Regulatory Mechanisms of Chondrogenesis and Implications for Understanding Articular Cartilage Homeostasis

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Abstract: Studies of chondrogenesis and embryonic limb development offer a wealth of knowledge regarding signals that regulate the behavior of chondrocytes. Many such chondrogenic regulators are upregulated in osteoarthritis-affected chondrocytes, suggesting a role in pathogenesis. Yet, some of the same factors also support adult articular cartilage homeostasis, and enhance neo-cartilage tissue formation in experimental models. In this review, we summarize many of the important regulatory mechanisms involved in chondrogenesis and examine how their disruption may contribute to functional changes in articular cartilage during osteoarthritis or aging.

INTRODUCTION

Understanding the mechanisms that control chondrogenesis is integral to the study of osteoarthritis (OA) and critical to the development of biological therapies for its treatment or prevention. Many molecules that regulate chondrogenesis such as members of the fibroblast growth factor (FGFs) or transforming growth factor β (TGFβ)/bone morphogenetic protein (BMP) superfamilies enhance the formation of cartilage-like tissue from pleuripotent stem cells or isolated chondrocytes. Such 'chondrogenic' factors may also be relevant for the treatment of OA, as cartilage, being avascular and aneural, cannot mount a vigorous endogenous repair reponse to injuries that predispose the joint to degeneration. The application of exogenous cells is already in clinical use for the repair of discrete cartilage lesions, and the judicious addition of 'chondrogenic' factors