the deep zone tend to be larger in size with hypertrophic appearance at the tidemark, the boundary articular cartilage, and the underlying subchondral bone (Broom and Poole 1982).

7.3 Developmental Processes of Joint Formation: An Overview

In the developing limb, skeletal elements are formed from a proximal to distal sequence, through temporally and spatially regulated processes that include mesenchymal condensation to give rod-shaped cartilage elements (anlagen), followed by elongation, branching, and/or segmentation (Sanz-Ezquerro and Tickle 2003a; Goldring 2012). For example, in the developing forelimb, the humerus element is formed first and then through segmentation and branching the radius and ulna, followed by the carpal and metacarpal elements, with the phalangeal elements forming last when a single skeletal condensation is segmented into 2 (thumb) or 3 (digits II to V) smaller segments through the formation of synovial joints (Hall and Miyake 2000). The general processes in joint development are depicted diagrammatically in Fig. 7.1a.

At the site of the future joint, the chondrogenic mesenchyme remained undifferentiated or undergoes a "dedifferentiation" process to form interzone regions, represented by a localized high-density region of cells. These cells appear flattened and begin to lose chondrogenic characteristics. With progression, the interzone further refines to a three-layered structure, with two outer layers of higher cell density flanking a central region of lower cell density where cells are thought to undergo apoptosis in some joints, forming a joint cavity. Cells within the high-density outer layers contribute to the formation of the future articular cartilage (Bland and Ashhurst 1996; Mitrovic 1978). The cartilage element proximal to the joint undergoes hypertrophy, initiating the process of endochondral ossification and the establishment of a growth plate at the epiphyseal regions. Importantly, growth plate chondrocytes appear not to be contributed by cells of the articular cartilage in development (Koyama et al. 2008). This is in support of the notion that the articular cartilage is structurally and functionally different to the growth plate cartilage.

While the morphological changes are well characterized and the role of interzone cells is established, the molecular regulation and changes in the differentiation and fate of interzone cells are not so clear (Pacifici et al. 2005; Decker et al. 2014). A study in mice using genetic activation of a reporter gene in interzone cells provided important clues to the progenitor status of interzone cells and their contribution to the articular cartilage and other structures of the joint (Koyama et al. 2008).

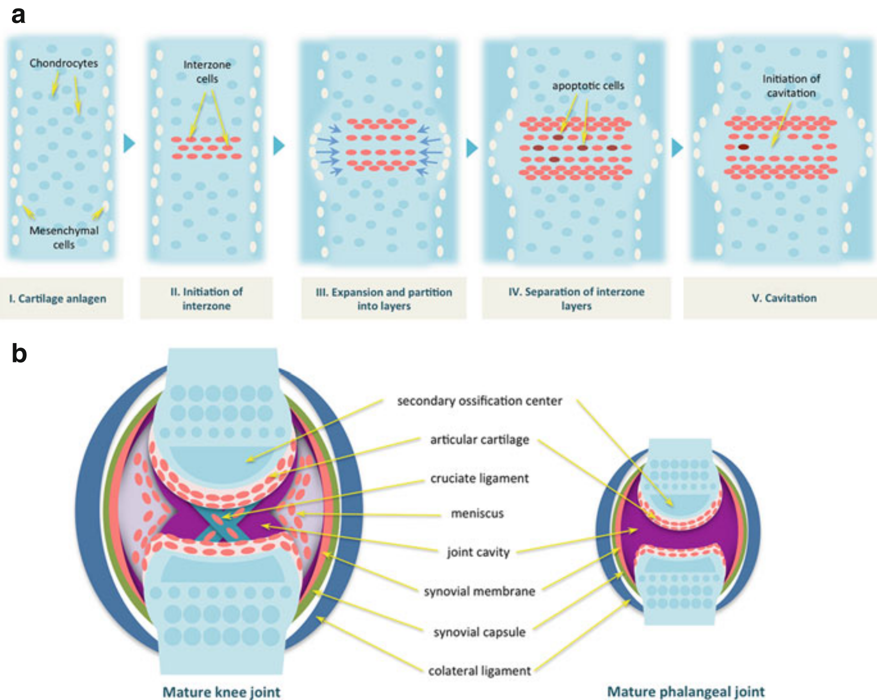

Fig. 7.1 Development of synovial joints. (**a**) Schematic representation of the developmental processes in synovial joint formation with the following steps: (*I*) condensation of mesenchymal cells in the formation of the cartilage anlagen; (*II*) cells at the future joint site undergo dedifferentiation that become flattened and arrange into layers to form an interzone; (*III*) the interzone expands by recruiting the cells from the surrounding mesenchyme and partitions into two outer regions with densely packed cells and the central/intermediate region with more loosely packed cells; (*IV*) initiating separation at the center of interzone with some cells that undergo apoptosis. (*V*) Cavitation with physical separation with cavity filled with synovial fluid, and the partitioned cells contribute to the formation of the articular cartilage, cruciate ligaments, synovial membrane, and meniscus. (**b**) Illustrations showing the anatomical structures of mature knee and phalangeal joints. The proposed contribution of interzone cells to the different structures is indicated by the position of the orange-colored interzone descendent cells

Such studies will provide further insights to more thorough understandings of the key events in joint formation.

7.4 Skeletal Patterning Through Formation of the Interzone Regions

7.4.1 Morphological Changes

Skeletal patterning in limb development is regulated in part by the formation of interzone regions within cartilage elements that will become future joints. The role of the interzone cells in joint formation is clear as their removal by microdissection

in the developing chick embryo in ovo resulted in the loss of the joint in the specified region (Holder 1977).

Cartilage elements in the developing limbs are formed through chondrogenic processes involving condensation of the mesenchyme and differentiation of mesenchymal cells toward the chondrocyte lineage under the regulation of key transcription factors such as *Sox9* in the initial stage and *Sox5/6* at a later stage (Akiyama et al. 2002; Archer et al. 2006), with specific changes in the extracellular matrix proteins and cell adhesion molecules, such as type I collagen and N-cadherin (DeLise et al. 2000) CD44 (Toole 1991), hyaluronic acid (HA), tenascin, and fibronectin (Dessau et al. 1980; Pitsillides et al. 1995).

The mechanism by which the interzone regions are initiated is not clear. It is likely to be complex involving intrinsic and extrinsic factors that may vary depending on the skeletal element concerned. While there may be some common factors in initiating interzone formation, studies have shown that the specificity of joints formed is dependent on additional autonomous and nonautonomous factors in directing joint morphogenesis. In the formation of the stylopods (humerus and femur) and zeugopods (radius and ulna, tibia, and fibula), the formation of the three cartilage elements, in the respective forelimbs and hind limbs, appears to be as discrete entities forming a Y-shape structure, and with elongation of the anlagen, a region of prechondrogenic cells is retained at the intersect that will become the interzone region corresponding to the future elbow and knee joints (Hamrick 2001; Hinchliffe and Johnson 1980). This is common from studies in mouse and chick limb development.

Formation of the autopod (metacarpal and phalangeal) joints appears to differ slightly, with an initial mesenchyme condensation forming a continuous cartilage element for the metacarpal in the digital rays that are segmented sequentially with elongation of the digital rays and formation of the phalangeal joints. This is the consensus in mouse development that the interzones in autopod develop through dedifferentiation of chondrogenic cells at sites of the future joints. However, in the developing chick autopods, the sequential condensation of digital cartilage anlagen suggests a mechanism similar to elbow and knee joints with the appearance of an intervening region of prechondrogenic cells that will become the interzone regions concomitant with the sequential condensation of the distal cartilage elements, suggesting species variations in the initiation of digit joints. It was suggested that this difference could be due to the presence of a phalange-forming region at the tip of the developing digit that is responsive to BMP signaling in the avian system (Suzuki et al. 2008). However, a similar region can be identified in the mouse (Stricker and Mundlos 2011). Thus, while this region may be responsible for the recruitment of mesenchymal cells into the condensing mesenchyme as the cartilage anlagen grows and elongates, the initiation of phalangeal interzones is different between avian and mammal.

Irrespective to how the interzone cells may arise, they are presented as more flattened cells than the surrounding rounded chondrocytes in the cartilage anlagen. These flattened cells are organized into layers aligned perpendicular to the proximal-distal axis of the developing limb and begin to lose their chondrogenic characteristics with a downregulated expression of *Sox9* and a change in the extracellular

matrix from a type II collagen- to a type I collagen-rich environment (Craig et al. 1987). Once initiated, the interzone becomes an important signaling region and a source of progenitor cells for the subsequent formation of the joint structures. With development, the number of cells within the interzone increases; it does not appear to be due to active proliferation of the interzone cells, but rather further recruitment of mesenchymal cells from the surrounding tissues (Niedermaier et al. 2005).

7.4.2 Molecular Regulation of Interzone Initiation

The specification of skeletal elements along the proximal-distal axis in the developing limb is likely to have some predetermined cues influencing the initiation and position of the future joint. For example, as joints are formed around skeletal elements of the stylopod and zeugopod, the disruption of paralogous *Hox* genes (*Hoxa11* and *Hoxd11*) in mouse that lead to the loss of radius and ulna bones (Davis et al. 1995) will influence the formation of the elbow joint, and the *Hoxd13* mutant mice with skeletal abnormalities restricted to the autopod will affect phalangeal joint formation (Dolle et al. 1993). It has been shown that the proximal-distal progression in digit development can be influenced by the prolonged FGF signaling from the apical ridge that results in the formation of an extra phalangeal joint/bone and that an FGF receptor inhibitor can block its formation (Sanz-Ezquerro and Tickle 2003b). Similarly, an additional phalange can be induced in a chick toe if sonic hedgehog (SHH) proteins are placed in between developing digital rays (Sanz-Ezquerro and Tickle 2000; Dahn and Fallon 2000).

Interestingly, cell-matrix interaction can also be a contributing factor, as demonstrated by inhibition of α5β1 integrin signaling using specific antibodies or arginine-glycine-aspartic acid (RGD)-blocking peptides, with the formation of an ectopic interzone between proliferating chondrocytes and hypertrophic chondrocytes in forelimb cartilage elements in mouse embryos at E14.5 (Garciadiego-Cazares et al. 2004). The ectopic interzone expresses interzone markers (*Wnt9a/Wnt14*, *Gdf5*, *chordin*, *autotaxin*, *Col1a1*, and *CD44*), while chondrocyte markers (*Ihh* and *Col2a1*) are downregulated, consistent with the initial stages of joint formation. Clearly, the positional specification and signals that initiate interzone formation will be complex that require more detailed molecular genetic studies in chick and mouse development.

At the molecular level, joint formation correlates with downregulation of type II collagen (*Col2a1*) and aggrecan (*Acan/Agc1*) at the specified joint region, with concomitant expression of genes such as *Gdf5*, *Gdf6*, *Bmp7* and *Bmp2* (Francis-West et al. 1999b; Merino et al. 1999; Storm and Kingsley 1996, 1999), *Noggin* (Brunet et al. 1998), *Wnt9a*/(*Wnt14*) (Hartmann and Tabin 2001), *Wnt4* and *Wnt16* (Guo et al. 2004), *Gli3* (Spater et al. 2006a), *CD44* (Pitsillides 2003), and *Erg* (Iwamoto et al. 2000). Overexpression of *Bmp2*, *Bmp4*, *Bmp7*, and *Gdf5* genes in the limb bud or inactivation of BMP antagonist Noggin causes overgrowth of the cartilage and inhibition of joint formation (Brunet et al. 1998; Storm and Kingsley 1999; Duprez et al. 1996), whereas deletion of *Gdf5* or *Gdf6* results in fusion of joints (Settle et al. 2003).

Gdf5 and *Wnt9a* are two early markers of the interzone. Therefore, it has been proposed that they have a role in early interzone formation, and there is a balance between chondrogenic (GDF5) and anti-chondrogenic (WNT9a) signals that regulate the initiation and progression of interzone formation. GDF5 is a member of the TGF-β superfamily. Mutations in this gene are associated with acromesomelic dysplasia, Hunter-Thompson type, brachydactyly type C, and chondrodysplasia Grebe type (Baldridge et al. 2010; Mundlos 2009), consistent with its role in chondrogenesis and joint formation. Furthermore, inactivation of *Noggin*, a secreted BMP antagonist, results in the absence of joints (Brunet et al. 1998), and mutations in *Noggin* lead to multiple synostoses (Gong et al. 1999). However, loss of TGF-β responsiveness from inactivation of the TGF-β type II receptor gene (*Tgfbr2*) in limbs of mice resulted in the absence of interphalangeal joints (Spagnoli et al. 2007). Thus, TGF-β signaling is needed to promote joint formation as signaling via TGFBR2 regulates *Noggin*, *Wnt9a*, and *Gdf5* expression (Spagnoli et al. 2007). Given that overexpression of *Gdf5* fails to induce joint formation but results in overproduction of cartilage and loss of joints (Francis-West et al. 1999a), TGF-β signaling is likely to play a regulatory role that is necessary but not sufficient to induce joint formation.

Several Wnt genes, including *Wnt4*, *Wnt9a*, and *Wnt16*, are identified with overlapping and complementary expression in early interzone cells together with increased β-catenin level and activity; hence, Wnt signaling is likely to play an important role (Guo et al. 2004). Wnt4 was implicated in both canonical and noncanonical Wnt signaling, while Wnt9a and Wnt16 were implicated in the canonical β-catenin pathway (Guo et al. 2004). Many studies have shown that the canonical β-catenin pathway has a role, as removal of β-catenin in mesenchymal progenitor cells promoted chondrocyte differentiation and genetic removal in chondrocytes led to bone fusion (Guo et al. 2004; Spater et al. 2006a; Kahn et al. 2009).

Ectopic expression of *Wnt9a* or activation of the canonical β-catenin pathway induced ectopic joint-like structure, with expression of *Gdf5* (Guo et al. 2004; Hartmann and Tabin 2001; Tamamura et al. 2005). Thus, Wnt9a was considered to be critical in determining where the joint will be formed, and the Wnt/β-catenin signaling was shown to be necessary and sufficient to induce early steps of synovial joint formation (Guo et al. 2004). However, later studies find that Wnt/β-catenin signaling may not be required for induction but needed for the subsequent maintenance and cell fate, important for long-term joint integrity (Koyama et al. 2008; Spater et al. 2006a).

Gdf5 is expressed by condensing mesenchyme and immature chondrocytes (Kan et al. 2013); its expression in early interzone cells may represent an early sign of the dedifferentiation. It is clear that what signals are required for joint initiation is still poorly understood. Given that c-Jun can act at the enhancer level to regulate Wnt signaling at the initiation and joint progression (Kan and Tabin 2013), and *Sox11* expression, a transcription factor that becomes restricted to interzone cells in joint development, can stimulate expression of *Gdf5* (Kan et al. 2013), there are upstream regulators that form feedback loops in promoting/regulating the initiation process, likely to be a balance between TGF-β and Wnt signaling.

7.4.3 Interzone as a Reservoir of Progenitor Cells for Joint Formation

Given that the interzone emerges from sites that are previously occupied by chondrocytes (phalangeal joints) or chondrocyte precursors (knee and elbow joint), the consensus is that interzone cells are descendants of the dedifferentiated chondrocytes/chondrocyte precursors, with a history of expressing *Sox9* or *Col2a1*. In addition, *matrilin 1* (*Matn1*) is normally expressed in all chondrocytes except articular chondrocytes, and *Gdf5* is expressed at the onset of interzone formation; mice with *Cre* recombinase expressed under the transcriptional regulation of *Sox9* (Soeda et al. 2010), *Col2a1* (Sakai et al. 2001), *Matn1* (Hyde et al. 2007), and *Gdf5* (Rountree et al. 2004) have been used to perform cell lineage tracing-tracking for the interzone cells in joint development.

The fate of *Gdf5*-expressing cells that has been studied in a genetic cross between Gdf5-Cre and Rosa26-LacZ (R26R) mice (Rountree et al. 2004; Koyama et al. 2008) showed that descendants of *Gdf5*-expressing cells gave rise to many joint tissues, including articular cartilage, synovial membrane, and intra-joint ligaments (Koyama et al. 2008). Although the Gdf5-Cre used in this study was not an inducible *Cre*, and *Gdf5* expression remains till the formation of the articular cartilage, the data does provide the genetic evidence in support of the proposed lineage. This finding is in support of another study using the *Matn1-Cre*/R26R mice that showed cells of the developing articular cartilage are not tagged with *LacZ* expression, while chondrocytes in the rest of the cartilage element are positive for LacZ (Hyde et al. 2007). This is consistent with these cells not having a history of *Matn1* expression, suggesting early articular chondrocytes did not arise from chondrocytes of the cartilage anlagen.

However, cell tracing studies using the Sox9-CreERt2/R26R mice with CreERt2 induced with tamoxifen at E11.5 and embryos examined at E17.5 showed Sox9-descendant cells in articular and growth plate chondrocytes, as well as ligaments (Soeda et al. 2010), suggesting cells from these tissues originated from a common progenitor pool with a history of *Sox9* expression. This would be in line with the concept of interzone cells that arise from dedifferentiation of chondrocytes/chondrocyte precursors, expressing *Sox9* in the condensing limb mesenchyme. Similar finding in a study using endogenous doublecortin (*Dcx*) to drive expression of reporter genes (LacZ or GFP) supported this concept, showing expression of *Dcx* in much of the limb mesenchyme that later are restricted to interzone and articular cartilage (Zhang et al. 2010).

In a related study using the *Col2a1-Cre*, a cross with the *R26R* mouse revealed resident chondrocytes of the cartilage anlagen that have switch off expression of *Col2a1* contribute to the interzone at E13.5. However, following interzone formation, non-*Col2a1*-expressing cells migrate into the developing knee joint interzone that formed the lateral and outer medial meniscus, suggesting cells of the developing meniscus in the knee joint have a complex cell origin (Hyde et al. 2008).

Incorporating these cell tracing-tracking findings, it is clear that interzone cells have progenitor properties. Once established, these cells can contribute to the formation of the articular cartilage as well as non-cartilaginous structures such as synovium

and, in the knee, cruciate ligaments and meniscus (Fig. 7.1b). While these studies provided the genetic support for the proposed origin of the cells within the developing joint, the mouse tools used were not ideally designed for cell tracing, and more detailed and thorough analyses of the progenitor cell pools within the interzone await the availability of inducible joint tissue-/cell-specific Cre (such as the tamoxifen-inducible Cre) for in vivo “pulse-chase” style of cell lineage tracing-tracking studies.

7.4.4 Interzone as a Signaling Center

The interzone, once established, must be maintained and directed to progress along the correct lineage of cellular differentiation and organization to form the various tissues of the joint. While both TGF-β and Wnt signaling are known to be involved in joint initiation, the balancing act continues in the developmental process for the formation of cartilage and fibrous tissues of the joint. TGF-β signaling is clearly important as many of the genetic defects affecting joint formation, in particular brachydactyly (short digits) disorders. Recent advances in deciphering the molecular basis of these brachydactylies show that genes in the BMP/TGF-β signaling pathway are deregulated, suggesting this signaling pathway is pivotal for digit and joint development (Mundlos 2009).

As the interzone begins to organize into the zonal layers in preparation for the formation of the articular cartilage layers along a chondrogenic lineage, and cells within the middle/intermediate layer begin to organize along the lineage for fibrous tissues, it can be envisaged that nonskeletal signals should be maintained in this middle/intermediate layer that should be reduced with corresponding enhanced chondrogenic signals for the outer articular chondrocyte layers. Indeed, this was demonstrated in a recent gene expression analysis of the interzone layers through laser capture microdissection from a developing interzone of a knee joint from a mouse embryo at E15.5 (Jenner et al. 2014). This study showed a high expression level for genes related to chondrogenesis, endochondral ossification, and chondrocyte hypertrophic and cartilage matrix genes. Both BMP and Wnt signaling appear to be active for chondrocyte differentiation and maturation, respectively (Jenner et al. 2014). Within the intermediate layer, chondrogenic genes are not the main feature, although *Sox9* and *Sox6* are expressed and some matrix genes such as *Col2a1*, *Comp*, and *Agc1* suggest this layer may still possess some chondrogenic potential. This would be consistent with joint fusions in some disorders where the balance is tipped toward chondrogenesis. Interestingly, this study also shows a high level of expression for inflammatory genes in the intermediate layer. Their presence while interesting is not clearly understood, but perhaps as a response to remodeling processes in preparation for cavitation as suggested by the authors (Jenner et al. 2014).

The interzone as a signaling center is an interesting concept, not only within the developing joint, as well as its potential to influence development of adjacent tissues. It was postulated that the interzone might be an essential regulator of skeletal development, controlling chondrogenesis of the adjacent cartilage element (Hartmann and Tabin 2000). It was also proposed that signal emanating from the interzone could determine the position of the more distal joint. This proposal arises

from the study of *Wnt9a* mis-expression study, in which the endogenous joint formation was inhibited by the presence of an ectopic joint (Hartmann and Tabin 2001). How this functions is not clear, but a model for spacing of joint was proposed, where the initial step of joint formation involves induction of Wnt9a, and with the formation of the interzone, distinct gene expression patterns will occur that includes *Gdf5*, *chordin*, and other secondary signals secreted from the interzone, acting on neighboring cartilage elements to prevent the induction of a new interzone in the vicinity until their level is reduced to a permissive level (Hartmann and Tabin 2001). This model would be consistent with the observation of an additional joint form through elongation of the cartilage element with prolonged FGF signaling at the apical ectodermal ridge (Sanz-Ezquerro and Tickle 2003b). Thus, with elongation, the level of inhibitory factors from the proximal interzone region is reduced to a level permissive of another joint to form, and the length of the distal cartilage anlagen can be a determining factor.

Studies of IHH signaling within the developing joint also supported the interzone as a signaling center. In the developing interzone, HIP1, a hedgehog target and negative regulator of hedgehog diffusion, is expressed and localized to the margin between the proximal and distal cartilage anlagen (Gao et al. 2009). This was hypothesized to regulate a precise level of hedgehog signaling within the boundaries of the interzone, and one of the targets is PTHrP that signals to the distal tip where the receptor (Ppr) is located. Through a negative feedback loop similar to the growth plate, PTHrP regulates the level of *Ihh* expression in a group of cells at a distal region, a signal needed to regulate the recruitment of mesenchymal cells into the condensing mesenchyme for growth of the cartilage anlagen (Gao et al. 2009; Stricker and Mundlos 2011).

Missense mutations in the *IHH* gene are responsible for brachydactyly type A1 (BDA1) with affected individuals having shortened or missing middle phalangeal bones (Gao et al. 2009; Ma et al. 2011; Guo et al. 2010). In a mouse model for BDA1 with an E95K mutation in *Ihh*, it was shown that the interaction with the receptor patched 1 (PTCH1) and HIP1 was affected (Gao et al. 2009). A consequence is reduced signaling capacity and increased signaling range. Thus, in the BDA1 mouse, while the signaling capacity is reduced, the range is extended, and IHH signals much further into the developing interzone with enhanced PTHrP level, and PTHrP from this center signals to a distal group of cells expressing the receptor, and the level of IHH at this region is downregulated due to the negative feedback loop; the result is reduced recruitment of mesenchymal cells into the cartilage anlagen affecting joint formation further supporting the interzone that acts as a signaling center (Gao et al. 2009).

7.5 Joint Cavitation Formation

7.5.1 Progression to Cavitation

As the joint develops, interzone cells will become increasingly flattened, which attenuates with the continued expansion of the cartilage anlagens. With progression, the presumptive joint capsule establishing in concomitant with interzone becomes

vascularized, infiltrating to the periphery of the synovium. Tissue separation then begins within the center of the interzone that is avascular, and it would seem reasonable to propose that cells within this region would need to change in preparation for this event.

Morphologically, the middle/intermediate layer of the developing interzone can be considered as a "transition zone" and, in the knee, can contribute to the spindle-shaped cells lining the surface of the future joint and the formation of the joint menisci. It seems that the process is intrinsic to cells and tissues within the interzone, as the early process of interzone formation and progression is unaffected by removal of the surrounding cartilaginous tissues, but removal of the interzone region results in loss of a joint (Holder 1977). Thus, interzone cells receive intrinsic signals that influence progression, although these signals could be influenced by local cues such as mechanical loading that will be discussed later (Osborne et al. 2002).

In chicks, the homeobox-containing transcription factor, *Cux1*, was shown to be involved at the onset of joint formation, downregulating the expression of *Col2a1* and *Agc1*, consistent with interzone progression accompanied by reduced staining for cartilage matrix and changes in the extracellular matrix (ECM) (Lizarraga et al. 2002). Wnt9a should also have a role in directing the progression of joint formation as it can induce or maintained the expression of key joint-forming genes, including *autotaxin* (an enzyme for the synthesis of lysophosphatidic acid), *chordin* (a BMP antagonist), and *CD44* (receptor for HA) (Hartmann and Tabin 2001). Thus, *Wnt9a* may exert a later effect in joint formation by promoting the induction of *CD44* expression in the developing interzone and changes in the ECM. This would be expected, as the joint cavitation process must involve key changes in the local ECM environment and architecture for a separation to take place and contribution for the articular cartilage to take place.

Gdf5-expressing cells within the developing interzone also continue to have a role in regulating the formation of cartilaginous and fibrocartilaginous tissues. In addition, *Bmp4* and *Gdf6* are also expressed during joint development (Storm and Kingsley 1996; Zou et al. 1997), suggesting BMP signaling continues to modulate the joint developmental process. Thus, abnormal joint progression can be observed in mice when the receptor (*Tgfbr2*), BMP antagonist (*Noggin*), *Wnt4*, *Wnt9a*, or *ß-catenin* is inactivated in mice (Brunet et al. 1998; Spater et al. 2006a, b; Spagnoli et al. 2007; Koyama et al. 2008), caused by deregulated chondrocyte differentiation at the joint sites.

Indian hedgehog (Ihh) is better known for its role in regulating chondrocyte proliferation and differentiation in the cartilage growth plate (Kronenberg 2003). However, it also has a critical role in regulating synovial joint formation. Inactivation of *Ihh* in mice results in loss of phalangeal joints, but cells in the tissue surrounding the presumptive joint regions express *Gdf5* but the progress of joint formation is impaired (St-Jacques et al. 1999). Its relevance in digit joint formation is further supported by the identification of mutations in *IHH* causing brachydactyly type A1 (BDA1) (Gao et al. 2001), which was later shown to the range of IHH signaling with enhanced signaling into the developing interzone region, affecting the progression of joint formation, perhaps altering the balance between chondrogenic and

non-chondrogenic signals in cellular differentiation and ECM production, leading to the failure of joint formation in some cases (Gao et al. 2009). This notion is supported from a study of the *short digit* (*Dsh*) allele in mice, with an 11.7 Mb inversion in chromosome 5 encompassing the *Shh* locus (Niedermaier et al. 2005). The inversion alters the regulatory control of *Shh* expression, and the digit phenotype in heterozygous (*Dsh/*+) mice results from a dysregulated expression of *Shh* at E13.5 and E14.5 in the phalangeal anlagen with increased hedgehog signaling into the developing interzone altering chondrocyte differentiation and matrix production, resulting in a phenotype similar to BDA1 (Niedermaier et al. 2005).

7.5.2 Cavitation Process

For a synovial joint to function, smooth long-lasting articular cartilage surfaces at opposing ends of long bones need to be generated, separated by a cavity filled with synovial fluid for lubrication. This is an area of extensive research given it is of fundamental importance to the function of the joint. Morphologically, cavitation occurs within the interzone intermediate layer by separation of the elongated and spindle-shaped cells, accompanied by local change in ECM. Once cavitation occurs, the outer layer of spindle cells will localize to the future surface of the articular cartilage (Ito and Kida 2000; Kavanagh et al. 2002; Prehm 1984), and the cavity is filled with lubricants such as hyaluronic acid (HA) (Dowthwaite et al. 2003). HA is a long polymer of glycosaminoglycan units of molecular weight up to 10^7 daltons. Although non-sulfated, it is highly negatively charged due to the carboxyl groups present within the sugar moieties. It fills the cavity produced by attracting and holds water to create a hydrated environment for frictionless joint movements.

A number of cellular and biochemical events have been proposed to be associated with the cavitation process. These included cell death, remodeling of the extracellular matrix, and mechanical influences (Andersen 1961; Mitrovic 1971, 1972; Nalin et al. 1995). Cell death through apoptosis is a common occurrence in development, helping to shape and pattern tissues (Suzanne and Steller 2013). Indeed, some studies have reported cell death in the interzone just prior to cavitation. However, the extent was minimal. Furthermore, cell death was not observed in studies of the developing rat and rabbit knee (Ito and Kida 2000; Kavanagh et al. 2002). However, cell death does occur in interzones of phalangeal joints (Fernandez-Teran et al. 2006), and we have demonstrated this in the developing mouse digits (Fig. 7.2). Thus, the role of cell death is still not clear as it appears to not be essential for all developing synovial joints. This difference between joints may be due to size and shape requirements. Further, massive cell death may not be required, and a temporal cell death is sufficient to initiate the process, triggering downstream events to promote cavitation. Studies of genetic diseases with malformation in phalangeal joint development support the role of cell death, as in its absence, joint fusion and syndactyly occur (Niedermaier et al. 2005; Mundlos 2009).

Change in the ECM environment is observed with cavitation (Andersen 1961; Craig et al. 1987) suggesting the need for remodeling and the involvement of matrix

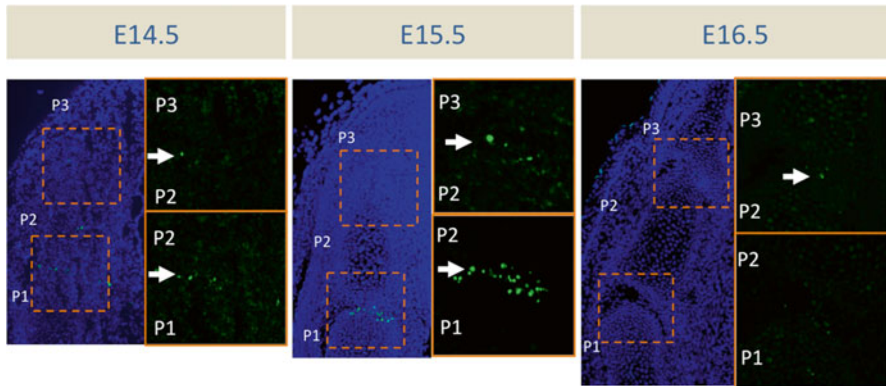

Fig. 7.2 Apoptosis and cavitation in mouse phalangeal joint development. Cellular apoptosis in phalangeal joint development. Apoptotic (TUNEL positive; GFP) cells are detected in the P1/P2 interzone (*white arrows*) of digit III at E14.5 that become more pronounced at E15.5 at the initial stage of cavitation, but with no apoptotic cells detected at E16.5 when a clear joint cavity is established. The distal P2/P3 interzone represents a later development that followed a similar trend. *P1* phalangeal bone 1, *P2* phalangeal bone 2, *P3* phalangeal bone 3. The *dotted boxes* represent magnified regions shown in the respective panels to the right of each of the developmental time points

metalloproteinases (MMPs). Interestingly, immunohistochemical studies show little evidence of MMPs in the interzone during cavitation (Edwards et al. 1994, 1996). MMP activity was detected post cavitation in articular cartilage (Gepstein et al. 2002) but likely to be involved in the establishment of the articular cartilage matrix. One possible way to enforce ECM changes without initial matrix degradation is to produce new matrix and physically push the "older matrix" apart. HA is produced at the plasma membrane of cells and is extruded directly into the extracellular space (Itano et al. 1999; Prehm 1984). Thus, in the developing interzone, it is possible that the presence of HA in the cavity would provide a swelling pressure to physically move the outer interzone layers apart. Furthermore, CD44 is a cell surface HA-binding protein and should have an important role in the cellular interactions with HA (Aruffo et al. 1990). HA-CD44 interaction can induce both cell adhesion and cell separation, depending on the HA concentration in the presence of receptor saturation (Toole 1991). During interzone cavitation, the increased HA synthesis binding to CD44 at cells of the intermediate interzone and cells at the surface of the future articular cartilage can further facilitate tissue separation. The relevance of HA-CD44 interaction is supported from exogenous application of HA oligosaccharides to displace the interaction of endogenous HA with cells that prevented joint cavitation in ovo (Dowthwaite et al. 1998).

Embryo movement and muscle contraction are known to play a role in joint development. Indeed, drug-induced muscle paralysis in chicken or mouse embryos inhibits joint formation (Fell and Robison 1934; Osborne et al. 2002). Muscle contraction is thought to influence the physical separation of the interzone, and HA has also been implicated in immobilization that inhibited cavitation in developing limb

joints (Osborne et al. 2002). Mechanical stimulus can increase the level and activity of the enzymes such as uridine diphosphoglucose dehydrogenase (UDPGD) activity and HA hyaluronan synthase (HAS), thereby increasing the level of HA (Mikic et al. 2000; Osborne et al. 2002). Furthermore, movement in joint development can stimulate the establishment of superficial cells of the articular cartilage with the production of HA and lubricin (Dowthwaite et al. 2003).

Genetic manipulations of muscle development and excitation-contraction coupling deficiency in mice provided mechanistic insights that show muscle contraction is needed to reinforce Wnt/β-catenin signaling, and its impairment in "muscleless" mouse embryos results in fused joints (Kahn et al. 2009). However, the specific mechanism by which muscle contraction regulates the levels of Wnt/β-catenin signaling in joint development is not known. Interestingly, while joint defects are observed in several limb joints, not all are affected with differences between joints in the fore- and hind limbs. For example, the elbow is affected but not the knee (Kahn et al. 2009). Intriguingly, in a gene profiling study for factors that direct development of the elbow and knee in mouse embryos, the authors found genes in the developing elbow joints are enriched in specification for muscle development genes (Pazin et al. 2012). Whether this has a direct link to the abnormal elbow joint development in the muscleless mouse embryos remains to be elucidated (Kahn et al. 2009).

7.6 The Joint Proper

Following cavitation, genetic cell lineage tracing experiments showed that cells in the outer interzone layers contribute to multiple joint tissues over time, including the future articular cartilage, synovial lining, and intra-joint ligaments that persisted into postnatal life (Decker et al. 2014). Cells in these tissues can be traced back to *Gdf5*-expressing dedifferentiation chondrocytes at the onset of interzone formation. Furthermore, expansion of the interzones included recruitment of mesenchymal cells from surrounding tissues of the joint sites into the *Gdf5*-expressing lineage (Niedermaier et al. 2005; Decker et al. 2014). Thus, cells outside the original cartilage anlagen contribute to interzone expansion and progression. These cells could be a population of *Tgfbr2*-expressing cells as demonstrated from a study using the *Tgfr2-LacZ* mice that are initially restricted to the surrounding tissues but, with time, contribute to the synovial lining, meniscal surface, outer ligaments, and groove of Ranvier (Spagnoli et al. 2007).

In mice, a thin layer of cells at the surface of the articular cartilage of the tibia at birth can be molecularly defined by the expression of genes such as collagen XXII (*Col22a1*) (Koch et al. 2006), lubricin (*Prg4*) (Rhee et al. 2005), and tenascin C (Mikic et al. 2000). These cells are derived from *Gdf5*-expressing cells from the interzone (Koyama et al. 2008; Rountree et al. 2004). By P10, the articular cartilage is thicker that can be roughly divided into three zones: a superficial zone of flattened cells at the surface, a middle zone of more rounded cells with typical chondrocyte appearance, and a deep zone of large cells more consistent with hypertrophic

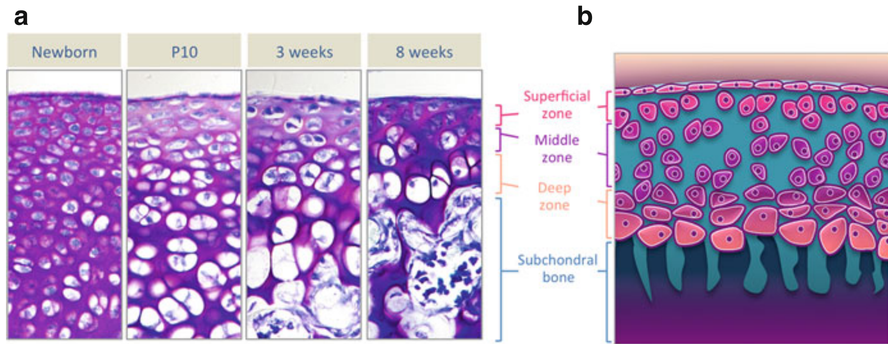

Fig. 7.3 Postnatal development and structure of articular cartilage. (**a**) Histological sections (toluidine blue staining) of the proximal tibia showing the progressive changes in the organization and differentiation of the chondrocytes in the articular cartilage from birth to 8-week-old mice. The progressive postnatal organization into the superficial, middle, and deep zone is illustrated. Cells in the middle and deep zone are progressively larger, consistent with hypertrophic chondrocytes. (**b**) Illustration shows the demarcated zonal organization of the articular cartilage in a mature joint

chondrocytes (Fig. 7.3). From 3 weeks, the articular cartilage exhibits a more mature organization with the onset of secondary ossification of the epiphyseal end of the proximal tibia that becomes a more permanent structure by 8 weeks of age (Fig. 7.3). Interestingly, a recent study tracing *Prg4*-expressing cells at the most superficial region of the articular cartilage of 1-month-old mice showed that they may serve as progenitor cells contributing to the cells of the deeper zones that persisted for at least up to 1 year (Kozhemyakina et al. 2015). Given that the origin of these *Gdf5*-expressing cells can be traced back to the interzone, it may be argued that interzone cells continue to play an important role in the growth and maintenance of the mature articular cartilage.

The joint is not complete without the capsule. It is a dense fibrous membrane attached to the whole circumference of each bone flanking the articular cartilage, forming a sleeve around the joint, providing a seal that keeps the lubricating synovial fluid within the joint space (Ralphs and Benjamin 1994). This capsule contributes to stability of the joint by limiting motion that is further supported by accessory ligaments inside and outside the capsule. It is also thought that the capsule itself may have a role in articulation function as part of the capsule is compressed during movement, which in time becomes more fibrocartilaginous as it adapts to compression. The capsule, despite its importance for joint function, is not well studied, in particular the developmental aspect.

7.7 Overall Perspectives

This chapter provided a brief account of our current understanding of synovial joint formation. With advances in mouse genetics and transcriptomic analyses, we have gained significant insights into the cellular origin of the joint tissues in development,

the molecular signals involved, and the fate of joint cells in adult life. It is a complex system of many players, with both intrinsic and extrinsic influences to the interzone. However, there remain many uncertainties and challenges that require detailed studies to better define the origin(s) of interzone cells. As the pool of Gdf5-expressing cells in the developing interzone is unlikely to be homogenous, there is a need to address potential subpopulations. Mice with better-defined cell-specific inducible *Cre* would be needed, and combining this with single-cell transcriptomics of interzone cells could provide vital clues to the contribution and presence of predetermined subpopulations. Further, more specific gene sets could be identified for the generation of *Cre* driver mice. For example, the Prg4-GFPCreERt2 mice presented as a useful tool to trace cells of the superficial zone in articular cartilage growth and maintenance, not only for the understanding of normal joint biology but also in disease states such as osteoarthritis. Similar *Cre* mice could be produced for the analysis of earlier developmental processes along the Gdf5 lineage toward articular chondrocytes and other joint tissues and to address their potential to become resident adult stem/progenitor cells in the joint (Dowthwaite et al. 2004; Candela et al. 2014). A big question in the field is to understand the reason behind the notoriously weak repair potential of adult cartilage. If there are stem/progenitor cells present in the joint, and they are similar to developmental progenitors "locked" away in a special niche, perhaps understanding the developmental cues may help to unlock these cells to enhance repair. In addition, we should not forget the power of mouse genetics with defined strains of mice with varying degrees of repair/regeneration potentials. For example, the recent genetic studies of the good (MRL, LG/J) and poor (C57Bl, SM/J) cartilage healer mice are aimed at discovering regenerative loci/genes that could promote repair (Rai and Sandell 2014), as the good healer mice can activate an effective repair of large defects in the articular cartilage (Fitzgerald et al. 2008). These mice are also good candidates to study reactivation of development progresses or the activation/mobilization of resident stem/progenitor cells in the repair process.

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Molecular Control of Cartilage Differentiation

8

Manuela Wuelling and Andrea Vortkamp

Abstract

The vertebrate skeleton is formed by two distinct processes; intramembranous and endochondral ossification. During intramembranous ossification, mesenchymal progenitor cells directly differentiate into bone-forming osteoblasts. In contrast, endochondral ossification is a two-step process during which bones are initially preformed as cartilage templates, which are subsequently replaced by bone tissue. During this process, the inner cells of the cartilage condensation differentiate into chondrocytes. These initially proliferate and subsequently exit the cell cycle to form hypertrophic chondrocytes producing a mineralized matrix, which serves as a template for the later ossification. The outer cells differentiate into fibroblastic cells froming the perichondrium, which surrounds the cartilage template and provides signals to organize chondrocyte differentiation. Cells in the perichondrium flanking the hypertrophic region differentiate into osteoblasts, which form the bone collar. Blood vessels from the bone collar invade the hypertrophic region in close association with bone-resorbing osteoclasts and bone-forming osteoblasts. The orchestrated formation, differentiation, and degradation of cartilage and bone are regulated by a multitude of signaling systems and transcription factors. The identified signaling molecules include Ihh, PTHrP, FGF, BMP, WNT, IGF, CNP, and CCN proteins. Mutation or deletion of these factors leads to skeletal malformations in humans and in transgenic mice. This chapter

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provides an overview of the function of the main regulators of chondrocyte differentiation focusing on what we know about the complex cross-talk between them. Furthermore, the impact of recent advances in the understanding of the genetic and epigenetic mechanisms governing chondrocyte differentiation will be discussed.

8.1 Endochondral Ossification: An Overview

The development of a bony skeleton is a hallmark of the evolution of vertebrates. Bones differentiate by two mechanistically distinct processes: intramembranous and endochondral ossification. During intramembranous ossification, mesenchymal progenitor cells directly differentiate into bone-producing osteoblasts forming the bones of the skull and parts of the facial bones and the clavicula. In contrast, the bones of the axial and appendicular skeleton develop by endochondral ossification, a process during which the later bones are preformed as cartilaginous templates, which are subsequently replaced by bone and bone marrow.

This process is initiated by the condensation of mesenchymal precursor cells into osteo-chondroprogenitors. Cells in the inner condensation differentiate into proliferating chondrocytes, while cells at the outside of the condensation form a fibroblastic cell sheet, the so-called perichondrium, which later will give rise to osteoblasts. The proliferating chondrocytes successively differentiate into highly organized subpopulations with distinctive morphological and molecular features summarized in Fig. 8.1 (for further information, see Chap. 4) (Kozhemyakina et al. 2015).

The first step is the differentiation of hypertrophic chondrocytes in the center of the cartilage element once it has reached a critical size. With the appearance of the hypertrophic zone, the flanking proliferating chondrocytes separate into two cell populations: at the ends of the skeletal elements, the chondrocytes remain round and are low proliferating, while the more central chondrocytes align into columns (columnar chondrocytes) and show a high proliferation rate. Once the three regions are established, the skeletal elements grow by continuous proliferation, especially of the columnar chondrocytes. These cells then exit the cell cycle to become prehypertrophic and hypertrophic chondrocytes. This differentiation is linked to a dramatic increase in cell size (Cooper et al. 2013) and the secretion of a specialized, mineralized matrix rich in Collagen type 10. The hypertrophic matrix provides a scaffold for the later forming osteoblasts, which replace the cartilage by bone tissue (Fig. 8.1). The hypertrophic cells either undergo chondroptosis, a specialized form of apoptosis (Roach et al. 2004), or transdifferentiate into bone-forming osteoblasts (for further information see Chap. 5).

Adjacent to the hypertrophic region, mesenchymal cells in the surrounding perichondrium differentiate into bone-forming osteoblasts producing a highly vascularized bone collar, which surrounds the hypertrophic region. Blood vessels from the periosteum subsequently invade the hypertrophic zone providing osteoclasts, which

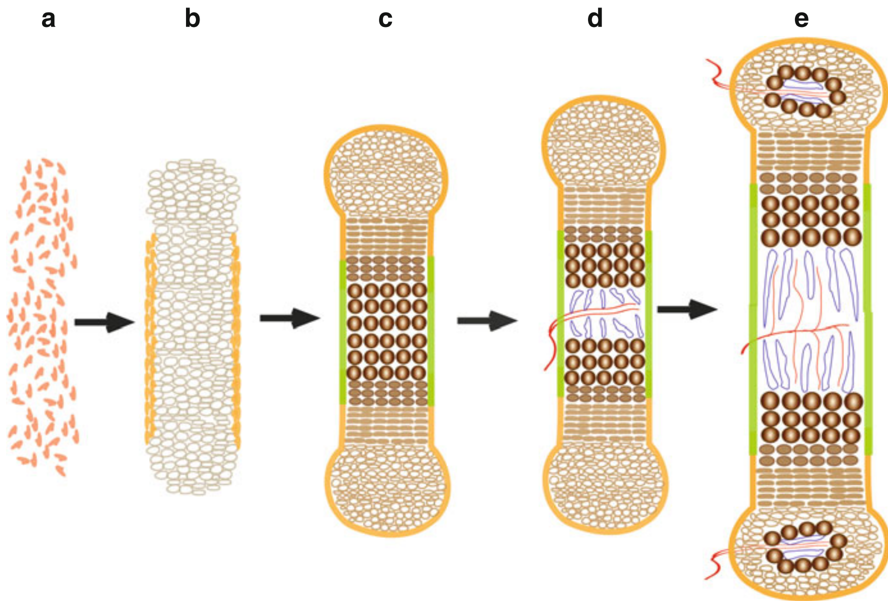

Fig. 8.1 Schematic overview over endochondral ossification. (**a**) Mesenchymal cells condense to form the cartilaginous anlagen (**b**), which are surrounded by the perichondrium (**b**). (**c**) In these anlagen, proliferating chondrocytes differentiate into round, low-proliferating chondrocytes ( ); columnar, high-proliferating chondrocytes ( ); and prehypertrophic ( ) and hypertrophic chondrocytes ( ), which mineralize their extracellular matrix. (**d**) Blood vessels invade the hypertrophic region transporting bone-forming osteoblasts and osteoclasts, which degrade the cartilage matrix and replace the cartilage with the bone and bone marrow ( ). Adjacent to the hypertrophic zone, cells in the perichondrium ( ) differentiate into osteoblasts forming the periosteum ( ). (**e**) After birth, a secondary ossification center is formed within the region of round chondrocytes near the joint. The remaining embryonic cartilage, the growth plate, is required to maintain longitudinal growth postnatally

resorb the calcified cartilage matrix, osteoblasts, which synthesize new bone trabeculae, and hematopoietic and mesenchymal stem cells, which together will form the bone marrow. Pre- and postnatally, the longitudinal growth of endochondral bones depends on distinct steps in the chondrocytes differentiation process. First of all, the proliferation rate of columnar chondrocytes, but also the timing when these cells exit the cell cycle, defines the number of cells available for differentiation. Next, the amount of hypertrophic differentiation and the increase in cell volume provide a tool to expand the bones. Last but not least, the pool of round chondrocytes has to be expanded to give rise to the secondary ossification center that is formed postnatally. All of these steps must be tightly controlled to balance elongation and ossification of the skeleton (Wuelling and Vortkamp 2011). In this chapter, we will discuss the main regulatory signaling systems and their downstream transcription factors that control and interconnect proliferation and differentiation of chondrocytes.

8.2 Transcriptional Control of Chondrocyte Differentiation

Mesenchymal cells in the cartilage condensation, the so-called osteochondroprogenitors, have the potential to differentiate into chondrocytes or osteoblasts. As mentioned above, chondrocytes will form in the center of the condensation, whereas the outer cells will initially form the fibroblastic perichondrium and later differentiate into osteoblasts. This decision is regulated by the interaction of the transcription factors Sox9, Runx2, and ß-Catenin.

In the mesenchymal condensation, ß-Catenin, the transcriptional mediator of canonical WNT signaling (see Chap. 10 for details) is co-expressed with Runx2 and Sox9, key regulators of cell differentiation. Chondrocyte differentiation is dependent on the expression of Sox9 and cells lacking Sox9 fail to form chondrocytes (Bi et al. 1999). Once Sox9 expression is induced in the inner cells of the condensation, it leads to the phosphorylation and subsequent degradation of ß-Catenin, whereas the outer cells maintain ß-Catenin expression and remain Sox9 negative (Bhattaram et al. 2014; Day et al. 2005; Topol et al. 2009). Elevated levels of ß-Catenin are required for the differentiation along the osteoblast lineage and mice carrying a deletion of ß-Catenin in the limb mesenchyme fail to develop bone. Instead, the mutants develop ectopic, Sox9 expressing, chondrocytes (Hill et al. 2005). In osteochondroprogenitor cells, expression of Sox9 and ß-Catenin is thus mutually exclusive, directing the cells to either differentiate along the chondrocyte or osteoblast lineage.

Osteoblast differentiation is later initiated by Ihh signals from the hypertrophic cells (see below), which induce high expression of Runx2, a key regulator of the subsequent osteoblast differentiation program in the perichondrium (Razzaque et al. 2005). Similar to the deletion of ß-Catenin, loss of Runx2 leads to a complete lack of osteoblasts in endochondral and intramembranous bones (Komori et al. 1997).

In the future chondrocytes, Sox9 activates the expression of two other members of the Sox transcription factor family, Sox5 and Sox6. These cooperate with Sox9 to establish and maintain the chondrocytic fate. Similar to loss of Sox9, deletion of both, Sox5 and Sox6, prevents chondrogenesis (Smits et al. 2001). Sox family proteins cooperate in the upregulation of structural components of the extracellular chondrocyte matrix, like Collagen type 2 and Aggrecan, and interact to prevent hypertrophic differentiation. Recently, genome-wide sequencing of Sox9/Sox5/Sox6 chromatin binding sites by ChIP-Seq provided evidence that Sox transcription factors cooperatively bind clustered enhancers, so-called super-enhancers, to activate chondrocyte-specific genes (Liu and Lefebvre 2015; Ohba et al. 2015).

Another essential target of Sox9 in the early condensation and in proliferating chondrocytes is the transcription factor Runx2, which is expressed at low levels in these cells (Dy et al. 2012). Runx2 and Sox9 interact on protein level through their conserved DNA-binding domains (Zhou et al. 2006). This interaction prevents binding of Runx2 to target promoters. As Runx2 induces osteoblast and hypertrophic chondrocyte differentiation, this interaction maintains the proliferative chondrocyte state.

During hypertrophic differentiation, chondrocytes start to express elevated levels of Runx2, reexpress ß-Catenin, and shut down the expression of Sox9. Loss of Runx2 and its homolog Runx3 prevents chondrocyte hypertrophy, whereas overexpression of Runx2 induces the differentiation process (Takeda et al. 2001; Yoshida et al. 2004). Ectopic expression of constitutively active ß-Catenin in chondrocytes causes an expansion of the hypertrophic zone, which is, at least in part, due to the upregulation of Runx2 expression (Dong et al. 2006).

As mentioned above, recent investigations demonstrate that hypertrophic chondrocytes can transdifferentiate into osteoblasts. Hypertrophic chondrocytes express several inducers of the osteoblast lineage, like Runx2, ß-Catenin, and Osterix. To facilitate terminal hypertrophic differentiation and presumably transdifferentiation, the Sox9 protein needs to be proteolytically degraded (Hattori et al. 2013). It thus can be speculated that Sox9 is required to prevent a premature cell fate switch into osteoblasts. This hypothesis is supported by the fact that mice deficient for Sox9 in hypertrophic chondrocytes show an increased expression of osteoblast markers in these cells (Dy et al. 2012).

8.3 Extracellular Signaling Factors Regulating Chondrocyte Differentiation

A *plethora* of growth factors have been identified as regulators of cartilage differentiation. Once the chondrogenic lineage is established (for details see Chaps. 4 and 7), the formation of different chondrocyte subpopulations, the proliferation rate, and the hypertrophic differentiation are regulated by several growth factor families, the best investigated being Indian hedgehog (Ihh), Parathyroid hormone-related protein (PTHrP), Bone morphogenetic proteins (BMPs), Fibroblast growth factors (FGFs), and Wingless/Int family proteins (WNTs). Additionally, recent studies identified Insulin-like growth factors (IGFs), CCN family member 2 (Ccn2), and C-natriuretic peptide (Cnp) as essential regulators of chondrogenesis (Fig. 8.3a).

8.3.1 Ihh Signaling

One of the key regulators of endochondral ossification is the secreted growth factor Ihh, which controls different steps of chondrocyte differentiation and synchronizes proliferation and differentiation of chondrocytes with the onset of osteoblast differentiation in the perichondrium (Wuelling and Vortkamp 2011). Ihh is expressed in prehypertrophic chondrocytes and signals to the proliferating chondrocytes and to the flanking perichondrium. Accordingly, the Ihh receptor complex composed of Patched1 (Ptch) and Smoothened (Smo) (see Chap. 9 for details) is mainly expressed in proliferating chondrocytes, the perichondrium, and osteoblasts. Overexpression of Ihh or its receptor in mouse chondrocytes identified Ihh as an activator of chondrocyte proliferation, which upregulates CyclinD1 expression (Long et al. 2001). Correspondingly, loss of Ihh in mice

leads to a reduced proliferation rate and dramatically shortened skeletal elements (St-Jacques et al. 1999). Moreover, Ihh-deficient mice show an accelerated hypertrophy with reduced regions of proliferating columnar cells, whereas over-expression of Ihh in chick or mouse results in increased regions of proliferating chondrocytes. These experiments identified Ihh as negative regulator of hypertrophic differentiation (Long et al. 2001). Molecular analysis revealed that Ihh signals to the round chondrocytes near the joint region, where it activates the expression of PTHrP. PTHrP is a secreted peptide hormone (see below), which signals back to the columnar chondrocytes preventing their mitotic exit and subsequent differentiation into prehypertrophic, Ihh-expressing cells. This negative interaction of the two signaling systems defines the length of the zone of proliferating, columnar chondrocytes (St-Jacques et al. 1999; Chung et al. 2001; Vortkamp et al. 1996).

A third function of Ihh is the initiation of osteoblast differentiation in the perichondrium flanking the prehypertrophic chondrocytes. In these osteoprogenitors, Ihh induces the expression of Runx2, which subsequently induces the transcription factor Osterix and other regulators of osteoblast differentiation (St-Jacques et al. 1999; Rodda and McMahon 2006). Deletion of Ihh signaling in the differentiating osteoblasts demonstrated that Ihh is not required for the differentiation process itself, but provides an inductive signal coupling the onset of chondrocyte hypertrophy to the onset of osteoblast differentiation (St-Jacques et al. 1999; Rodda and McMahon 2006).

Hedgehog signaling is mediated by transcription factors of the Gli family, Gli1, Gli2, and Gli3, which are expressed in proliferating chondrocytes and the perichondrium (Koziel et al. 2005). Dependent on Ihh signaling, Gli2 and Gli3 are proteolytically processed to function as an activator in the presence of Ihh signals or a repressor in the absence of Ihh signals. Gli1 is only expressed in cells receiving an Ihh signal and is thought to reinforce the activator function of the other family members. Its role in chondrocytes is not well understood and likely only minor. Single mutations of either Gli2 or Gli3 lead to mild proliferation defects (Mau et al. 2007; Miao et al. 2004). However, analysis of transgenic mice carrying a combined deletion of Ihh and Gli3 demonstrated that loss of Gli3 rescues the reduced proliferation rate and the accelerated hypertrophy of Ihh mutants. Gli3 seems thus to mainly act as a repressor of Ihh target genes in chondrocytes (Hilton et al. 2005; Koziel et al. 2005). In contrast, loss of Gli2 in Ihh mutants is not sufficient to rescue the Ihh phenotype. Double mutation of Gli2 and Gli3 mimics the Ihh;Gli3 phenotype, indicating that Ihh mainly acts by inhibiting the repressive effects of Gli3, whereas Gli2 does not act as a strong repressor in chondrocytes (Hilton et al. 2005; Kesper et al. 2010). Instead, detailed analysis of Gli2 and Ihh;Gli2 mutants revealed that the Gli2 activator function supports the Ihh-induced proliferation rate and is required for the induction of osteoblast differentiation in the perichondrium, which is not rescued by loss of Gli3 (Joeng and Long 2009; Kesper et al. 2010).

Comprehensive investigation of Gli3-deficient mice and mice overexpressing Ihh revealed that the zone of round chondrocytes is reduced in both mutants,

indicating that the balance between the Gli activator and repressor function defines the size of this chondrocyte population (Chung et al. 2001; Koziel et al. 2005). Interestingly, loss of Trps1, a zinc finger transcription factor of the GATA family that binds to the activator form of Gli3, leads to a similarly reduced zone of round chondrocytes (Wuelling et al. 2009). The mechanisms by which these factors act on a molecular level are not fully understood, yet.

8.3.2 PTHrP Signaling

As explained above, PTHrP interacts with Ihh to inhibit the onset of hypertrophic differentiation. PTHrP is expressed in round chondrocytes, while its receptor, the PTH/PTHrP receptor (Ppr1), is expressed at low levels in columnar and at high levels in prehypertrophic chondrocytes. Deletion of PTHrP or Ppr1 in mice leads to a reduced chondrocyte proliferation rate and an accelerated onset of hypertrophy (Karaplis et al. 1994; Lanske et al. 1996; for review: Kronenberg 2006), whereas constitutive activation of Ppr1 or treatment of limb explant cultures with PTHrP results in increased proliferation and an expansion of the zone of columnar chondrocytes due to a delayed onset of hypertrophy (Schipani et al. 1997). A similar phenotype is found in human patients with Jansen metaphyseal dysplasia, which results from an activating mutation in Ppr1 (Schipani et al. 1999).

Binding of PTHrP to Ppr1 activates different G-proteins, $G\alpha_s$, $G\alpha_q$, and $G\alpha_{11}$. Intracellularly, $G\alpha_q$ and $G\alpha_{11}$ activate Protein kinase C and calcium signaling to oppose $G\alpha_s$ function. $G\alpha_s$ signals induce adenylate cyclase-dependent cAMP production, which activates Protein kinase A (PKA) (Guo et al. 2002; Kronenberg 2006). Activation of PKA has several biological consequences: first it results in the activation of Sox9, which is phosphorylated at distinct serine residues, resulting in increased Col2 expression and repression of the hypertrophy-inducing factor Runx2 (Huang et al. 2001).

Additionally, PKA activates Protein phosphatase 2a (PP2a), which dephosphorylates the Histone deacetylase 4 (Hdac4). Dephosphorylated Hdac4 translocates to the nucleus, where it binds to and inhibits the activity of Mef2c, a transcription factor of the Myocyte enhancer factor family, which induces chondrocyte hypertrophy by activating Runx2 expression (Kozhemyakina et al. 2009). Loss of PTHrP or Ppr1 in the respective mutants would then result in decreased PKA-dependent PP2a levels. Consequently, Hdac4 remains phosphorylated, and the inhibition of Mef2c by Hdac4 is released, leading to the observed accelerated onset of hypertrophy.

Besides the PKA-dependent effects, PTHrP activates the expression of at least one other transcription factor, Zfp521, which is preferentially expressed in prehypertrophic chondrocytes. Mice in which Zfp521 is deleted in chondrocytes resemble PTHrP-deficient mice. Zfp521 has been shown to interact with and stabilize Hdac4, thereby augmenting the repressive effect of Hdac4 on Mef2c-dependent Runx2 expression (Correa et al. 2010).

In addition to the effect of PTHrP on chondrocyte differentiation, PTHrP induces chondrocyte proliferation by directly acting on cell cycle regulators. PTHrP

activates the expression of CyclinD1 and CyclinA by activation of the cAMP response element-binding protein (Creb) (Ionescu et al. 2001) and Activating transcription factor 3 (Atf3) (James et al. 2006). Atf3 directly binds to the CRE in the CyclinD1 and CyclinA promoter, thereby activating chondrocyte proliferation (Beier 2005; Beier et al. 1999). Cyclins activate the progression from G1 to S-phase by association with Cdks, which phosphorylate pRB family proteins including p107 and p130. Phosphorylation separates pRB from E2F transcription factors, which in turn induce cell cycle progression. Besides its role as a cell cycle regulator, CyclinD1, in a complex with Cdk4, phosphorylates Runx2 leading to its degradation and inactivation (Zhang et al. 2009; Shen et al. 2006).

Recent studies identified an additional function of $G\alpha_s$ in the maintenance of the pool of round chondrocytes. The deletion of this subunit leads to an increased proliferation and differentiation of round into columnar chondrocytes (Chagin and Kronenberg 2014). If $G\alpha_s$ acts independent of Ihh/Gli3 in these cells or mediates, their function remains to be addressed.

8.3.3 FGF and Cnp Signaling

Several FGF proteins and their receptors are expressed in distinct chondrocyte populations: FGF receptor 1 (Fgfr1) is mainly found in round chondrocytes, Fgfr2 in hypertrophic chondrocytes and the bone, and Fgfr3 in proliferating and prehypertrophic chondrocytes (Fig. 8.2). Studies of human patients identified mutations in all three Fgfrs as cause for skeletal malformations. Mutation of Fgfr1 and Fgfr3 results in craniosynostosis, while inactivation of Fgfr1 or Fgfr2 disturbs chondrocyte hypertrophy and osteoblast formation (for details on FGF signaling, see Ornitz and Marie 2015). Gain of function mutations in Fgfr3 are the cause for hypochondroplasia, achondroplasia. and thanatophoric dysplasia (Shiang et al. 1994; Tavormina et al. 1995; Bellus et al. 1995). These chondrodysplasia phenotypes are characterized by mild to severe shortening of endochondral bones, especially of the long bones. Molecular analysis revealed that the severity of the phenotype is dependent on the degree of receptor activation (Naski et al. 1996). Mice carrying the human achondroplasia mutation in Fgfr3 show decreased chondrocyte proliferation rates and a reduced region of hypertrophic chondrocytes (Naski and Ornitz 1998; Li et al. 1999). In contrast, loss of Fgfr3 increases the region of proliferating and hypertrophic chondrocytes (Colvin et al. 1996; Deng et al. 1996). Therefore, Fgfr3 signaling represses the proliferation of columnar chondrocytes and diminishes the zone of hypertrophic chondrocytes.

While several FGFs are expressed in the developing bone, deletion studies in mice identified Fgf9 and Fgf18 as the critical ligands in endochondral bones. Both proteins are expressed in the perichondrium and periosteum (Fig. 8.2). Loss of either FGF results in increased regions of proliferating and hypertrophic chondrocytes, mimicking the phenotype of Fgfr3-deficient mice. In addition, the Fgf9 and Fgf18 mutants display distinct ossification defects that are dependent on Fgfr1 and Fgfr2 signaling (Hung et al. 2007; Liu et al. 2007; Ohbayashi et al. 2002).

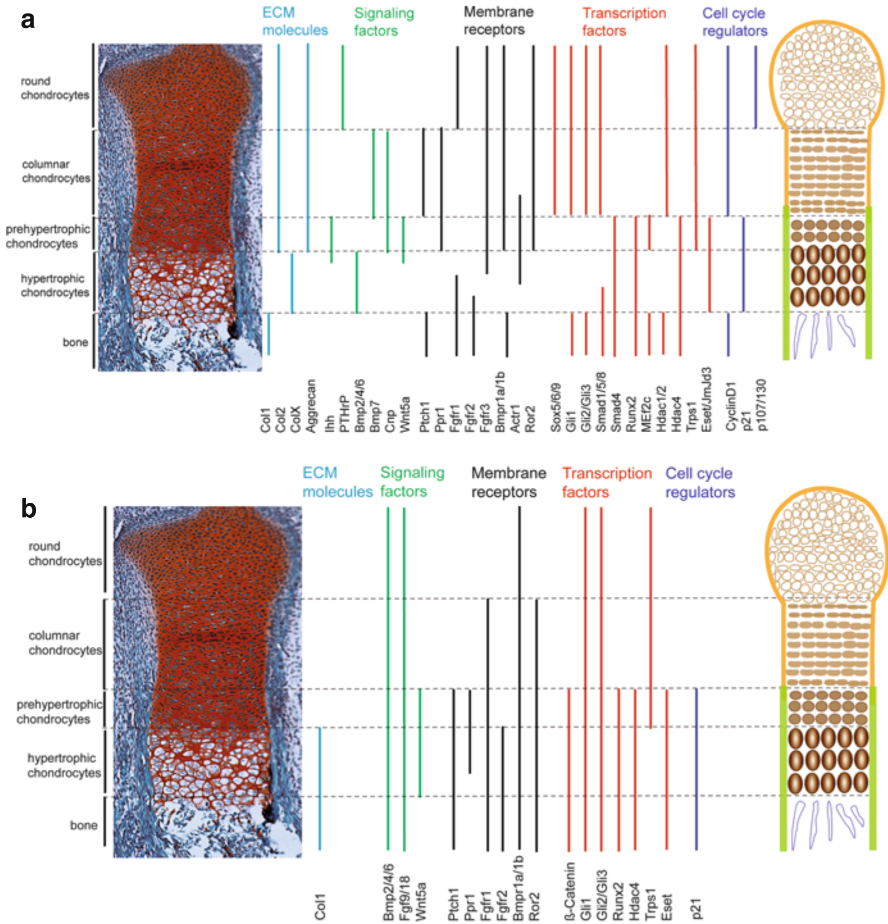

Fig. 8.2 Gene expression in chondrocytes. Distinct chondrocyte populations express different regulators of chondrocyte differentiation as outlined in the text. Safranin-Weigert staining of a longitudinal section of the ulna from a 16.5 dpc mouse embryo shows the morphological feature of the different cell types. (**a**) Genes expressed in chondrocytes. (**b**) Gene expression in the perichondrium and periosteum. *Bars* represent the length of the respective expression domain. *Dotted lines* demarcate the borders of the different chondrocyte populations

The anti-proliferative effect observed in chondrocytes contradicts the effect of FGFs in other cell types, where they function as a mitogens. Exchanging the intracellular domain of Fgfr3 and Fgfr1 revealed that the anti-proliferative function is not dependent on the receptor subtype, but an intrinsic reaction of the proliferating chondrocytes (Wang et al. 2001). How and when the response of the cell changes is not completely clear. It seems, however, to be linked to the establishment of the embryonic growth plate as at the earliest cartilage differentiation stages, FGF signals support chondrogenesis and proliferation (Yu and Ornitz 2008).

In proliferating chondrocytes, FGF signaling leads to the activation of different intracellular signaling pathways including the activation of Stat1, PP2a, and the MAP kinase pathway. Interestingly, chondrocyte proliferation and hypertrophic differentiation seem to be controlled by independent mechanisms. FGF treatment has been shown to activate PP2a to dephosphorylate p107, which then inhibits cell cycle progression of chondrocytes (Kolupaeva et al. 2008, 2013). Surprisingly, this effect has not been observed downstream of PTHrP, which seems to activate PP2a leading to an increased proliferation (Kozhemyakina et al. 2009). If this discrepancy depends on differences in the composition of the catalytic PP2a complex needs to be addressed in detailed molecular studies.

Besides regulating PP2a, FGF signaling activates the transcription factor Stat1 (Sahni et al. 2001), which subsequently activates the expression of the CyclinD/Cdk inhibitor p21 (de Frutos et al. 2007). A direct inhibition of cell cycle regulators has also been observed in chondrocyte cell lines, in which FGF treatment leads to reduced CyclinB/Cdk1 activity, main regulators of mitotic entry (Tran et al. 2010). FGF signaling thus seems to regulate not only G1-/S-phase progression but also mitotic division, to control cell cycle progression in chondrocytes.

The impact of FGF function on hypertrophic chondrocytes is mainly mediated by activation of the MAP kinase pathway. Mouse mutants expressing constitutive active Mek1 (caMEK; mitogen-activated protein kinase/Erk kinase1) develop an achondroplasia-like phenotype with premature hypertrophy. Introducing the caMEK1 allele into Fgfr3-deficient mice rescued their skeletal overgrowth by accelerating the delayed onset of hypertrophy. Furthermore, whereas loss of Stat1 rescues the proliferation defect of achondroplasia mice, it does not rescue the phenotype of caMEK mutants supporting the hypothesis that Stat1 regulates proliferation, while and MAP kinase controls hypertrophic differentiation downstream of Fgfr3 (Murakami et al. 2004).

Importantly, FGF signaling has been identified as an essential regulator of Ihh expression. Ihh expression is downregulated in mice carrying an activated Fgfr3 mutation (Liu et al. 2007; Naski and Ornitz 1998). This effect is likely mediated via p38 and Erk1/Erk2 signaling, which stimulates the expression of Ihh in vitro (Lai et al. 2005). The reduced domain of proliferating chondrocytes observed in the achondroplasia mice might thus be due to the reduced Ihh and subsequent PTHrP expression. Accordingly, treatment with PTH or PTHrP rescues the reduced zone of proliferating chondrocytes in these mutants further supporting the epistatic interaction of the pathways (Xie et al. 2012).

Another signaling factor which rescues the consequences of activated FGF signaling is the C-type natriuretic peptide (Cnp). Cnp is expressed at high levels in chondrocytes and deletion of Cnp, or the natriuretic peptide receptor 2 (Nrp2) leads to shortened skeletal elements, while Cnp treatment induces an expansion of the zone of proliferating and hypertrophic chondrocytes (Yasoda et al. 1998). Overexpression of Cnp in chondrocyte rescues the achondroplasia phenotype in constitutive active Fgfr3 mutants by inhibition of MAPK signaling (Yasoda et al. 2004). Recently, a stabilized form of Cnp, BMN111, has been approved for clinical

trials on human achondroplasia patients (Lorget et al. 2012; Wendt et al. 2015; Klag and Horton 2015).

8.3.4 BMP Signaling

Bone morphogenetic proteins (BMPs) belong to the Transforming growth factor ß (TGFß) family of secreted proteins. BMPs have originally been identified by their bone inductive function when overexpressed or exogenously applied to connective tissues (Urist 1965). In vivo several BMP proteins, antagonists, and their respective receptors are expressed in overlapping domains in the developing skeleton and have numerous roles in different chondrocyte populations (Fig. 8.2). Overexpression of BMPs, activated BMP receptors, or inactivation of the Bmp inhibitor Noggin in early limb mesenchyme or cell cultures results in ectopic or overgrown cartilage structures, whereas inhibiting BMP signaling impedes cartilage formation (Brunet et al. 1998; Capdevila and Johnson 1998; Duprez et al. 1996; Kawakami et al. 1996; Yoon and Lyons 2004; Zou et al. 1997). Importantly, in the early condensation, BMP signaling is required for the expression of Sox9, Sox5, and Sox6 and thereby induces chondrocyte formation (Gao et al. 2013). At post-condensation stages, activation of BMP signaling in limb explant cultures induces chondrocyte proliferation and hypertrophic differentiation (Kobayashi et al. 2005; Minina et al. 2001).

Binding of BMP induces the dimerization of two types of the serine-threonine receptors, the TGFß/BMP receptors type I and type II, which activate transcription factors of the Smad family. While Smad2 and Smad3 are the main receptor-activated Smads downstream of TGFß signaling, BMP induces phosphorylation and activation of Smad1, Smad5, and Smad8. Subsequently, the activated Smads form complexes with Smad4 and enter the nucleus to regulate target gene expression (Yoon and Lyons 2004).

Inactivating single components of the BMP signaling system, like single BMPs, BMP receptors (Bmpr), or Smads, in the developing mouse skeleton leads to mild, but distinct, chondrodysplasia phenotypes (Yoon and Lyons 2004). The combined loss of the type I receptors, Bmpr1a and Bmpr1b or the combined deletion of Smad1, Smad5, and Smad8 results in the absence of endochondral bones (Retting et al. 2009). These phenotypes demonstrate that BMP signaling is required to induce chondrocyte differentiation in endochondral bones, but that single components have a highly redundant function in this process. Surprisingly, chondrocyte-specific deletion of the common activator, Smad4, leads only to mild alterations of chondrocyte differentiation including an expansion of the zone of round chondrocytes in expense of columnar chondrocytes, a reduced proliferation rate, and ectopic hypertrophic differentiation (Zhang et al. 2005). These data challenge the view that Smad4 acts as the general mediator of BMP signaling and prompted the search for Smad4-independent signaling pathways in chondrocytes.

One pathway discussed is the MAP kinase pathway, which seems to boost Smad function. BMP signaling has been shown to activate p38 through activation of TGFß-activated kinase 1 (Tak1). Tak1 phosphorylates Smad1/Smad5/Smad8 in

prehypertrophic and hypertrophic chondrocytes to amplify their activity toward Ihh (see below) and other Smad target genes (Greenblatt et al. 2010).

While receptor-activated Smad1, Smad5, and Smad8 are expressed throughout the growth plate, inhibitory Smads, Smad6 and Smad7, are expressed in prehypertrophic and hypertrophic chondrocytes (Sakou et al. 1999). Overexpression of Smad6 in chondrocytes reduced phosphorylation of Smad1/Smad5/Smad8, thereby delaying chondrocyte hypertrophy (Horiki et al. 2004).

A novel regulator of the BMP pathway is the membrane protein Neogenin. Neogenin binds to BMP proteins and Bmpr1a (Hagihara et al. 2011) and regulates hypertrophic differentiation by facilitating Smad1/Smad5/Smad8 phosphorylation without activating p38 kinase (Zhou et al. 2010).

BMP signaling through Smad1/Smad5/Smad8 is also essential for the activation of Ihh expression, leading to an expanded domain of columnar chondrocytes. Accordingly, Ihh expression is lost in Smad1-/Smad5-/Smad8-deficient mice (Retting et al. 2009). The effect of BMP signaling on chondrocyte proliferation and Ihh expression seems thus to oppose the function of FGF signaling. This hypothesis is supported by experiments demonstrating that BMP treatment rescues chondrocyte proliferation and Ihh expression in mice carrying an activated Fgfr3 mutation at least in explant cultures (Kobayashi et al. 2005; Minina et al. 2002; Yoon and Lyons 2004). Furthermore, FGF signals oppose the induction of Ihh expression downstream of BMPs by phosphorylation of Smads by Erk1/Erk2, identifying Smad proteins as an interface between the BMP and FGF signaling pathways (Retting et al. 2009).

An additional cross-talk exists between BMP and PTHrP signaling in reducing Runx2 levels. While PTHrP represses Runx2 expression, BMP stabilizes Runx2 protein in prehypertrophic chondrocytes by inhibiting the formation of CyclinD/Cdk4 complexes, which target Runx2 for degradation (Shu et al. 2011). These data not only support multiple functions of BMPs at different steps of chondrocyte differentiation but also the complex interplay between the signaling systems.

Besides Bmpr1 and Bmpr2, the Activin receptor type I (Acvr1) is expressed in chondrocytes and can be activated by BMP signals. Loss of Acvr1 in chondrocytes leads to malformations of the skull and the vertebral column indicating receptor-dependent differences in BMP responses (Rigueur et al. 2015). Moreover, mutations in Acvr1 have been identified as the molecular basis of human fibrodysplasia ossificans progressiva, which is characterized by heterotopic ossification of skeletal muscle and soft tissue (Hatsell et al. 2015).

8.3.5 CCN/CTGF and IGF Signaling

CCN family member 2 (Ccn2 or CTGF) is a secreted protein expressed in prehypertrophic chondrocytes. In vitro, Ccn2 increases proliferation and hypertrophic differentiation of chondrocytes (Nakanishi et al. 2000). In vivo, overexpression of Ccn2 has no obvious effect during embryogenesis, but bone length is increased in adult mice (Tomita et al. 2013). Deletion of Ccn2 reduces chondrocyte

proliferation, while Ihh and Collagen type X expression is not obviously altered in embryonic chondrocytes (Kawaki et al. 2011). Interestingly, the expression of Aggrecan and other structural ECM proteins is reduced in the Ccn2 mutants, indicating that the availability of growth factors is altered in these mutants (Ivkovic et al. 2003). While Ccn2 has been identified as a ligand of the low-density lipoprotein receptor 1 (Lrp1), recent data provides evidence that it also binds to Fgfr2 and Fgfr3 in chondrocytes. In vitro phosphorylation of Erk1/Erk2 was increased by cooperative binding of Ccn2 and FGF proteins to the FGF receptors (Aoyama et al. 2012). The increased hypertrophic differentiation after Ccn2 treatment in chondrocytes might thus be a consequence of enhanced FGF signaling, but this needs to be verified by in vivo analysis of the molecular cross-talk of both pathways.

Overexpression of Ccn2 also increases the expression of Insulin-like growth factor 1 and 2 (Igf1, 2). Whereas IGFs were initially believed to function as paracrine signals during skeletal development, the lack of skeletal defects in mouse mutants carrying a deletion of IGF in hepatic cells, the main producers of circulating IGF, provided first evidence for an autocrine function of IGFs in the skeletal system. Igf1 is expressed in osteoblasts. In chondrocytes, Igf2 and Igf receptor 2 (Igf2r) expression levels are high in proliferating and low in hypertrophic chondrocytes, while Igf1r shows reciprocal expression, low in proliferating and high in prehypertrophic chondrocytes (Wang et al. 1995). Deletion of Igf1, Igf2, or Igf1r in murine chondrocytes reduces the size of the skeletal elements, due to a reduce proliferation rate and a delayed onset of hypertrophy (Liu et al. 1993; Wang et al. 2011). Postnatal deletion of Igf1r in chondrocytes increases PTHrP expression, which might account for the delayed hypertrophy. However, Ihh expression was not affected by loss of IGF signaling (Wang et al. 2011), and combined deletion of Igf1r and the Ihh receptor Smo indicates that both signaling pathways independently regulate skeletal growth (Long et al. 2006).

8.3.6 WNT Signaling

As mentioned previously, canonical WNT signaling mediated by ß-Catenin is essential for the differentiation of osteo-chondroprogenitor cells in the early condensation. In addition, it supports hypertrophic differentiation and possibly the transdifferentiation of chondrocytes into osteoblasts (see Chap. 10 for details).

Noncanonical WNT signaling, which activates the WNT/calcium and planar cell polarity (PCP) pathway, has additional functions during chondrogenesis (Bradley and Drissi 2010). Wnt5a is expressed in prehypertrophic chondrocytes overlapping with Ihh expression and in the periosteum. Wnt5a-deficient mice develop shorter limbs due to a reduced proliferation rate and loss of hypertrophic chondrocytes. Overexpression of Wnt5a results in an expanded zone of round chondrocytes, decreased proliferation, delayed hypertrophy, and loss of Ihh expression in prehypertrophic chondrocytes (Yang et al. 2003). Additionally, in columnar chondrocytes noncanonical WNT signaling through the WNT receptor frizzled7 (Fzd7) and the co-receptor Ror2 organizes the alignment of columnar chondrocytes by directing the

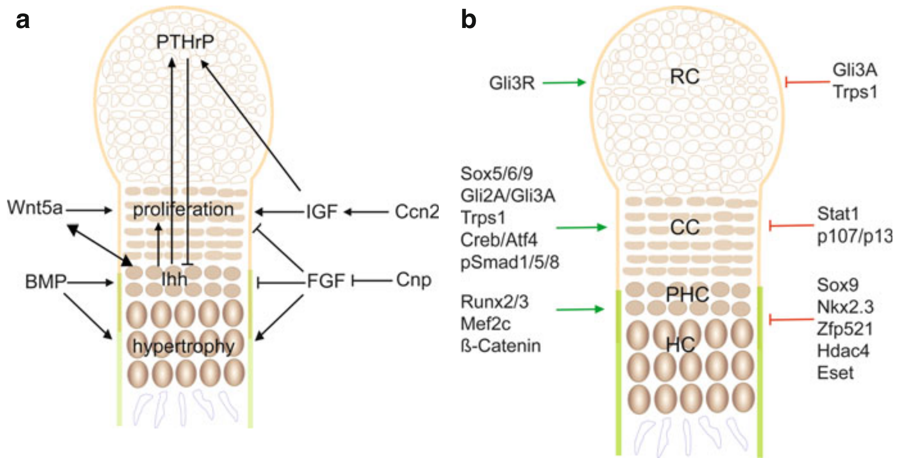

Fig. 8.3 (**a**) Signaling mechanisms orchestrating proliferation and differentiation of chondrocytes. Ihh is expressed in prehypertrophic chondrocytes and activates proliferation of columnar chondrocytes. In round chondrocytes, Ihh induces the expression of PTHrP, which signals back to inhibit hypertrophic differentiation of columnar chondrocytes. Ihh expression is negatively regulated by FGF signaling and activated by BMP signaling. In parallel, BMP and FGF signals regulate proliferation and hypertrophy independent of Ihh. Cnp inhibits FGF function in proliferating and hypertrophic chondrocytes. Wnt5a and Ihh interact in a positive feedback loop to activate each other. Ccn2 activates chondrocyte proliferation and PThrP expression via activation of IGF signaling independent of Ihh. (**b**) Transcription factors and epigenetic modifiers either activate (*left*) or inhibit (*right*) distinct differentiation steps in chondrocytes. *RC* round chondrocytes, *CC* columnar chondrocytes, *PHC* prehypertrophic chondrocytes, *HC* hypertrophic chondrocytes

plane of cell division relative to the longitudinal axis of the bone (Gao et al. 2011; Li and Dudley 2009). Balanced Wnt5a levels are thus necessary for the coordinated differentiation of chondrocytes along the axis of the skeletal elements, and both, deficiency and ectopic expression of Wnt5a, interfere with this balance.

Moreover, Wnt5a induces the proteolytic degradation of the transcription factor Nk3 homeobox2 (Nkx3.2). Nkx3.2 is expressed in chondro-progenitors and proliferating chondrocytes and inhibits chondrocyte hypertrophy by repression of Runx2 (Provot et al. 2006). Nkx3.2 levels are elevated in Ihh-deficient mice, and downregulation of Wnt5a mediates Nkx3.2 degradation in vitro (Choi et al. 2012), again showing the complex cross-talk between different signaling pathways in the developing growth plate (Fig. 8.3a).

8.4 Epigenetic Mechanisms Regulating Bone Growth

In the last decade, genetic analysis gene expression and cell lineage differentiation revealed a substantial impact of the chromatin organization. Epigenetic chromatin modifications include the acetylation and methylation of histone proteins, which change the chromatin conformation to provide a platform for specific transcription

factor binding. Histone acetylation by Histone acetyltransferases (Hats) opens the chromatin structure, while deacetylation by Histone deacetylases (Hdacs) and subsequent methylation by Histone methyltransferases (Hmts) leads to gene silencing. Several histone-modifying enzymes are expressed during endochondral ossification and genetic inactivation of single genes or inhibition of different Hdac classes using small-molecule inhibitors resulted in severe skeletal malformations based on defects in chondro- and osteogenesis (Paradis and Hales 2015; reviewed in Bradley et al. 2015).

Although the comprehensive analysis of epigenetic changes has only been initiated recently, several results support their importance for chondrocyte differentiation. During chondrogenesis histone-modifying enzymes interact with various master transcription factors, including Sox9, Smad1, and Runx2. In the cartilage condensation, BMP signaling induces Sox9 and Fgfr3 expression by recruiting p300 to their promoter regions (Pan et al. 2009; Sun et al. 2009). Furthermore, Sox9 and p300 cooperatively bind to and induce hyperacetylation and activation of the Col lagene type 2 promoter (Furumatsu et al. 2005). Surprisingly, although histone deacetylases and methyltransferase are thought to transfer repressive marks, the Hdac Sirt1 and the methyltransferases Set7/Set9 also interact with the histone acetyltransferase p300 on the Col lagen type 2 promoter in human chondrocytes, resulting in increased H3K4 trimethylation and H3K9 and H4K16 acetylation and increased Col lagen type 2 expression (Oppenheimer et al. 2014).

Besides such specific effects on single promoters, recent experiments revealed that the transcription factor Trps1 binds to Hdac1 and Hdac4 thereby activating the global histone deacetylation activity (Wuelling et al. 2013). While the disturbed mitotic progression observed in Trps1 mutants seems to be due to the global increase in acetylation, it is not clear if other aspects of the Trps1 phenotype, like the disturbed onset of hypertrophic differentiation, are also dependent on overall changes in chromatin acetylation or if Trps1 directly acts on promoters of key regulators. It is interesting to note that Trps1 regulates Runx2 expression (Napierala et al. 2008). If this repression is mediated by epigenetic changes or if Trps1 interaction stabilizes the repressive Hdac4/Mefc2 complexes (see above) has not been investigated.

Recent data also provide new insight into the role of Hmts in chondrocytes. Several Hmts including Eset, G9a Setdb1, Prdm2, and Suv39 H1 and H2 as well as their target, methylated H3K9, are detected at higher levels in prehypertrophic and hypertrophic chondrocytes (Ideno et al. 2013). Inactivation of Eset in mice disturbs growth plate formation, leading to an accelerated onset of hypertrophic differentiation (Ideno et al. 2013; Yang et al. 2013). Eset interacts with Hdac4 to repress Runx2 activity, which might account for the accelerated hypertrophic differentiation in Eset mutants. Eset mutants also show an extended domain of Collagen type 10 expression, while Ihh levels are reduced indicating that histone methylation independently controls Ihh expression and hypertrophic differentiation (Yang et al. 2013). Interestingly, the demethylase Jmjd3 also interacts with Runx2, and deletion of Jmjd3 leads to reduced proliferation and a reduced zone of hypertrophic chondrocytes (Zhao et al. 2015). However, the functional consequences of the interaction of Runx2 with several histone-modifying enzymes need to be deciphered in more detail.

Conclusion

The formation of endochondral bones is coordinated by a complex network of signaling systems, transcription factors, and epigenetic modifiers. These have to be tightly controlled to organize chondrocyte condensation, proliferation, and the differentiation of osteo-chondroprogenitor cells into chondrocytes and osteoblasts. Furthermore, the single control systems interact at different levels to coordinate distinct differentiation steps. While many single factors have been identified based on phenotypes in man and mouse, their interaction is far less understood. One critical aspect of their interaction seems to be the maintenance of the chondrocytic fate. This is determined by Sox9, and different signaling systems interact to maintain Sox9 expression. Another step is the decision between cell cycle progression and cell cycle exit, and most signals like Ihh, PTHrP, BMPs, and FGFs act on different components of the cell cycle to drive or inhibit proliferation (Fig. 8.3a). Cell cycle exit and hypertrophic differentiation seem to be dependent on the level of Runx2 as the action of various signals either inhibit (PTHrP, FGF, Wnt5a) or support (BMP) its expression and stability. In addition, the domain of proliferating chondrocytes is determined by the negative feedback interaction between Ihh and PTHrP, with PTHrP levels being the measure for the initiation of hypertrophy. Interestingly, several signaling systems like FGF and BMP act on Ihh expression and proliferation, thereby coupling the proliferation rate to the size of the domain of proliferating cells (Fig. 8.3a).

Downstream of these signaling network, transcription factors interact to translate the information into specific gene expression changes (Fig. 8.3b). We are, however, just beginning to understand the underlying molecular mechanisms in detail. Moreover, the function of histone-modifying enzymes and their interaction with chondrocyte-specific transcription factors add further complexity to the regulation of cell differentiation. A comprehensive, combined analysis of both systems is thus required to fully understand how extracellular signals regulate chondrocyte differentiation on transcriptional level.

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Hedgehog Signaling in Chondrocytes

9

Kay Grobe

Abstract

The skeleton develops from a densely packed, avascular mesenchyme, called the skeletal blastema. Chondrogenesis from this mesenchyme requires a balance between negative and positive maturational factors during initial chondrocyte proliferation and differentiation, as well as during postnatal chondrocyte development and homeostasis. Accurate regulation of this developmental program is crucial for the ultimate size of skeletal elements, as premature or delayed maturation often results in their severe shortening. One essential group of regulators of chondrogenesis comprises members of the Hedgehog (Hh) morphogen family. Hh's act as long-range morphogens during chondrocyte development and endochondral ossification. Mutations in Hh effectors, receptors, and co-receptors, as well as in ciliary proteins that act as modulators of Hh reception, result in skeletal and craniofacial deformities. In addition to their essential roles in chondrogenesis, both Sonic Hh and Indian Hh family members serve as crucial regulators of endochondral ossification, a process in which calcified hypertrophic cartilage is resorbed and replaced by bone. Finally, dysregulated Hh signaling contributes to cartilage and bone pathologies in the adult. This chapter summarizes the current understanding of Hh production and signaling in chondrocytes in development and disease.

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9.1 Hedgehog Functions in Chondrocyte Biology

Two distinct processes form vertebrate skeletons during development: intramembranous and endochondral ossification. Intramembranous bones are directly formed by specialized, terminally differentiated mesenchymal stem cells called osteoblasts. Osteoblasts synthesize densely cross-linked collagen and specialized proteins, including osteocalcin and osteopontin, to form the basis of the cranial vault, some facial bones, and parts of the mandible and clavicle. Endochondral ossification that forms the rest of the skeleton during development, in contrast, relies on the replacement of a cartilaginous template with bone (Yoshida et al. 2004). As a first step in this process, the cartilaginous template (or cartilage anlage) generated from mesenchymal progenitors expresses collagens I, III, and V as a result of mesenchymal cell condensation and chondroprogenitor cell differentiation, called chondrogenesis (Goldring et al. 2006). Fibroblast growth factor 8 and Sonic hedgehog (Shh) (Kmita et al. 2005) are two essential modulators of cell proliferation within this cartilage template (Hall and Miyake 2000), and bone morphogenetic protein (BMP; most BMPs are transforming growth factor beta family members) signaling contributes to the formation of precartilaginous condensations and subsequent chondrocyte differentiation (Yoon et al. 2005). The BMP antagonist, Noggin, further permits precartilage cell differentiation into chondrocytes (Yoon and Lyons 2004; Pizette and Niswander 2000). This process is marked by the expression of cartilage-specific collagens II, IX, and XI. The proliferation of these cells requires Indian hedgehog (Ihh) signaling in parallel with BMP function or BMP signaling acting as a modulator of Ihh function (Minina et al. 2001; Vortkamp 2001). Finally, cells undergo terminal differentiation, or chondrocyte hypertrophy, and apoptosis in the intervening interzone. During chondrocyte hypertrophy, there is a notable increase in cell size, up to 20-fold of its initial resting size. The hypertrophic zone is further characterized by the expression of collagen X and alkaline phosphatase and the subsequent calcification of the matrix (St-Jacques et al. 1999). This process involves matrix remodeling by matrix metalloprotease (MMP)-9, MMP-13, and MMP-14 and vascularization mediated by vascular endothelial growth factor (VEGF) activity. In this process, the hypertrophic cartilage is finally replaced by bone, except for resting chondrocytes embedded in a dense extracellular matrix (ECM) lacking blood vessels, nerves, or lymphatics at the ends of (opposing) bones (called articular cartilage).

A similar sequence of chondrocyte proliferation and differentiation occurs in the postnatal growth plate, leading to rapid growth of the skeleton (Onyekwelu et al. 2009). At birth, the articular cartilage of many joints in humans and mice is still indistinguishable from the epiphyseal growth plate. Soon after birth, however, a secondary ossification center appears within the epiphyseal cartilage, dividing it into the future metaphyseal growth plate proximally and the articular surface distally (for further details, see Chap. 4). Ihh that is still produced in the metaphyseal growth plate directly and indirectly induces parathyroid hormone-related peptide (PTHrP) expression in periarticular resting zone chondrocytes (Karaplis et al. 1994; Lanske et al. 1996; Vortkamp et al. 1996; Chung et al. 2001; Kronenberg 2006) (Fig. 9.1). PTHrP, in turn, induces continued proliferation and inhibits the

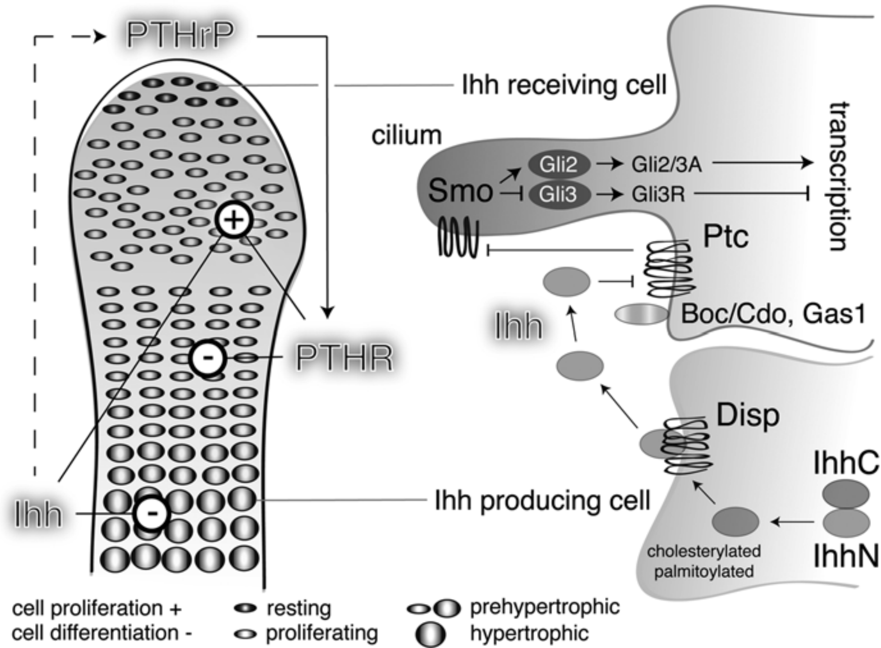

Fig. 9.1 Overview of bone development and Hh signaling. *Left*: Ihh and PTHrP/PTHR regulate chondrocyte proliferation and differentiation during endochondral bone formation. PTHrP is synthesized by resting chondrocytes and perichondrial cells. Secreted PTHrP diffuses toward the prehypertrophic zone, where it binds to and activates its receptor, PTHR. PTHR activity maintains chondrocyte proliferation (+) and delays chondrocyte differentiation (–) into prehypertrophic and hypertrophic chondrocytes. After chondrocytes stop proliferating at the transition from a proliferating into a hypertrophic phenotype, they start to synthesize Ihh, which indirectly increases the synthesis of PTHrP. Ihh and PTHrP thus participate in a negative feedback loop that regulates the proliferation rate of growth plate chondrocytes. Besides increasing PTHrP synthesis, Ihh also stimulates the proliferation of chondrocytes and directly inhibits their terminal differentiation. *PTHrP* parathyroid hormone-related protein, *Ihh* Indian Hedgehog, *PTHR* parathyroid hormone receptor. *Right*: The Ihh ligand in producing cells undergoes a series of autoprocessing/lipidation reactions that result in its secretion in multimeric, dual-lipidated form and its firm tethering to the cell surface. All Hh family members are then released from the cell surface via the activity of the 12-span transmembrane protein Dispatched (*Disp*). Ihh binding to the receptor Patched (*Ptc*) on the receiving cell releases the 7-pass transmembrane protein Smo from constitutive inhibition, allowing for Smoothened (*Smo*) translocation to the primary cilium. This activates glioblastoma (Gli)2/Gli3 transcription factors (Gli2/3A) and inhibits the generation of Gli3 repressors (Gli3R). In the presence of Hh, the co-receptors Boc, Cdo, and Gas1 assist in the release of Smo from Ptc inhibition and thereby contribute to Hh pathway activity

progression to maturation in proliferating and prehypertrophic chondrocytes. This process, in turn, maintains the length of chondrocyte columns and thus the architecture of the epiphyseal growth plate. In addition, Ihh acts independently of PTHrP on the periarticular chondrocytes and regulates the differentiation of columnar chondrocytes within the proliferative zone (Kobayashi et al. 2005). In postnatal joints,

PTHrP and Ihh remain expressed in a zone-specific manner (Onyekwelu et al. 2009), potentially regulating mineralization by chondrocytes at the osteochondral interface of the immature joint (Jiang et al. 2008). Here, Ihh expression is particularly strong in chondrocytes at the articular surface, indicating a role in resisting chondrocyte hypertrophy, mineralization, and/or ossification.

Local chondrocytes in the postnatal skeleton are also influenced through endocrine hormones (particularly thyroid hormone and estrogen). Ihh signaling is further known to induce BMP-4, a mitogenic factor, in a PTHrP-independent manner. Notably, this induction depends on mechanical stimulation (Wu et al. 2001). Cyclic mechanical stress induces Ihh expression in chondrocytes; gadolinium, an inhibitor of stretch-activated channels, inhibits Ihh induction. This suggests that the Ihh gene is mechanoresponsive because of the involvement of primary cilia in Hh signaling (discussed in more detail below). Finally, functional interactions between Ihh and the Wingless/Wnt pathways regulate cartilage growth plate control and joint segmentation (Spater et al. 2006). Loss of activity of Wnt9a (a secreted signaling molecule) transiently downregulates Ihh expression and reduces Ihh signaling activity in prehypertrophic chondrocytes; in vivo chromatin immunoprecipitation revealed a direct interaction between the β-catenin/lymphoid enhancer-binding factor 1 (constituting part of the Wnt receptor/signal transduction complex) and the Ihh promoter. Another report demonstrated that Ihh, Wnt5b, and Wnt11 control chondrogenesis in parallel pathways (Church et al. 2002) and that Ihh can cause parallel inhibition of Lrp (Wnt co-receptor) and Sfrp (Wnt antagonist) in chondrocytes (Choi et al. 2012). The conclusion that Wnt5a signaling in the prehypertrophic zone of the cartilage growth plate may be increased, however, is not supported because of the unchanged Wnt5a levels in Ihh mutant mice in vivo (St-Jacques et al. 1999; Long et al. 2001).

9.2 Hedgehog Morphogen Production and Reception

Vertebrates produce three structurally and functionally closely related Hh's (Sonic Hh (Shh), Indian Hh (Ihh), and Desert Hh). Of these, the function of Shh has been best characterized (reviewed by McMahon et al. 2003), including its role in the development of the head process and in the development of limbs: limb budding, anterior/posterior patterning of the limb skeleton, and specification of vertebrate digit identities (Capdevila and Johnson 2000). Shh expressed in the forebrain also mediates the development of the mid- and upper face, the frontonasal process, and the maxillary processes (Byrnes et al. 2009). Dysregulation of the Shh pathway therefore results in a wide and complex array of skeletal and craniofacial defects, including syndactyly, holoprosencephaly, hypotelorism, cleft palate, and cyclopia (Belloni et al. 1996; Chiang et al. 1996). Desert Hh is expressed in peripheral nerves and in male gonads (Bitgood and McMahon 1995), suggesting a functional role restricted to these tissues. The third vertebrate Hh family member is Ihh. Both Ihh and Shh functions have been studied in cartilage and bone patterning throughout the axial, appendicular, and facial skeletons (Hammerschmidt et al. 1997; Capdevila

and Johnson 2000; Chai and Maxson 2006), as well as in calvarial ossification and suture morphogenesis (Pan et al. 2013). Ihh is mainly produced by post-mitotic prehypertrophic chondrocytes adjacent to the proliferative zone that express the parathyroid hormone (PTH)/PTHrP receptor (PTHR) and stimulate the proliferation of chondrocytes at the growth plate and later in development. Ihh further regulates chondrocyte hypertrophic and osteoblast differentiation, either directly or via PTHrP (Nakamura et al. 1997; Mak et al. 2008; Vortkamp et al. 1996) (Fig. 9.1). In the latter system, a negative feedback loop between Ihh and PTHrP regulates the rate of chondrocyte differentiation: Ihh produced by prehypertrophic chondrocytes induces PRTrP expression, which prevents further differentiation of chondrocytes expressing PTHR. Ihh knockout mice show appositional chondrocyte differentiation and loss of PTHrP and either die during mid-gestation because of yolk sac defects or die at birth because of rib cage deformities and respiratory failure (Byrd et al. 2002). Chondrocyte-specific (*Col2α1Cre;Ihhd/Ihhd*) mice also die at birth, showing delayed chondrocyte hypertrophy, reduced calvarial bone size and ossification, abnormal mineralization of axial and appendicular bones, and widened cranial sutures (Razzaque et al. 2005). These findings demonstrate that chondrocyte-derived Ihh not only is responsible for the regulation of the endochondral skeleton by regulating both chondrocyte proliferation and differentiation, but it is also essential for osteoblast differentiation. Ihh expression in chondrocytes depends on the runt-related transcription factors (Runx)2 and Runx3 (Yoshida et al. 2004). Runx2−/− mice die after birth and completely lack bone formation due to absence of osteoblast differentiation and delayed chondrocyte maturation (Komori et al. 1997; Otto et al. 1997), and Runx3−/− mice show mildly reduced chondrocyte maturation. Runx2/3 compound mutant mice completely lack Ihh expression (Yoshida et al. 2004).

All Hh homologs undergo the same three-step conserved maturation pathway in producing cells (Fig. 9.1). Production of the active Hh protein begins with auto-cleavage of a HhNC precursor protein into a N-terminal (HhN) signaling domain and the HhC autoprocessing domain. This cleavage reaction is linked to the covalent attachment of a cholesterol moiety to the carbonyl of the C-terminal HhN glycine residue (Porter et al. 1996a, b; Cohen 2003). In a second step, Hh acyltransferase attaches a palmitoyl group to the NH_2-terminal cysteine of the Hh signaling domain (Pepinsky et al. 1998). The dually lipidated molecule constitutes the active morphogen (Jacob and Lum 2007; Taylor et al. 2001). Upon secretion to the cell surface, lipidated Hh's multimerize prior to their release (Dierker et al. 2009a) and transport to cells expressing the Hh receptor Patched (Ptc) (Panakova et al. 2005; Zeng et al. 2001). The paradoxical situation is that a membrane-tethered molecule serves as a long-range morphogen; this requires specific mechanisms for its release and transport. The 12-pass transmembrane protein Dispatched (Disp) is essential for the release of lipid-modified Hh's (Burke et al. 1999; Caspary et al. 2002; Kawakami et al. 2002). Disp is therefore critical for full signaling within the chondrocyte target field in developing bones and consequently for the establishment of a normal skeletal growth plate (Tsiairis and McMahon 2008). The exact mechanism of Disp-dependent release of lipidated Hh, however, is not yet resolved. Other suggested players in Hh transport include Hh micelle formation by unknown mechanisms

(Zeng et al. 2001), Hh transport together with lipoprotein particles (Panakova et al. 2005; Eugster et al. 2007), Hh transport on filopodia (called cytonemes) (Bischoff et al. 2013; Roy et al. 2011), Hh association with the soluble glycoprotein Scube2 (Creanga et al. 2012; Hollway et al. 2006; Johnson et al. 2012; Kawakami et al. 2005; Tukachinsky et al. 2012; Woods and Talbot 2005), or simple diffusion of solubilized Hh after its proteolytic processing from the cell surface (called shedding) (Dierker et al. 2009b; Ohlig et al. 2011; Ohlig et al. 2012). Notably, cytonemes have not been reported on chondrocytes, making this transport mechanism unlikely. Moreover, the very dense extracellular matrix (ECM) of the developing skeleton makes most of these suggested mechanisms—in particular Hh transport on filopodia and transport via large lipoprotein particles or exosomes—hard to envision. It has been firmly established, however, that Hh long-range function depends on the expression of heparan sulfate proteoglycans (HSPGs). Again, the underlying mechanism of HSPG-mediated Hh transport is not clearly defined, but it is assumed that these versatile molecules somehow aid Hh transport by "facilitated diffusion" or Hh stabilization against degradation (Lin 2004; Muller et al. 2013).

In contrast to the components that act to release Hh, Hh signaling components in receiving cells have been studied in more detail (Cohen 2003; Robbins et al. 2012; Ingham and McMahon 2001). Hh proteins induce signaling on receiving cells by direct binding to the Hh receptor Ptc, a 12-pass transmembrane protein (Fuse et al. 1999). The amount of Hh available for Ptc binding is regulated by other Hh-binding proteins, such as Hh-interacting protein (Chuang and McMahon 1999) and growth arrest-specific protein 1 (Gas1) (Evangelista et al. 2006; Lee et al. 2001). Furthermore, the Interference Hh protein family (Ihog in *Drosophila* and CDO and BOC in humans) (Wilson and Chuang 2006; Kavran et al. 2010) and HSPGs (Bornemann et al. 2004; Beckett et al. 2008) modulate Hh signaling. Hh binding to Ptc (together with Hh binding to Boc/Cdo and Gas1 (Allen et al. 2011)) induces internalization of the receptor/ligand complex and relieves Ptc-mediated catalytic inhibition of the seven-pass transmembrane protein Smoothened (Smo) (Taipale et al. 2002). Active Smo then transduces the Hh signal to the cytoplasm, resulting in processing and activation of the glioblastoma (Gli) family of transcription factors (Gli1–Gli3) (Hatsell and Frost 2007). Gli1, in contrast to Gli2 and Gli3, lacks an amino-terminal repressor domain and thus represents a constitutive activator of the Hh pathway (Hatsell and Frost 2007; Hynes et al. 1997; Karlstrom et al. 2003). Yet, in mouse development, Gli1 is not essential since Gli1−/− mutants survive from birth to adulthood with a normal phenotype (Park et al. 2000). In contrast, Gli2 and Gli3 are required for mouse development and carry an N-terminal repressor domain in addition to the C-terminal activator domain and thus can act as both activators and repressors (Sasaki et al. 1999; Ruiz i Altaba 1999). Their bifunctionality is determined by the presence of Hh signaling: the absolute concentration of Hh ligands specifically induces defined Gli transcription factor activation, resulting in Hh concentration-dependent activation of target genes (Ogden et al. 2004; Harfe et al. 2004). In the absence of Hh signaling, Gli3 is complexed with suppressor of fused (SuFu), which leads to Gli3 phosphorylation by several kinases. This targets Gli3 for proteolytic processing into the truncated repressor form (Gli3R) that locates

to the nucleus and inhibits transcription of target genes (Persson et al. 2002). Upon Smo activation in the presence of Hh signaling, however, SuFu is sequestered away from Gli3, proteolytic processing is inhibited, and full-length Gli3 (Gli3A) induces target gene transcription. Gli2 can likewise be converted into a repressor by proteolytic processing in the absence of Hh signaling and is activated by high levels of Hh. However, Gli2 C-terminal processing is less effective than that of Gli3. Therefore, Gli2 mostly remains transcriptionally active even at low levels of Hh signaling in vivo (Fuccillo et al. 2006).

Gli-regulated Hh-dependent target genes include Wnts, BMP, and the Ptc receptor itself. Importantly, upregulation of Ptc in response to Hh signaling constitutes a negative feedback loop by increasing the relative amount of free Ptc on the cell surface, which in turn inhibits Smo activity and signaling. In addition, Ptc directly reduces Hh levels in the ECM by ligand internalization upon binding (Jeong and McMahon 2005). In chondrocytes, another direct consequence of Ihh signaling is the Wnt5A-dependent, yet PTHrP-independent, degradation of Nkx3.2 proteins that are normally expressed in chondrocyte precursor cells and in early-stage chondrocytes (Choi et al. 2012). In these cells, Nkx3.2 proteins enhance chondrocyte differentiation and survival while inhibiting chondrocyte hypertrophy and apoptosis.

9.3 Primary Cilia in Hedgehog Perception

Primary cilia are involved in the regulation of Hh signal transduction, although the precise mechanisms are not fully elucidated (Tran et al. 2008). A primary cilium consists of a singular, immotile organelle, which is present on most cells, including chondrocytes, during interphase (Scherft and Daems 1967). Currently, it is thought that primary cilia provide an environment that facilitates interactions between different Hh pathway components (Ruat et al. 2012), such as Ptc, Smo, and Gli proteins that require ciliary transport in order to activate Hh-dependent gene expression (Keady et al. 2012). Upon Hh binding to Ptc and following Smo stimulation, Smo is translocated to the cilium and subsequently interacts with Gli's, leading to their activation. Gli's then move down the cilium to enter the nucleus and transduce the Hh signal (Huangfu and Anderson 2005, 2006).

For these reasons, the targeted inactivation of intraflagellar transport (IFT) proteins, such as components of the kinesin-like protein motor complex and retrograde dynein motors, has been found to affect Hh signal transduction (Ruat et al. 2012). Conditional inactivation of the Kif3a subunit of the kinesin-2 intraflagellar transport motor in mesenchymal skeletal progenitor cells, for example, resulted in severe patterning defects in the craniofacial area, the formation of a split sternum, and the development of polydactyly, deformities reminiscent of those described in mice with deregulated Hh signaling (Koyama et al. 2007).

In Kif3a-deficient mesenchymal tissues, both the repressor function of Gli3 and the activation of the Shh transcriptional targets Ptc and Gli1 are compromised (Kolpakova-Hart et al. 2007). This is consistent with the finding that Gli signaling

depends on Kif3a function (Haycraft et al. 2005; Huangfu and Anderson 2005). Kif7, which plays a role in the turnover of Sufu and the exclusion of Sufu-Gli complexes from the primary cilium, regulates the activity of Gli transcription factors through both Sufu-dependent and Sufu-independent mechanisms (Hsu et al. 2011). Mutations in the IFT protein DynC2H1 cause short-rib polydactyly syndrome, a lethal autosomal recessive condition that features cerebral and skeletal abnormalities, including appendicular malformations (Dagoneau et al. 2009; El Hokayem et al. 2012; Merrill et al. 2009). Finally, partial mutation of intraflagellar transport 80 (IFT 80) in humans causes Jeune asphyxiating thoracic dystrophy and short-rib polydactyly syndrome. IFT80 is mainly expressed in growth plate chondrocytes, and IFT80 knockdown impairs chondrocyte cilia formation and chondrogenic differentiation in mouse bone marrow-derived stromal cells by downregulating Hh signaling (Wang et al. 2013). In addition to merely acting as a location for Hh signaling regulation, the primary cilium also plays a role in mechanosensitive Hh signaling in adult articular chondrocytes (Thompson et al. 2014). Mechanical strain promotes Ihh expression and Hh pathway activation; cilia disassembly due to high-magnitude strain prevents this process. However, in comparisons of Ihh- and Kif3a-deficient mice, chondrogenesis differs significantly, indicating that Ihh actions may not solely depend on molecular association of Hh reception components with cilia (Koyama et al. 2007; Kolpakova-Hart et al. 2007).

9.4 Hedgehog Functions in Chondrocyte Pathobiology

Osteoarthritis (OA) is linked to the irreversible degeneration of articular cartilage in adult joints, often due to initial injury. In this disease, articular cartilage chondrocytes undergo phenotypic and gene expression changes that resemble their end-stage differentiation in the growth plate during skeletal development, suggesting that Ihh and the Ihh/PTHrP axis continue to play a role in OA. Indeed, Ihh expression is upregulated in human OA cartilage, and this upregulation correlates with OA progression and changes in chondrocyte morphology. Consistent with this observation, transgenic mice with induced Ihh expression exhibit increased chondrocyte hypertrophy and cartilage damage resembling human OA. In these mice, higher levels of Hh signaling in chondrocytes caused a more severe osteoarthritic phenotype (Lin et al. 2009). Two other genetic studies in mice confirmed this finding, showing that conditional deletion of Ihh in chondrocytes attenuates OA progression (Zhou et al. 2014a, b). Only mild OA changes were observed in Ihh-deficient mice, while control mice displayed significantly more cartilage damage. OA markers such as collagen X and MMP-13 were decreased in Ihh-deficient mice, and the activity of cathepsins and MMPs in knee joints of animals with deletion of Ihh was decreased. Consistent with this finding, PTHrP inhibits mineralization in articular cartilage that is associated with OA (Terkeltaub et al. 1998), and histone deacetylase four was suggested to have chondroprotective properties by inhibiting the Ihh transcription factor Runx2 (Cao et al. 2014). Therefore, the PTHrP/Ihh axis continues to participate in the maintenance of articular cartilage, and dysregulation of this system likely contributes to the pathogenesis of OA.

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Wnt Signaling in Cartilage Development 10

Christine Hartmann

Abstract

Cartilage is a type of connective tissue where the cells within – the chondrocytes – produce a matrix rich in type II collagen and proteoglycans. With a few exceptions in the skull and part of the clavicles, the skeletal elements (bones) are formed from cartilaginous templates that prefigure the future bones in shape and size. The cartilaginous templates eventually need to be replaced in a process referred to as endochondral ossification. Cartilage development is controlled at multiple levels: condensation of mesenchymal cells, differentiation of mesenchymal cells into chondroblasts and chondroblasts to chondrocytes, maturation of proliferating chondrocytes to postmitotic prehypertrophic and then to hypertrophic chondrocytes, and finally the remodeling at the transition from hypertrophic chondrocytes to trabecular bone. All these steps need to be controlled and Wnt signals play important regulatory roles in those processes. There are 19 different Wnt ligands in vertebrates, which can utilize diverse signaling pathways acting either positively or negatively on chondrogenesis and during cartilage development.

10.1 Introduction

Hyaline cartilage is a type of connective tissue that together with bone comprises the skeleton. The hyaline cartilage matrix is produced by chondrocytes and is rich in type II collagen and proteoglycans. One distinguishes between so-called

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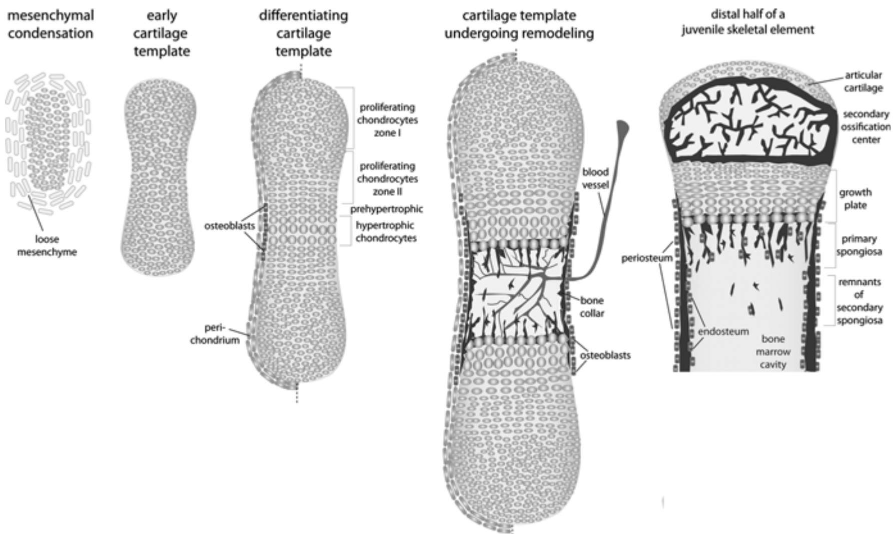

Fig. 10.1 Scheme of the endochondral ossification process; mesenchymal cells condense to form the cartilage template. Chondrocytes within the cartilage template undergo differentiation from proliferating to prehypertrophic to hypertrophic chondrocytes in the center of the anlage. Coupled to the occurrence of hypertrophic chondrocytes is the differentiation of osteoblasts from cells within the perichondrium in the region adjacent to prehypertrophic and hypertrophic chondrocytes. Finally the cartilage template undergoes remodeling; mature hypertrophic chondrocytes, which produce a mineralized matrix, are either undergoing apoptosis and are removed by chondro-/osteoclasts or transdifferentiate into osteoblast precursors. The mineralized matrix that stays behind is used by the osteoblast precursors that originate either by transdifferentiation or are migrating in along with blood vessels from the perichondrium to lay down nonmineralized bone matrix (osteoid). Postnatally a true growth plate can be found which is sandwiched between the secondary ossification center in the articular region and the primary ossification center, forming primary and secondary spongiosa. Note: For simplistic purposes the osteoblasts and/or the perichondrium only is indicated on the left side in some of the schematically depicted differentiation stages

replacement cartilage (hyaline cartilage of, e.g., long bones in the appendicular skeleton) and permanent cartilage (articular hyaline cartilage of the synovial joints). The latter is normally not remodeled into bone in healthy individuals. In contrast, the embryonic skeleton, where the majority of the individual skeletal elements are prefigured by hyaline cartilaginous templates, needs to be remodeled into bone in a process referred to as endochondral ossification. Endochondral ossification is a multi-step, tightly regulated process that requires the coordination of the differentiation processes of distinct cell types (Fig. 10.1). Firstly, chondrocytes in the cartilage template need to undergo a differentiation/maturation program from proliferating, to prehypertrophic, and finally to hypertrophic chondrocytes. The latter mature further to produce a mineralized matrix. This mineralized matrix is left behind after the disappearance of hypertrophic chondrocytes and is used by osteoblasts as a template to form trabecular bone, also known as primary spongiosa. Secondly, a subpopulation of the cells in the perichondrium surrounding the cartilage template is

induced to differentiate into osteoblast precursors and finally into osteoblasts that form the bone collar encasing the central part of the cartilage anlage. The timely coordination of the first two processes is achieved by a signal produced by prehypertrophic chondrocytes, Indian hedgehog (Ihh), which signals to and induces osteoblastogenesis in cells of the adjacent perichondrium (St-Jacques et al. 1999; Chung et al. 2001). In addition, Ihh influences the pace of chondrocyte proliferation/maturation by two mechanisms: (a) via a feedback loop regulating parathyroid hormone-like hormone (Pthlh/Pthrp) and (b) by directly regulating chondrocyte proliferation (Kronenberg 2003). Thirdly, although the cartilage is avascular, hypertrophic chondrocytes produce vascular endothelial growth factor (VEGF), a signal required to attract blood vessels to the chondro-osseous front (Gerber et al. 1999). Along with the blood vessels, perichondrial-derived osteoblast precursors and osteoclast precursors enter the remodeling zone (Maes et al. 2010). (For further information see Chap. 6.) Recently it has been shown that trabecular and endosteal osteoblasts can also originate from chondrocytes, most likely from type X collagen positive, hypertrophic chondrocytes (Yang et al. 2014a, b; Zhou et al. 2014; Park et al. 2015). The latter process probably ensures that osteoblast precursors are readily available during the early cartilage into bone remodeling phase. Thus, hypertrophic chondrocytes eventually undergo one of two different fates: they undergo apoptosis and are actively removed by osteoclasts and chondroclasts or give rise to osteoblasts and potentially other cell types of the bone marrow (Shapiro et al. 2005; Yeung Tsang et al. 2014; Yang et al. 2014a, b; Park et al. 2015).

Many different signals influence cartilage formation and the differentiation and maturation of chondrocytes during embryonic skeletal development, among them Wnt signaling. In the vertebrate genome, a total of 19 different Wnt ligands are encoded (see also Wnt Homepage: http://web.stanford.edu/group/nusselab/cgi-bin/wnt/main). Wnt ligands are secreted, cysteine-rich molecules that need to be posttranslational modified by palmitoylation and glycosylation (Doubravska et al. 2011; Komekado et al. 2007; Kurayoshi et al. 2007; Willert and Nusse 2012). Wnt's can interact with different receptors and signal through different intracellular pathways (Fig. 10.2) (van Amerongen 2012; Niehrs 2012). On the basis of receptors and intracellular signaling molecules, or the process they are involved in, they can be subdivided into pathways affecting either translation such as the Wnt/TSC2/mTOR-mediated pathway or the cytoskeleton, such as the Dvl/aPKC (atypical protein kinase C), the Wnt/GSK3-mediated, and the planar-cell-polarity (PCP) pathway (Inoki et al. 2006; Zhang et al. 2007; Salinas 2007; Sebbagh and Borg 2014). The latter can also affect transcription. Transcriptional responses are also evoked by the Ca^{2+} pathway, the Wnt/Ror2 pathway, and the Wnt/β-catenin pathway (often referred to as the canonical Wnt pathway) (Kohn and Moon 2005; Schambony and Wedlich 2007; Clevers and Nusse 2012; Green et al. 2014).

Wnt signaling is also modulated extracellular by several antagonist and a few agonists (for a comprehensive review see (Cruciat and Niehrs 2013)). R-spondins belong to the extracellular agonist of Wnt signaling that in a receptor-interaction-dependent manner can potentiate different pathways. For example, binding to Syndecan-4 potentiates the PCP pathway, while binding to members of the

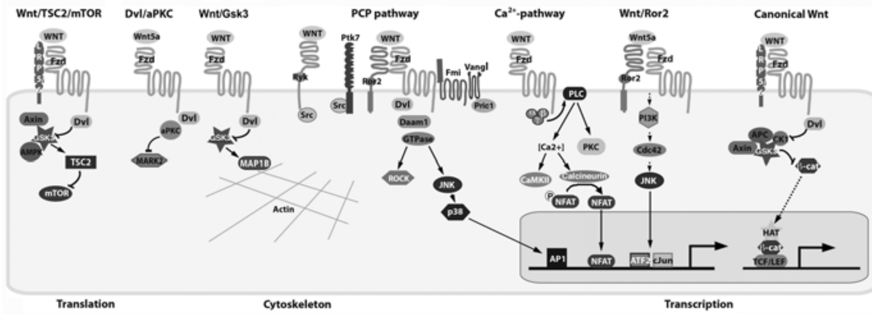

Fig. 10.2 Schematic overview over the diverse Wnt pathways distinguished on the basis of receptors, intracellular components, and cellular processes, such as effects on translation, cytoskeleton, and transcription. Abbreviations: *AMPK* AMP-activated protein kinase, *AP1* activator-protein 1, *APC* adenomatous polyposis coli protein, *aPKC* a-typical protein kinase C, *α,β,γ* heterotrimeric G-protein subunits, *ATF2* activating transcription factor 2, *β-cat* β-catenin, *CaMKII* calcium/calmodulin-dependent protein kinase II, *Calcineurin* calcium-dependent phosphatase 3, *Cdc42* cell division control protein 42, *CK1* casein kinase 1, *c-Jun* AP1 family member, *LEF* lymphoid-enhancer binding factor, *LRP* low-density lipoprotein receptor, *Daam1* dishevelled-associated activator of morphogenesis 1, *Dvl* dishevelled, *Fmi* flamingo, *Fzd* frizzled, *GSK3* glycogen synthase kinase 3, *HAT* histone acetyltransferase, *JNK* c-Jun N-terminal kinase, *mTOR* mammalian target of rapamycin, *MARK* microtubule affinity-regulating kinase, *MAP1B* microtubule-associated protein 1B, *NFAT* nuclear factor of activated T-cells, *PI3K* phosphoinositide 3-kinase, *PKC* protein kinase C, *PLC* phospholipase C, *Pric1* Prickle1, *Ptk7* protein tyrosine kinase, *p38* p38-mitogene activated kinase, *Ror2* receptor tyrosine kinase-like orphan receptor 2, *Rock* Rho-associated coiled-coil kinase, *Ryk* receptor-like tyrosine kinase, *TCF* T-cell factor, *TSC* tuberous sclerosis complex, *Vangl* van Gogh-like, *Src* Proto-oncogene, non-receptor tyrosine kinase, *GTPase* Guanosine triphospate hydrolyzing enzyme

*L*eucin*e*-rich *r*epeat-containing *G*-protein-coupled receptor (Lrg) family (Lrg 4-6) potentiates the Wnt/β-catenin pathway (de Lau et al. 2012, 2014; Ohkawara et al. 2011). The PCP-, the Wnt5a/Ror2-, the Ca^{2+}-, and the Wnt/β-catenin pathways have been functionally implicated in chondrogenesis, and their roles will be discussed in the following paragraphs. As a clear distinction between the first two pathways is not possible, the roles of their pathway components will be discussed in the following chapter.

10.2 PCP- and Wnt5a/Ror2-Pathway Components and Their Roles in Chondrogenesis

10.2.1 Receptors and Associated Molecules

Human mutations in components of the PCP pathway, such as WNT5a and Ror2, have been associated with Robinow syndrome (RRS) and/or brachydactyly type B1, characterized among others by short-limbed dwarfism and shortened or missing distal phalanges, respectively (Afzal et al. 2000; Person et al. 2010; Schwabe et al. 2000; van Bokhoven et al. 2000; Oldridge et al. 2000). For the alternative Wnt co-receptor Ryk, which has also been implicated in PCP, no

mutations have yet been found in patients with autosomal-dominant RRS (Mazzeu 2013; Green et al. 2014). Loss-of-function mutations in several PCP pathway components, such as Wnt5a, Ror2, Vangl2, Prickle1, and Ryk, result in RRS-like phenotypes in mice (Liu et al. 2014; Wang et al. 2011; Gao et al. 2011; Andre et al. 2012; Macheda et al. 2012; Takeuchi et al. 2000; DeChiara et al. 2000; Oishi et al. 2003; Yang et al. 2013). Functional studies in mice suggest further that the PCP pathway components play multiple, locally distinct roles in early chondrogenesis. Wnt5a is highly expressed in the distal limb mesenchyme, and loss of Wnt5a or combined loss of Ror2 and Vangl2 results in a distal upregulation of anti-chondrogenic Wnt/β-catenin signaling and suppression of chondrogenesis (Topol et al. 2003; Gao et al. 2011; Yamaguchi et al. 1999). Based on in vitro data, it was suggested that Ror2 is involved in mediating the inhibitory effect of Wnt5a on Wnt/β-catenin signaling (Gao et al. 2011; Mikels and Nusse 2006; Mikels et al. 2009). Interestingly, in the absence of either Ror2 or Ror2 and Vangl2, Wnt3a-mediated β-catenin transcriptional activity is no longer inhibited by Wnt5a instead it is enhanced (Gao et al. 2011). However, loss of Ror2 or Vangl2 alone does not lead to ectopic activation of Wnt/β-catenin signaling in the distal mesenchyme (Gao et al. 2011). These results have been challenged by the work of Ho and colleagues, which showed that Wnt5a still inhibits canonical signaling in *Ror1/2* double knockout cells (Ho et al. 2012). The work by Ho and colleagues also questioned the involvement of JNK as an intracellular signaling component in the Ror2/Wnt5a pathway (Ho et al. 2012). The related receptor Ryk does not seem to be involved in mediating suppression of Wnt/β-catenin signaling in the distal mesenchyme. This conclusion is based on the loss-of-function phenotype of Ryk alone or in combination with Vangl2 (Andre et al. 2012). The close homolog of Ror2 and Ror1 has not been clearly associated with PCP signaling. While Ror2 seems to regulate chondrocyte maturation at the level of prehypertrophic chondrocytes (Schwabe et al. 2004), mice lacking Ror1 function survive and show postnatal growth retardation associated with changes in their growth plates, but no embryonic alterations in long bone growth (Lyashenko et al. 2010). Despite the lack of a clear embryonic phenotype associated with chondrogenesis, loss of Ror1 in a Ror2 mutant background augments the Ror2$^{-/-}$ appendicular skeletal phenotype, primarily in the proximal elements, such as the humerus and femur, suggesting that Ror1 signaling can in part compensate for the loss of Ror2 (Nomi et al. 2001). A process, which may also require conversion-extension movement and PCP signaling, is the formation of the sternum where two anlagen need to fuse and the cells probably need to intercalate along the ventral midline (Chen 1952). This process is disturbed by the loss of Ror1 and further abrogated by the additional loss of Ror2 (Lyashenko et al. 2010; Nomi et al. 2001).

In *Drosophila* the frizzled (Fzd) receptors, seven transmembrane domain-containing proteins are at the core of PCP signaling (Carvajal-Gonzalez and Mlodzik 2014). So far a link of vertebrate Fzd proteins to polarity in chondrocytes has only been established in chicken and in vitro using rat chondrocytes. In both systems, overexpression studies provided evidence that noncanonical Fzd signaling is

involved in regulating the polarity of chondrocytes (Li and Dudley 2009; Randall et al. 2012). However, chondrocyte-associated polarity phenotypes have not yet been described for any of the published Fzd loss-of-function mutants in mice.

10.2.2 Wnt Ligands Associated with PCP

Recent studies suggested that the distal expression of Wnt5a is also involved in establishing polarity within the chondrocytes of the newly forming digit anlagen thereby influencing cartilage elongation (Gao et al. 2011; Kuss et al. 2014). In line with this, it has been shown in chicken that interference with noncanonical Wnt signaling, probably with the PCP pathway, affects the polarity of the proliferating chondrocytes (Li and Dudley 2009). Previous loss- and gain-of-function studies implicated Wnt5a signaling also in chondrocyte proliferation and differentiation (Yang et al. 2003; Hartmann and Tabin 2000; Kawakami et al. 1999; Yamaguchi et al. 1999). The gain-of-function effect of Wnt5a on differentiation observed in vivo can also be recapitulated in vitro in limb bud long-term micromass cultures (Daumer et al. 2004). The analysis of the Wnt5a mutant skeletal elements suggests that Wnt5a is required for the transition from proliferative to prehypertrophic chondrocytes and that it acts on proliferation of more distal chondrocytes (Zone II), overlapping with the region where it is expressed (Yang et al. 2003). As Wnt5a signaling has been shown to downregulate Wnt/β-catenin signaling, part of the observed phenotypes may be associated with a misregulation of that pathway as well (Mikels and Nusse 2006; Topol et al. 2003). To discriminate in vivo between direct Wnt5a PCP and indirect effects will, however, be difficult and a future challenge, particularly as in vitro work by the Nusse lab, suggests that Wnt5a cannot only repress but also activate canonical Wnt signaling (Mikels and Nusse 2006; van Amerongen et al. 2012).

A closely related family member Wnt5b is expressed in a domain partially overlapping with Wnt5a in the mouse long bones, with Wnt5b being expressed in more mature chondrocytes (Witte et al. 2009; Yang et al. 2003). In contrast, in the chicken long bones, the two Wnt genes are expressed in different domains (Witte et al. 2009; Hartmann and Tabin 2000; Yang et al. 2003). In the chick, Wnt5a expression is restricted to the perichondrium, while Wnt5b is expressed in prehypertrophic and early hypertrophic chondrocytes (Hartmann and Tabin 2000). Overexpression of Wnt5a in the chicken is associated with upregulation of Wnt5b (Hartmann and Tabin 2000; Church et al. 2002). Wnt5b overexpression in chick also resulted in shortening of the long bones and may affect a different population of chondrocytes than Wnt5a, prehypertrophic in addition to hypertrophic (Hartmann 2002). However, different phenotypic effects have been reported between different experimentators (Church et al. 2002; Hartmann 2002), which may be due to total differences in the overall infection efficiency or local infection efficiency, soft tissue versus cartilage. Similar dose-dependent effects to the overexpression studies in chicken have been reported in transgenic studies in mouse due to varying levels of transgene expression (Yang et al. 2003). The

overexpression studies in mouse expressing either Wnt5a or Wnt5b under the col2a1 promoter/enhancer element revealed that the two related Wnt molecules regulate proliferation of discrete chondrocyte populations and that this is probably mediated via two distinct, but yet not unambiguously identified pathways (Yang et al. 2003). In vitro data using chondroprogenitor cells suggest that Wnt5a and Wnt5b can activate calcium-dependent signaling pathways, leading to an increase in the nuclear location of NFAT and they also activate NF-κB signaling (Bradley and Drissi 2010, 2011). NFAT positively affects chondrogenesis regulating Sox9 levels, while the authors associated NF-κB signaling with the inhibitory effect of Wnt5a on chondrocyte hypertrophy, by downregulating Runx2 – a transcription factor that positively regulates hypertrophy (Bradley and Drissi 2010; Yoshida and Komori 2005). NF-κB activation is mediated by PI3K/Akt signaling, and Wnt5a signaling has been associated with this pathway in different human cells (Kawasaki et al. 2007; Zhang et al. 2014; Zhao et al. 2015). However, there is also one very recent report showing that in cardiac cells signaling by Wnt5a and another noncanonical Wnt, Wnt11, leads to Akt degradation (Bisson et al. 2015). Thus, these cell-type specific differences may be credited to yet unidentified, cell-type specific factors. In addition, Wnt5b has been shown in vitro to activate on one hand the kinase JNK, an intracellular component of the PCP, and the Wnt5a/Ror2 pathway, regulating cell migration, and on the other hand downregulates the levels of cadherin associated β-catenin and thereby decreases cell adhesion (Bradley and Drissi 2011). The potential to activate JNK is, however, not limited to Wnt5b as both Ror2 overexpression and Wnt5a or even Wnt3a treatment lead to increased JNK activity in embryonic fibroblasts (Oishi et al. 2003; Yamanaka et al. 2002; Ho et al. 2012). Although the in vivo and in vitro overexpression data are suggestive of a role for Wnt5b in chondrogenesis, actually Wnt5b mutant mice have no reported skeletal phenotype and are viable (Agalliu et al. 2009). Thus, its function may be compensated by functionally related Wnt molecules, such as Wnt5a. Furthermore, a functional in vivo analysis of the different potential downstream pathway components on their role in chondrogenesis is still outstanding. The latter will be difficult to achieve as the implicated intracellular components are also associated with the activity of other signaling molecules.

Another Wnt ligand associated with the PCP pathway and signaling through Ror2 based on studies in zebra fish is Wnt11 (Ulrich et al. 2003; Bai et al. 2014). In long bones of chicken and mouse, Wnt11 is expressed in prehypertrophic chondrocytes, overlapping with the expression of Wnt5b (Witte et al. 2009; Church et al. 2002). In humans WNT11 is expressed in the perichondrium and maps to a chromosomal region associated with high bone mass (Lako et al. 1998). Retroviral-mediated overexpression of Wnt11 in chicken leads to a slight shortening of the long bones, however, without delaying chondrocyte maturation (Church et al. 2002). Mice lacking Wnt11 die within 2 days after birth for unknown reasons and no skeletal phenotype has yet been reported in the mutants (Majumdar et al. 2003). If Wnt11 plays a role in the postnatal skeleton remains to be determined.

10.3 The Wnt/Calcium Pathway

The Wnt/calcium-signaling pathway was first identified in *Xenopus* and zebra fish (Kuhl et al. 2000). Components of this pathway have also been associated with chondrogenesis. One of the enzymes acting downstream of phospholipase C is the protein kinase C (PKC). Eleven different PKC isoforms exist that can be subdivided into three groups: calcium-dependent "conventional" cPKC (α, βI, βII, γ), calcium-independent "novel" nPKC (δ, ε, η, θ), and "atypical" aPKC (ζ and ι/λ) (Steinberg 2008). In the chicken limb mesenchyme, the following PKCs have been reported to be expressed: cPKCα, nPKCε, and the aPKCs ζ and ι/λ (Choi et al. 1995; Nicolin et al. 2004). Limb mesenchymal cells have the potential to spontaneously undergo chondrogenic differentiation in culture. Interestingly, the total level of cPKCα and nPKCε increased during the culture period of 5 days, while the level of the aPKCs stayed the same (Choi et al. 1995; Yang et al. 1998). Furthermore, PKC activity steadily increased during the first 3 days, correlating with the onset of chondrogenesis (Sonn and Solursh 1993). Fractionation experiments have shown that the alteration in levels occurs primarily in the particulate membrane fraction, which may be associated with activation of PKCs (Yang et al. 1998). In contrary to the chick, in the mouse PKCβ and to some extend PKCζ have been reported to be expressed in growth plate chondrocytes (proliferative and hypertrophic) (Bareggi et al. 1994). Whether this reflects species-specific differences or stage-specific differences has so far not been clarified. Only the cPKC class members can be activated by binding of diacylglycerol (DAG) and phospholipids and are calcium dependent. There are numerous in vitro reports on the role of PKCs in chondrocytes, which are primarily based on the use of agonists and antagonists, and the interpretation of these data is somewhat complicated by side effects of the compounds and differential effects depending on cell density and/or differentiation status of the culture (Matta and Mobasheri 2014). Treatment with the phorbol ester PMA, a DAG mimetic and an activator of cPKCs, inhibited chondrogenesis, probably because prolonged treatment leads to PKC (α, γ, ε) downregulation (Choi et al. 1995). However, there are conflicting reports to whether PKCs are required for initiation of chondrogenesis (commitment phase) or for chondrocyte differentiation (phenotypic expression of chondrogenic markers and matrix accumulation) (Choi et al. 1995; Garrison et al. 1987). In micromass cultures from chicken wing anlagen, it has been shown that Wnt5a, but also Tgfβ3, treatment stimulated PKCα activity facilitating chondrogenesis (Jin et al. 2006). In the mesenchymal cells of the chicken leg anlage, PKCδ was reported to act as a positive mediator of chondrogenesis (Matta et al. 2011).

Another molecule activated by PLC and increased intracellular calcium levels is the calcium/calmodulin-dependent kinase II (CaMKII), a multimeric protein consisting of up to 12 subunits. CaMKII is activated by calcium/calmodulin binding which leads to a conformational change releasing the active site from the inhibitory domain and subsequent autophosphorylation of the enzyme. The latter step renders the enzyme independent from calcium/calmodulin (Hudmon and Schulman 2002). All four CaMKII genes, α, β, γ, and δ, which encode multiple splice isoforms are expressed in chicken and mouse chondrocytes and are activated in prehypertrophic

chondrocytes (Taschner et al. 2008; Li et al. 2011; Li and Dudley 2009). Misexpression of a constitutively active, phosphomimetic version of CaMKII in chicken limbs resulted in elongation of the skeletal elements and affected expression of cell cycle-related genes in the infected chondrocytes (Taschner et al. 2008). Furthermore, a subset of the infected chondrocytes within the upper (zone I) and lower (zone II) proliferative zones undergoes premature differentiation (Taschner et al. 2008; Li et al. 2011). Li and colleagues proposed that a certain threshold of CaMKII activity needs to be reached in order to overcome an inhibitory gradient within the growth plate and to activate the hypertrophic program (Li et al. 2011). According to the findings by Li and colleagues, activated CaMKII signaling may regulate chondrocyte hypertrophy in chicken through the upregulation of Runx2 and β-catenin (Li et al. 2011). In contrast to the studies in frog and zebra fish which placed CaMKII activation downstream of Wnt5a and Wnt11 (Kuhl et al. 2000), the studies in chicken revealed a negative effect of noncanonical Wnt signaling on phospho-CaMKII levels, suggesting that they act as antagonists (Li et al. 2011). Other downstream components of the Wnt/calcium pathway are members of the transcription factor family, nuclear factor of activated T-cells (NFAT), and encompassing four independent genes (Saneyoshi et al. 2002). The activity of NFAT is regulated in part by its nuclear translocation controlled by the phosphorylation status of NFAT. Phosphorylated NFAT resides in the cytoplasm; upon calcium mobilization, the phosphatase calcineurin is activated and dephosphorylates NFAT, which is then translocated into the nucleus (Rao et al. 1997). Nuclear activity of NFAT transcription factors has been reported to promote chondrogenesis, or to maintain chondrocyte nature, but certain family members, such as NFATc1 (NFATp/NFAT1), may even repress chondrogenesis (Bradley and Drissi 2010; Tomita et al. 2002; Wang et al. 2009; Ranger et al. 2000). Interestingly, in cardiac myocytes, it has been proposed that CaMKII counteracts calcineurin activity by phosphorylating NFAT (MacDonnell et al. 2009). Furthermore, Wnt5a/Ca^{2+}-signaling pathway has been implicated in negatively regulating canonical Wnt/β-catenin signaling through CaMKII/TAK1 (transforming growth factor beta-activated kinase 1)/NLK (nemo-like kinase) in human embryonic kidney (HEK293) cells (Ishitani et al. 2003).

The Wnt ligand, Wnt3a, is always referred to as one of the ligands that signal through the canonical Wnt/β-catenin pathway (Shimizu et al. 1997). However, there is evidence that Wnt3a can activate different pathways even in the same cell (Tu et al. 2007; Hwang et al. 2005). A solution for this conundrum was recently presented – this ligand may activate different pathways in a concentration-dependent manner. The work was performed in vitro using human articular chondrocytes, and it was shown that low concentrations activated the Ca^{2+} pathway, while high concentrations preferentially the canonical Wnt/β-catenin pathway (Nalesso et al. 2011). On the basis of their observations, Nalesso and colleagues proposed that the loss of differentiation markers upon Wnt3a treatment was dependent on CaMKII signaling (Nalesso et al. 2011). Similar conclusions have also been drawn on the basis of Wnt3a treatment during mesenchymal stem cell (MSC) differentiation (Qu et al. 2013). Other investigators proposed that at least in chicken, Wnt3a may inhibit onset of chondrogenesis via a c-Jun/AP-1-mediated pathway (Hwang et al. 2005).

A similar mode of action has also been proposed for the inhibitory effect of Wnt7a on chicken mesenchymal limb bud cells (Tufan et al. 2002). As in another system, a possible link between CaMKII, JNK, and c-Jun has been established and this may be a common intracellular pathway downstream of a certain Wnt-receptor combination, or alternatively both pathways may be activated in parallel (Wu et al. 2009; Li et al. 2013).

10.4 The Canonical Wnt/ß-Catenin Pathway

Numerous roles for Wnt/β-catenin pathway in chondrogenesis have been proposed based on in vitro and in vivo experiments, which will be discussed in more detail in this paragraph. The central intracellular mediator of this pathway is β-catenin, encoded by the *Ctnnb1* gen. In the absence of an adequate Wnt ligand, intracellular β-catenin is associated with the so-called destruction complex. This complex is composed out of the scaffolding proteins adenomatous polyposis coli (Apc) and Axin and the two kinases, glycerol synthase kinase (GSK) and casein kinase 1 (CK1), facilitating the stepwise phosphorylation of β-catenin associated with the complex. Upon these *N*-terminal phosphorylation events, β-catenin is ubiquitinated and degraded via the proteasome pathway. In the presence of a suitable Wnt ligand binding to a receptor complex composed out of the low-density lipoprotein, receptor-related proteins 5 or 6 (LRP 5/6) and one of the members of the Fzd receptors, LRP5 or LRP6, are being phosphorylated and internalized by caveolin-dependent endocytosis as part of the so-called signalosome (a complex of p-LRP5/6, Axin and GSK3) (Taelman et al. 2010). As such the destruction complex is inactivated and unphosphorylated β-catenin can translocate into the nucleus. Here it acts as a transcriptional co-activator together with members of the T-cell factor/lymphoid-enhancer factor-1 (TCF/LEF-1) family, encoding transcription factors with an HMG-box DNA-binding motif. In the absence of β-catenin, TCF/LEF-1 transcription factors interact with the corepressor Groucho. Dependent on the presence of either the co-activator β-catenin or the corepressor Groucho, the complex recruits additional co-activators or corepressors, respectively, among them histone-modifying enzymes leading to chromatin remodeling (Clevers and Nusse 2012; Arce et al. 2006; Clevers 2006; MacDonald et al. 2009).

Based on in vitro and in vivo data modulating β-catenin activity in the mouse limb mesenchyme, it has been proposed that canonical Wnt/β-catenin signaling negatively regulates the early differentiation step from a mesenchymal cell to a chondrocyte (Hill et al. 2005; Guo et al. 2004). Wnt's expressed by and signaling from the ectoderm activating the Wnt/β-catenin pathway are probably involved in restricting the chondrogenic differentiation within the limb bud mesenchyme by silencing the expression of the transcription factor Sox9 – a factor essential for chondrogenesis (Hill et al. 2005; ten Berge et al. 2008; Kumar and Lassar 2014; Akiyama 2008). In chicken Wnt6 has been identified as such an inhibitory ectodermal signal (Geetha-Loganathan et al. 2010). Numerous other Wnt ligands, such as Wnt1, Wnt3a, Wnt7a, Wnt4, Wnt9a (formerly Wnt14), as well as β-catenin

activation, can inhibit chondrogenesis in vitro in micromass culture (Spater et al. 2006a; Rudnicki and Brown 1997; Surmann-Schmitt et al. 2009; Church et al. 2002; Hosseini-Farahabadi et al. 2013; Tufan and Tuan 2001; Hartmann and Tabin 2001). Secreted Wnt inhibitors, such as Frzb-1/sFrp3 and Wif-1, which are expressed in and around the condensing chondrogenic region, probably counteract the anti-chondrogenic activity of certain Wnt ligands (Surmann-Schmitt et al. 2009; Wada et al. 1999). In addition, Sox9 itself mediates degradation of β-catenin (Topol et al. 2009). Modulation of the expression of LRP4, a negative regulator of canonical Wnt signaling, in vitro influences the expression of extracellular matrix proteins (Asai et al. 2014). Collectively, these results suggest that canonical Wnt/β-catenin signaling negatively regulates the step from a mesenchymal cell to a chondroblast. As such the observation that mouse embryonic fibroblasts that lack the Wnt-antagonist sFrp1 show an increased potential to undergo chondrogenesis in the presence of bone morphogenetic protein 2 (Bmp2) is somewhat surprising. In particular, as sFrp1−/− cells have decreased phospho-β-catenin levels, normally a sign for β-catenin stabilization (Gaur et al. 2006). The Wnt/β-catenin pathway continues to negatively influence chondrogenesis beyond this initial step of chondroblast formation; stabilization of β-catenin or overexpression of certain Wnt's, such as Wnt3a and Wnt9a, in differentiated cartilage anlagen or differentiated sternal chondrocytes results in chondrocyte dedifferentiation (Guo et al. 2004; Spater et al. 2006a; Miclea et al. 2009). Similarly, dedifferentiation of rabbit articular chondrocytes was associated with increased levels of β-catenin, which decreased upon redifferentiation (Ryu et al. 2002). Furthermore, transgenic overexpression of a constitutive active LEF-1 (CA-LEF, a fusion protein of the transactivation domain of β-catenin and the DNA-binding domain of LEF-1) under the Col2a1 promoter/enhancer also resulted in disorganized, partially dedifferentiated cartilage anlagen with chondrocytes failing to mature (Tamamura et al. 2005). One of the transcription factors involved in executing the chondrogenesis-inhibitory activity of Wnt/β-catenin signaling is probably Twist1 (Reinhold et al. 2006).

During chondrocyte maturation, however, the situation turns around and canonical Wnt signaling is now playing a positive role. Retroviral-mediated expression of a constitutively active form of β-catenin, lacking the *N*-terminus, accelerated chondrocyte maturation (Hartmann and Tabin 2000). A similar phenotype was observed upon misexpression of Wnt4 and Wnt8, as well as upon conditional stabilization of β-catenin using a Col2a1-CreER line (Enomoto-Iwamoto et al. 2002; Hartmann and Tabin 2000; Dao et al. 2012). Conditional inactivation of *Ctnnb1* on the other hand resulted in a delay of chondrocyte maturation (Hu et al. 2005; Hill et al. 2005; Akiyama et al. 2004; Dao et al. 2012). Mutants lacking both co-receptors of Wnt/β-catenin signaling, LRP5 and LRP6, displayed a phenotype very similar to *Ctnnb1*-deficient animals, including delayed maturation of hypertrophic chondrocytes (Joeng et al. 2011). In line with this retroviral overexpression of the inhibitor Frzb1/sFrp-3 or a dominant-negative form of the transcription factor, LEF-1 also delayed chondrocyte maturation and Frzb1 counteracted the promoting activity of Wnt8 (Enomoto-Iwamoto et al. 2002). Mechanistically the positive effects of

Wnt/β-catenin signaling on the maturation of hypertrophic chondrocyte have been explained by the following observations: temporal downregulation of Ihh associated with a delay in chondrocyte maturation was observed in mutants lacking the Wnt-ligand Wnt9a (Spater et al. 2006b). This Wnt9a mutant phenotype was augmented by the additional loss of Wnt4 or the additional loss of one copy of *Ctnnb1* and involves probably direct regulation of Ihh expression by a β-catenin/LEF-1 transcriptional complex (Spater et al. 2006b). Furthermore, Wnt/β-catenin signaling enhances Bmp2-induced *Runx2* expression through direct interaction with the *Runx2* promoter thereby accelerating chondrocyte maturation (Dong et al. 2006). Another mechanism proposed is that chondrocyte maturation by Wnt/β-catenin signaling through LEF-1 is Sox9-mediated (Yano et al. 2005). This proposed mechanism seems to be contradictory to the results mentioned earlier, as activation of Wnt/β-catenin signaling was shown to be associated with a downregulation of Sox9 transcription (Hill et al. 2005; ten Berge et al. 2008; Kumar and Lassar 2014). Furthermore, it has been published that interaction between β-catenin and Sox9 can lead to degradation of the latter (Akiyama et al. 2004), although these results have not been confirmed in an independent study (Topol et al. 2009).

Wnt/β-catenin signaling also plays a role in mature chondrocytes. There is evidence that β-catenin is required for the repression of the expression of *receptor activator of nuclear factor kappa-B ligand* (*Rankl*) in chondrocytes (Golovchenko et al. 2013; Wang et al. 2014). Rankl is a positive regulator of osteoclastogenesis (Kong et al. 1999). Wang and colleagues also proposed that Wnt/β-catenin signaling in chondrocytes positively regulates osteoprotegerin (Opg), a negative regulator of osteoclastogenesis; however, this was not observed in two independent studies (Golovchenko et al. 2013; Wang et al. 2014) . Based on the overexpression of the intracellular inhibitor of β-catenin ICAT, it has also been proposed that β-catenin is involved in the positive regulation of VEGF and the *matrix metalloproteinase 13* (*Mmp13*) transcription, two factors which are produced by late hypertrophic chondrocytes (Chen et al. 2008). A slight downregulation of both factors has also been reported upon loss of β-catenin activity in hypertrophic chondrocytes (Golovchenko et al. 2013). Thus, the following picture emerges: canonical Wnt/β-catenin signaling needs to be tightly controlled and fine-tuned during the different steps of chondrogenesis. The levels need to be low during the early differentiation steps from mesenchymal cells to chondroblasts, and chondroblasts to chondrocytes, while slightly increased levels of Wnt/β-catenin signaling appear to be beneficial for chondrocyte maturation, and in hypertrophic chondrocytes the pathway appears to be involved in regulating osteoclastogenesis locally at the chondro-osseous junction (Golovchenko et al. 2013; Wang et al. 2014). In addition, β-catenin is required in hypertrophic chondrocytes for the transdifferentiation of chondrocyte-derived osteoblast precursors, as seen by the dramatic drop of trabecular chondrocyte-derived osteoblasts upon β-catenin inactivation in hypertrophic chondrocytes (A. Houben and C. Hartmann, unpublished observation). In summary, the scenario of Wnt/β-catenin signaling during chondrogenesis is somewhat similar to the one regarding the different roles of Wnt/β-catenin during osteoblastogenesis. In the latter Wnt/β-catenin signaling acts as a

permissive signal for osteoblast differentiation, where it is required to repress the chondrogenic potential of precursors. For further osteoblast maturation, its levels need to be decreased and increased again in mature osteoblasts to regulate factors involved in bone remodeling control (reviewed in (Hartmann 2006; Baron and Kneissel 2013)).

10.5 Does the Origin/Nature of the Primary Chondrocyte Matter?

Phenotypic differences with regard to the activity of certain Wnt molecules have been observed using primary chondrocytes of different anatomical origin as well as from different species. For example, Wnt5a retroviral overexpression did not inhibit chondrogenesis in chicken limb bud micromass cultures – on the contrary it may even have a positive effect (Church et al. 2002; Hartmann and Tabin 2001; Jin et al. 2006; Tufan and Tuan 2001). However, in mandibular micromass cultures from chicken cultured up to 8 days, it was observed that the chondrocyte matrix degraded starting at day 6 of culture (Hosseini-Farahabadi et al. 2013). In contrast, Wnt5a treatment of mouse limb bud micromass cultures from days 3 to 21 did not affect matrix production or maintenance (Bradley and Drissi 2010). Differential effects have also been observed on more maturated chondrocytes: Wnt5a treatment of rabbit articular chondrocytes inhibited collagen type II expression (Ryu and Chun 2006), while no dedifferentiation effects or downregulation of collagen type II was observed when Wnt5a was overexpressed in chicken sternal chondrocytes or upon transgenic overexpression of Wnt5a under the Col2a1 promoter in mice (Yang et al. 2003; Spater et al. 2006a). Hence, these observations can be interpreted in the following ways: (a) differences in the experimental setup, treatment using either recombinant protein or conditional medium versus vector-mediated overexpression (retroviral or transgene) may account for the divergent results; (b) the same pathway is capable of eliciting different responses in cells of different origins, or (c) the same Wnt ligand can activate different pathways dependent on the receptor profile of the responding cell. For the latter this has been demonstrated for the ligand, Wnt5a, a ligand that often is referred to as a noncanonical acting Wnt ligand. However, if certain Fzd's are present, this ligand can also activate the canonical Wnt/β-catenin pathway (He et al. 1997; Mikels and Nusse 2006). As mentioned earlier, in the case of Wnt3a, it has been shown that it can activate noncanonical and canonical Wnt/β-catenin signaling in a concentration-dependent manner in human articular chondrocytes, whether this is mediated through the same receptor complexes and whether the same holds true for chondrocytes of different origin has not yet been determined (Nalesso et al. 2011). Furthermore, in osteoblasts, which share a common progenitor with chondrocytes, Wnt3a can also activate $G_{\alpha q/11}$/PKCδ signaling (Tu et al. 2007). Hence, it is probably important to know exactly which receptors are expressed by the cell type that is treated with a particular Wnt ligand and ligand concentration may matter as well.

10.6 Issues Arising Concerning Conditional Mutants in Chondrocytes

One of the possible problems arising with regard to the interpretation of conditional knockout data is the specificity of Cre lines. Although Col2a1-Cre and Aggrecan-Cre lines and the inducible versions thereof have previously been considered to be chondrocyte specific, this assumption has been put into question by a recent report showing that these promoters are also active in mesenchymal precursors giving rise to osteoprogenitors (Ono et al. 2014). As such, some of previously published loss-of-function phenotypes as well as transgenic overexpression/misexpression phenotypes using Col2a1 or Aggrecan promoters may in part result from a loss or gain of activity in mesenchymal precursors distinct from chondrocytes. In addition, given the recent in vivo findings of osteoblast precursors arising by transdifferentiation from hypertrophic chondrocytes, genetic alterations in chondrocytes will eventually also inflict functionally on osteoblasts that are directly derived from chondrocytes (Yang et al. 2014a, b; Zhou et al. 2014; Park et al. 2015) (for further information see Chap. 5). Hence, phenotypes which have previously been postulated to arise from chondrocyte-specific gene inactivation may have to be re-evaluated and potentially reinterpreted, particularly in those cases where osteoblast differentiation is also affected.

10.7 Interplay of Wnt Signaling with Other Signaling Pathways

As mentioned Wnt9a/β-catenin signaling temporally regulates the expression of Ihh, a central regulator of skeletogenesis (Kronenberg 2003; Spater et al. 2006b). These two pathways also intersect in other processes (Mak et al. 2006). Wnt signaling also differentially interacts with PthrP signaling controlling chondrocyte hypertrophy and final maturation – possibly, by multiple mechanisms. One of them may even involve direct interaction of β-catenin with the intracellular domain of the receptor favoring the $G_{\alpha q}/Ca^{2+}$ differentiation over the $G_{\alpha s}$/cAMP-proliferation pathway (Guo et al. 2009; Yano et al. 2013). The latter mechanism, however, has been proposed to occurring independent of transcriptional activation of the Wnt/β-catenin pathway (Yano et al. 2013). Wnt signaling also interacts with Bmp signaling in chondrogenesis (Papathanasiou et al. 2012; Gaur et al. 2006; Fischer et al. 2002a, b; Chen et al. 2008). Using human mesenchymal stem cells, synergistic effects of Tgfβ and Wnt/β-catenin signaling on chondrogenic differentiation have been reported (Zhou et al. 2004). In contrast, Fgf- and Wnt/β-catenin signaling have been reported to have opposing effects on early chondrogenesis in the developing limb (Kumar and Lassar 2014).

10.8 Differences in Wnt Signaling in Cartilage-Associated Disease of the Joint

The articular cartilage of the joints in the adult skeleton is subjected to disease-related degenerative changes. Alterations in Wnt signaling have been associated with these changes: the R-spondin co-receptor, Syndecan-4, has been implicated in cartilage

breakdown in osteoarthritis (OA) (Echtermeyer et al. 2009). Antagonists such as FrzB1/sFRP-3 and Dickkopf-1 have also been implicated in OA (Lories et al. 2009); for review see (Corr 2008; Blom et al. 2009; Luyten et al. 2009; Schett et al. 2008). Alterations in Wnt ligands and Wnt-signaling pathway molecules have been found in single nucleotide polymorphism association studies, and dysregulation of Wnt ligands and Wnt-signaling pathway molecules have been reported comparing chondrocytes from OA and rheumatoid arthritis patients (Garcia-Ibarbia et al. 2013; Imai et al. 2006; Sen et al. 2000). In an ex vivo articular cartilage injury study, the Wnt ligand Wnt16 and the Wnt-antagonist FrzB have been reported to be differentially expressed, and Wnt16 and β-catenin are dramatically upregulated in human OA joint areas showing moderate to severe damage (Dell'accio et al. 2008). Whether the observed dysregulation in Wnt-signaling pathway members is causal for the degeneration of the articular chondrocytes or occurs as part of a repair mechanism remains to be shown in the future.

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Cartilage Differentiation and the Actin Cytoskeleton

11

Frank Beier

Abstract

Chondrogenesis, e.g., the formation of cartilage from precursor cells, is characterized by drastic changes in cell shape and size. This involves major reorganization of the cytoskeleton, in particular the actin network. However, we have known for several decades that the actin cytoskeleton does not merely and passively respond to upstream signals but instead actively controls chondrocyte cell fate and gene expression. Recent years have provided new insights into the regulation of actin organization both during chondrogenesis (in particular through signaling proteins of the Rho GTPase family) and into the downstream mechanisms connecting actin dynamics to chondrocyte gene expression (e.g., through the chondrocyte master transcription factor Sox9). These insights increase our understanding of the fundamental processes controlling skeletal development and are also highly relevant to disturbances of normal chondrocyte function in diseases such as chondrodysplasias and osteoarthritis.

11.1 Chondrogenesis and Chondrocyte Differentiation

The vast majority of the vertebrate skeleton forms through the process of endochondral ossification where future bones are first laid down as cartilage precursors, before replacement of most of this cartilage template by bone tissue and bone marrow (Kozhemyakina et al. 2015; Kronenberg 2003). This process is reviewed in detail elsewhere in this book and will therefore not be covered extensively (for

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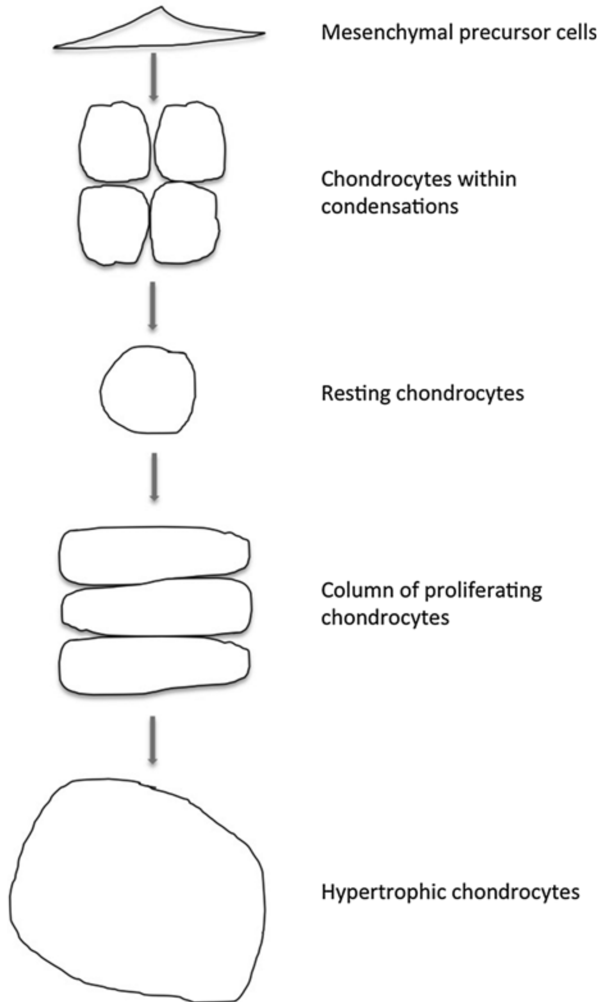

Fig. 11.1 Morphological changes during chondrocyte differentiation. Mesenchymal precursor cells have a fibroblast-like, elongated shape which changes to the cobblestone-like appearance of early chondrocytes in condensations and to rounding of resting chondrocytes. Proliferating chondrocytes form columns of flat cells parallel to long axis of the developing bone. Hypertrophic chondrocytes are characterized by a large increase in cell volume

further details, see Chaps. 4, 5, 6, and 7). However, it is important to point out the essential role of chondrocytes and their precursor cells in this process. The condensation of the earliest progenitor cells determines the location and shape of the later bones, while the cellular processes of proliferation, cell growth (e.g., hypertrophy), and extracellular matrix secretion within the cartilage growth plates control the rates of longitudinal growth of these structures. These processes are accompanied by a highly regulated, stepwise progression through a complex cellular differentiation program (Fig. 11.1).

In the most studied models of endochondral ossification, the long bones of the limbs, mesenchymal precursor cells condense and form cell-to-cell adhesions during fetal development (Woods et al. 2007a). These cells subsequently differentiate into cartilage cells, called chondrocytes (sometimes also called chondroblasts, although most people use these terms interchangeably during the early stages of cartilage formation). These cells are marked by the expression of the "Sox Trio," e.g., the transcription factors Sox5, 6, and 9, as well as their target genes collagen II and aggrecan, whose gene products form the major components of the cartilage extracellular matrix (Lefebvre and Smits 2005). These cells then form the chondrocytes in the resting zone of the growth plate. Some of these cells then accelerate their rate of proliferation; interestingly, this occurs largely along the longitudinal axis of the anlage, in a spatially controlled process. Here, cell division occurs in a plane perpendicular to the longitudinal axis of the bone, followed by rearrangement of the daughter cells in a column parallel to that longitudinal axis ("chondrocyte rotation"; for further details, see Chap. 4).

Continued proliferation of chondrocytes along this axis results in the very characteristic columns of cells, resembling stacks of coins, in the cartilage growth plate. Under the control of exogenous factors such as Indian hedgehog, parathyroid hormone-related peptide, thyroid hormones, as well as endogenous transcriptional programs, chondrocytes ultimately withdraw from the cell cycle. At this time, they begin what was, until recently, considered the terminal step in their differentiation process. They gradually increase cell size to give rise to large hypertrophic chondrocytes, thereby contributing greatly to the elongation of the skeletal elements (Sun and Beier 2014). At the same time, these differentiating cells change their gene expression patterns, inducing the expression of type X collagen (the classical marker of hypertrophic chondrocytes) and genes involved in matrix mineralization (alkaline phosphatase, bone sialoprotein [BSP]), matrix remodeling (matrix metalloproteinase 13 [MMP13]), and tissue turnover (vascular endothelial growth factor [VEGF], receptor of activator of nuclear factor kappa-B ligand [RANKL]). This is mediated by a distinct set of transcription factors including Runx2/3, MEF2C, and FoxA2/3 (Sun and Beier 2014).

Until recently, the most common view was that hypertrophic chondrocytes ultimately undergo apoptosis to make space for invading bone cells (osteoblasts) and the resulting formation of trabecular bone (Tsang et al. 2015). However, strong evidence from lineage studies in mice have challenged this view and suggest that at least a subset of these hypertrophic chondrocytes gives rise to osteoblasts and their descendants, osteocytes (for further details, see Chap. 5). While not everyone has embraced this concept and the mechanisms involved are still unclear, further studies will undoubtedly resolve the prevalence and significance of this phenomenon and greatly influence our understanding of endochondral ossification.

11.1.1 Pathological Chondrogenesis and Chondrocyte Hypertrophy

The processes described above are essential for normal skeletal growth and development. Disturbance by gene mutations (e.g., in chondrodysplasias) or

environmental factors (diet, injuries, medications, mechanical loading, etc.) can all lead to skeletal dwarfism and deformities (van der Eerden et al. 2003; Ballock and O'Keefe 2003). Thus, understanding the mechanisms that control chondrogenesis and chondrocyte differentiation is essential for a better understanding, prevention, and treatment of these diseases.

Moreover, improper skeletal differentiation has been implicated in a large number of other diseases. During adulthood, the cartilage remains in articular joints (e.g., articular cartilage) and is essential for normal joint movement. The chondrocytes of articular cartilage are similar, although not identical, to the chondrocytes of the resting zone of the growth plate described above. They usually have a stable phenotype and don't undergo rapid proliferation or hypertrophy. However, it has been recognized now that under certain conditions – for example, after cartilage or joint injury – these cells can undergo a phenotypic change and adopt a growth plate phenotype, involving proliferation (leading to chondrocyte clusters) followed by hypertrophy (Dreier 2010; Pitsillides and Beier 2011; van der Kraan and van den Berg 2012). Molecules secreted by hypertrophic chondrocytes such as matrix metalloproteinase (MMP)-13 and vascular endothelial growth factor (VEGF) then initiate events associated with osteoarthritis (such as extracellular matrix breakdown, angiogenesis, and ultimately osteogenesis). Thus, this ectopic adoption of a growth plate phenotype appears to be one of the pathways leading to cartilage degeneration in osteoarthritis. Ectopic endochondral ossification in other joint structures such as ligaments, meniscus, etc. has also been observed. For example, work by us and others has shown that loss of the signaling protein Mig-6 in mice leads to massive endochondral ossification in many structures surrounding the knee and other joints (Staal et al. 2014; Pest et al. 2014; Jin et al. 2007; Zhang et al. 2005).

Ectopic endochondral ossification has also been shown outside the skeletal system itself. One rare but extremely severe example is the genetic disease fibrodysplasia ossificans progressiva (FOP) (Kaplan et al. 2012). Due to an activating mutation in a gene for a bone morphogenetic protein (BMP) receptor, nonskeletal tissues (such as muscle) in these patients gradually turn into bone, leading to paralyses and ultimately death. Much more common, but equally devastating, is ectopic endochondral ossification in cardiovascular tissues, leading to vascular calcification, which in turn is a major risk factor for many cardiovascular diseases (Demer and Tintut 2014). All these examples highlight that better understanding of the pathways controlling chondrogenesis and chondrocyte differentiation is essential for improved prevention and management of many diseases. However, while we have made substantial progress over recent decades, much remains to be learned about the regulatory pathways controlling each step in this complex process.

11.2 The Actin Cytoskeleton in Chondrogenesis

When examining chondrocyte differentiation, one of the most obvious features is the striking change in cellular shape and appearance at each stage. Mesenchymal precursor cells are elongated and fibroblastoid (Fig. 11.1). Upon chondrogenesis,

the cells take on a rounded to cobblestone-like appearance and are initially closely packed within the condensations, with cell-cell adhesions through cadherins and other adhesion molecules (Woods et al. 2007a). In the resting zone of the growth plate, cells remain rounded but became separated from one another, as they secrete large amounts of extracellular matrix. In contrast, in the proliferative zone, cells adopt a flattened appearance, with close contacts to their clonal neighbors within each column (but separated laterally from other columns by septa of extracellular matrix). Finally, in the hypertrophic zone, cells undergo a large expansion in cell volume while returning to a round to polygonal shape. If hypertrophic chondrocytes indeed undergo a further switch (e.g., trans-differentiation or asymmetric division) to an osteoblastic cell, this step would require another extensive change in cellular morphology.

Strikingly, these changes in cell shape, size, and organization appear to be correlated to changes in their differentiation, as judged by their gene expression patterns. When chondrocytes are plated in monolayer culture on plastic, they rapidly loose their chondrogenic phenotype and revert to a fibroblastoid, elongated appearance and loose expression of chondrocyte marker genes such as type II collagen and aggrecan (Dessau et al. 1981; von der Mark and Conrad 1979; von der Mark et al. 1977; Grundmann et al. 1980). At the same time, fibroblast markers such as collagen I and tenascin C are upregulated under these conditions (Parreno et al. 2014). However, these molecular changes can (at least at early stages/passages) be reverted by different culture methods that do not allow the adoption of the fibroblastoid cell shape, such as suspension culture or three-dimensional culture in gels, beads, or micromasses. These data point to a strong connection between cell shape and chondrogenic gene expression (Daniels and Solursh 1991; Solursh et al. 1982). Thus, the question is whether these morphological changes (1) are secondary to changes in gene expression; (2) appear in parallel but independent to gene expression changes, in response to some common upstream signal; or (3) play a more central role in regulating molecular events in chondrocyte differentiation. Cell shape is regulated by the interplay between extracellular forces on the cell, including cell-matrix and cell-cell contacts, and the cytoskeleton, in particular the actin-myosin component. Thus, research over several decades has tried to elucidate the links between actin organization and chondrogenic cell differentiation (Daniels and Solursh 1991).

Early work demonstrated that “dedifferentiation” of chondrocytes to fibroblast-like cells was accompanied by reorganization of the actin cytoskeleton (Zanetti and Solursh 1984). Differentiated chondrocytes display relatively little filamentous actin that is organized in a cortical fashion, e.g., at the cell periphery directly underneath the cell membrane. Upon dedifferentiation in monolayer culture, actin polymerization increases and leads to the formation of stress fibers, thick actin cables that transverse the cells and are typical of fibroblasts (Zanetti and Solursh 1984).

The logical next experiments were then to interfere with actin polymerization and examine how this affects the maintenance or loss of the chondrocyte phenotype.

Several drugs allow the suppression of actin polymerization, including latrunculin B, cytochalasin D, and dihydrochalasin B. While these compounds act through partially different mechanisms, one common and maybe surprising feature is that they generally support the reestablishment of the chondrocyte phenotype along with chondrogenic gene expression and extracellular matrix synthesis patterns (Zanetti and Solursh 1984; Benya and Padilla 1993; Brown and Benya 1988; Benya 1988; Benya and Shaffer 1982; Benya et al. 1978, 1988). These data strongly suggest that suppression of changes in actin organization – and the subsequent changes in cell morphology – can prevent chondrocyte dedifferentiation. Thus, the actin cytoskeleton appears to not just be a simple effector of upstream signals but an active regulator of cell fate during chondrogenesis. This scenario does not only apply to dedifferentiated chondrocytes (which might have an epigenetic memory of their previous phenotype) but also holds true to the chondrogenic differentiation of mouse embryonic stem cells (Zhang et al. 2006).

One open question is how changes in the actin cytoskeleton lead to altered gene expression. One key factor appears to be the master chondrogenic transcription factor Sox9, along with Sox5 and Sox6. Our data show that cytochalasin D treatment induces Sox9 expression and activity in micromass cultures, although the mechanisms mediating this induction remain unknown (Woods and Beier 2006; Woods et al. 2005). More recently, Parreno et al. demonstrated that myocardin-related transcription factor (MRTF) mediates induction of fibroblast markers under dedifferentiation conditions, which can be reversed by the actin polymerization inhibitor latrunculin B (Parreno et al. 2014). Thus, some insights into the molecular mechanisms connecting actin dynamics and chondrogenic gene expression are emerging, although much work remains to be done. Moreover, it needs to be recognized that the situation is more complex than it appears at first glance. For example, cytochalasin D effects on chondrogenic gene expression appear to be context – and culture model dependent (Woods and Beier 2006; Woods et al. 2005). Moreover, the actin stabilizer jasplakinolide (which would be expected to have opposite effects as the actin polymerization inhibitors) can also promote a chondrogenic phenotype under certain conditions (Woods and Beier 2006; Woods et al. 2005). Clearly, we are just scratching the surface when it comes to the understanding of the molecular links between the actin cytoskeleton and chondrocyte biology.

Actin dynamics have also linked to later stages of chondrocyte differentiation. We have performed microarray experiments on the effects of cytochalasin D treatment on chondrocyte gene expression and compared these data to previous studies on in vivo and in vitro models of chondrocyte hypertrophy (Woods et al. 2009). Indeed, our data suggest that this actin polymerization inhibitor promotes the hypertrophic phenotype, as judged by gene expression changes. When used in an embryonic tibia organ culture model as a model of endochondral bone growth, cytochalasin D caused (maybe not surprisingly) a loss of the normal structure of the growth plate (Woods et al. 2009). However, treated chondrocytes appeared larger, and the tibiae grew significantly more in the presence of this compound. This suggests a continued role of actin dynamics as chondrocytes mature to

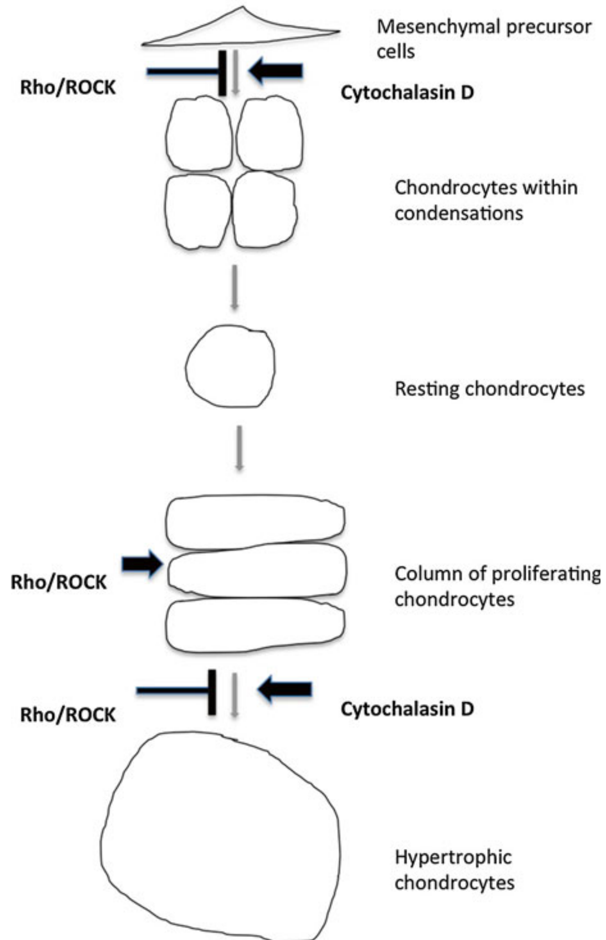

Fig. 11.2 Effects of actin polymerization inhibitors and RhoA/ROCK signaling on chondrocyte differentiation. Cytochalasin D and other actin polymerization inhibitors promote both early chondrogenesis and hypertrophic chondrocyte differentiation. In contrast, activation of the RhoA/ROCK pathway suppresses both of these processes but stimulates chondrocyte proliferation

hypertrophy. Effects of actin modulators on chondrocyte differentiation are summarized in Fig. 11.2.

Finally, analyses of mutations in mouse and human genes encoding actin-interacting proteins provide further evidence for a key role of actin dynamics in cartilage development. In humans, mutations in the filamin A and B genes give rise to skeletal diseases including chondrodysplasias (Bicknell et al. 2005; Krakow et al. 2004; Robertson et al. 2003; Feng and Walsh 2004), while mice deficient for adservin, filamin B, or formin 1 show growth plate phenotypes (Zhou et al. 2007; Zheng et al. 2007; Nurminsky et al. 2007; Hu et al. 2014). As described above, the precise links between the roles of these proteins in actin organization and in cartilage development remain to be elucidated, but these phenotypes highlight the importance of proper regulation of actin dynamics in skeletal development.

11.3 Actin-Regulatory Pathways in Cartilage Development: Rho GTPases

Another important question is what regulates the organization and dynamics of actin during chondrocyte differentiation. Many studies in multiple tissues and cell types have established a family of small signaling proteins, the Rho GTPases, as the master regulators of the actin cytoskeleton (Vega and Ridley 2008; Heasman and Ridley 2008). Rho GTPases, similar to the related Ras and Rab proteins, act as molecular switches. In their GTP-bound form, they are active and stimulate a large number of downstream pathways, including cytoskeletal pathways, transcription, cell cycle progression, and vesicular traffic (Fig. 11.3). Hydrolysis of the GTP to GDP leads to their inactivation, a process that can be accelerated by GTPase-activating proteins or GAPs. Exchange of GDP to GTP, and thereby reactivation of the GTPases, is catalyzed by guanine nucleotide exchange factors or GEFs. There are numerous different GEFs and GAPs for the Rho family of GTPases. Their activities are controlled by upstream signals acting through various receptors, including G-protein-coupled receptors, receptor tyrosine kinases, and integrins. Thus, the

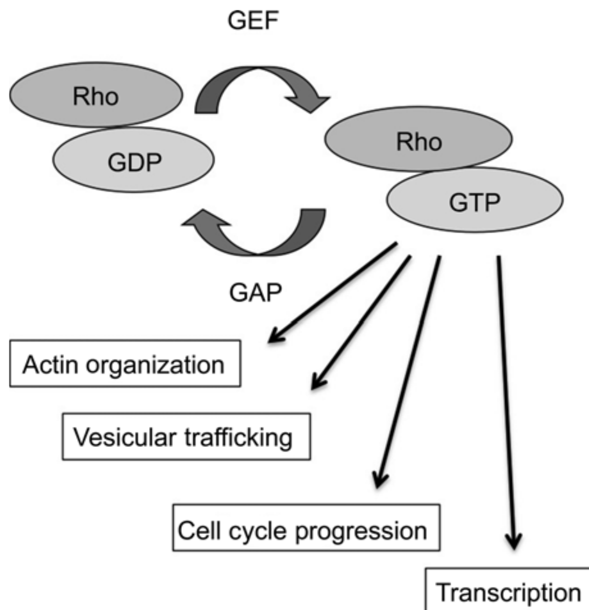

Fig. 11.3 Rho GTPase cycle. Rho GTPases such as RhoA, Rac1, and Cdc42 are active when they are bound to GTP and stimulate a number of cellular activities, including (but not limited to) actin organization, vesicular trafficking, cell cycle progression, and transcription. Inactivation of the Rho GTPases occurs by hydrolysis of GTP to GDP, which is enhanced by GAPs (GTPase-activating proteins). Reactivation requires exchange of GDP to GTP, which is catalyzed by guanine nucleotide exchange factors (*GEFs*). Both GAPs and GEFs are directly controlled by cell surface receptors, providing a link between extracellular signals and Rho GTPase activity

GEF and GAP proteins connect Rho GTPases to upstream signals from growth factors, hormones, extracellular matrix components, and likely biomechanical signals. The three prototypic Rho GTPases RhoA, Rac1, and Cdcd42 have been investigated in the context of chondrogenesis.

11.3.1 RhoA and Its Downstream Kinases ROCK1/2

RhoA can induce stress fibers, in part through the downstream kinases ROCK1 and ROCK2 (Riento and Ridley 2003). Based on these activities and the negative correlation of stress fiber formation and chondrogenesis discussed above, we postulated that Rho/ROCK signaling would suppress chondrogenesis. Indeed, overexpression of RhoA in the murine chondrogenic cell line ATDC5 suppressed markers of chondrogenesis such as collagen II and aggrecan expression as well as glycosaminoglycan (GAG) synthesis (Woods and Beier 2006; Woods et al. 2005). In contrast, inhibition of ROCK signaling by the small molecule Y27632 promotes round cell shape, cortical actin organization (Fig. 11.4) and chondrogenic gene expression in primary chondrocytes in monolayer culture. This effect appears to be mediated by induction of Sox9, similar to that of cytochalasin D. Similar results were subsequently obtained by others (Kumar and Lassar 2009; Matsumoto et al. 2012). Y27632, as well as the RhoA-inhibiting toxin C3, also blocks stress fiber formation induced by transforming growth factor alpha (TGF-α) (Appleton et al. 2010), a member of the epidermal growth factor family that we have implicated in osteoarthritis pathogenesis (Appleton et al. 2007). In addition, Y27632 blocked the breakdown of cartilage extracellular matrix proteins induced by TGF-α in an explant model of rat articular cartilage (Appleton et al. 2010).

Rho/ROCK inhibition therefore appears to promote chondrogenesis, suggesting that it could have a therapeutic application in diseases such as osteoarthritis (while actin-modifying drugs might have to widespread effects to be considered in this context). A recent study indeed suggested that a different ROCK inhibitor has some beneficial effects in a rat model of osteoarthritis (Takeshita et al. 2011). However, we have also shown that ROCK inhibition does not only promote chondrogenesis but also later stages of the chondrocyte life cycle such as hypertrophy (Wang et al. 2004). Given the likely pathogenic role of ectopic chondrocyte hypertrophy in osteoarthritis discussed above, it remains to be seen whether this pathway is a reasonable target for long-term treatments.

11.3.2 Rac1/Cdc42

In many tissues, the activities of RhoA are antagonized by signaling from the other prototypic Rho GTPases Rac1 and Cdc42. Data from our and other labs suggest a similar scenario for cartilage. Overexpression of Rac1 or Cdc42 indeed induces both: early chondrogenesis and hypertrophic differentiation in ATDC5 cells (Woods et al. 2007b; Wang and Beier 2005), the opposite phenotype of what we saw upon

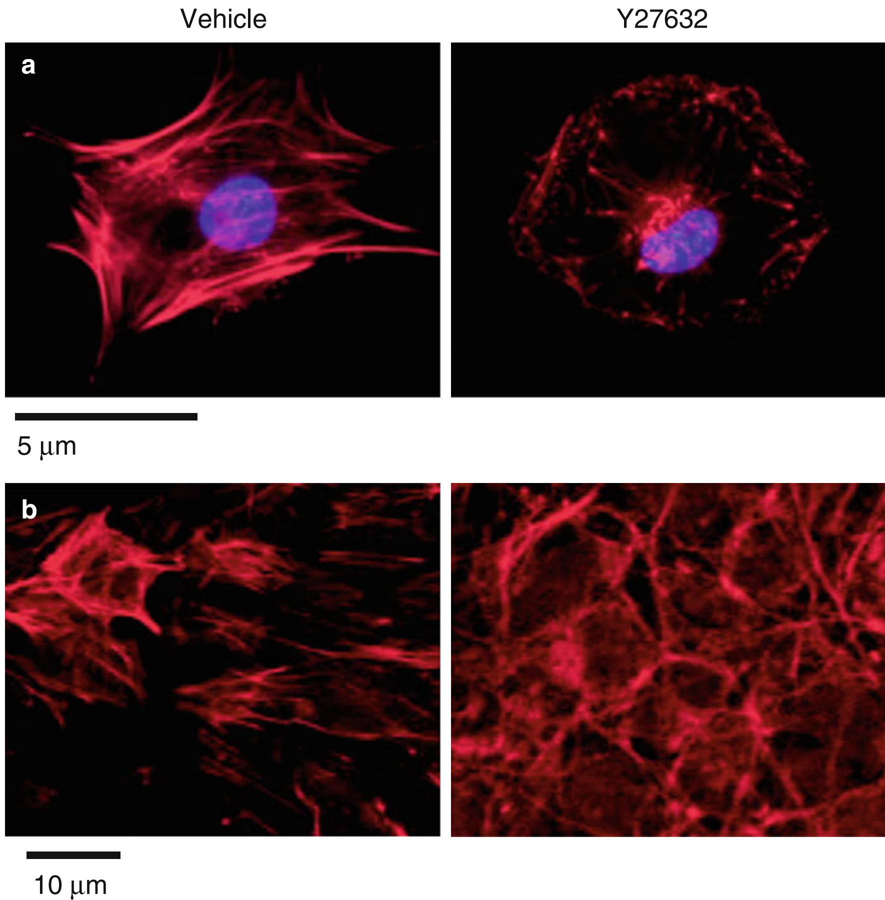

Fig. 11.4 Effects of ROCK inhibition on the chondrocyte actin cytoskeleton. Primary mouse chondrocytes in monolayer culture (**a**) or mouse mesenchymal limb bud cells in micromass culture (**b**) were treated with vehicle (DMSO) or 10 microM Y27632, a ROCK inhibitor, and stained with rhodamine phalloidin (*red*) for the actin cytoskeleton and Hoechst for nuclei (*blue*). Compared to vehicle-treated cultures, ROCK-inhibition reduces the number of stress fibers and increases cortical actin staining as well as cell rounding (Adapted from Woods et al. 2005)

overexpression of RhoA. These activities appear, at least partially, to be mediated by the p38 MAP kinase pathway, a known promoter of chondrogenic differentiation (Bobick and Kulyk 2008). Similar findings were reported by others (Kerr et al. 2008).

However, these findings were obtained in vitro, with cell lines and primary cells. More recently, we and others generated conditional mouse mutants for the *Rac1* and *Cdc42* genes that suggest a somewhat more complex role of these genes in vivo. Cartilage-specific Rac1 “knockout” mice, generated using a collagen II Cre driver line, display a high degree of perinatal lethality (Wang et al. 2007). Surviving mice are dwarfed with numerous abnormalities in the axial and appendicular skeleton.

Their growth plates are strikingly disorganized and hypocellular, with reduced chondrocyte proliferation, decreased expression of the cell cycle promoter cyclin D1, and increased rates of apoptosis. However, the expected delay or reduction of hypertrophic differentiation, which we had postulated based on our in vitro results, was not observed. Instead, accelerated cell cycle exit based on reduced cyclin D1 expression seemed to result in premature onset of hypertrophic differentiation. Primary chondrocytes from these mice showed altered cell shape, actin organization, and adhesion to extracellular matrix proteins, suggesting a disruption of adhesive signaling (Wang et al. 2007). Indeed, the growth plate phenotype of these mice demonstrated substantial similarities to that of cartilage-specific knockout mice for beta1-integrin and integrin-linked kinase (ILK) (Terpstra et al. 2003; Grashoff et al. 2003; Aszodi et al. 2003).

Similar phenotypes were seen in mice where the *Rac1* gene was deleted using the Prx1 Cre driver line which is active in limb mesenchyme, in addition to other tissues (Suzuki et al. 2009). Due to the wider and earlier expression of this Cre driver, the authors also noticed additional phenotypes, such as syndactyly due to loss of apoptosis in the interdigital areas. The same authors subsequently generated *Cdc42* conditional knockout mice, using both Prx1 and collagen II Cre driver lines (Aizawa et al. 2012; Suzuki et al. 2015). The phenotypes of these mice resembled that of the *Rac1* mutants described above, with syndactyly, greatly reduced skeletal size, disorganized growth plates, and shorter proliferative zones. Interestingly, the hypertrophic zone was enlarged, but expression of hypertrophic marker genes such as collagen X and MMP13 was reduced. These data demonstrate essential but overlapping roles of Rac1 and Cdc42 in cartilage; analyses of double mutants would be of great interest, although a very severe phenotype is to be expected.

Based on these findings, it is not surprising that researchers have started to examine the role of Rac1 in osteoarthritis. Both fibronectin fragments and epidermal growth factor ligands activate Rac1 in articular chondrocytes, and Rac1 inhibition blocks the expression of MMP13, the key collagenase in cartilage breakdown in this disease (Long et al. 2013). In vivo studies suggest that Rac1 inhibition by lentiviral delivery of a dominant-negative form or by the small molecule inhibitor NSC23766 slowed osteoarthritis in a mouse model, whereas overexpression of an activated form accelerated cartilage breakdown (Zhu et al. 2015b). A follow-up study by the same authors suggest that in human osteoarthritis, expression of OCRL1, a GAP (inhibitor) for Rac1, is reduced, leading to aberrant activation of Rac1 signaling and thus cartilage breakdown (Zhu et al. 2015a). Thus, Rac1 inhibition appears to be protective in the context of osteoarthritis, which is somehow surprising given the strong skeletal phenotype of *Rac1* knockout mice. Generation of inducible (adult) cartilage-specific knockout mice will be required to address this issue further and is under way in our laboratory.

In addition, comparable studies for Cdc42 and RhoA should be performed. Moreover, we need to remember that these Rho GTPases act through multiple downstream pathways, not only the actin cytoskeleton. For example, we have recently identified the iNOS gene as an important mediator of Rac1 signaling in the regulation of chondrocyte proliferation (Wang et al. 2011).

11.4 Open Questions

While it is now clear that the actin cytoskeleton is an important driver of cartilage development and chondrocyte differentiation, numerous questions remain. Downstream of actin, the mechanisms connecting cytoskeletal dynamics to gene expression and cell maturation are still poorly understood. Upstream, the actin-dependent and actin-independent roles of Rho GTPases need to be clarified further. Other, non-Rho pathways controlling actin turnover mechanisms need to be examined. More generally, the links between extracellular signals, both from biochemical and biomechanical factors, and the organization of the actin cytoskeleton have to be examined in much more detail, ideally in real time in living organisms, in the authentic three-dimensional tissue architecture. This is extremely challenging from a technical perspective, but recent and forthcoming advances in imaging, genetic engineering and other techniques raise the hopes for rapid progress in this area.

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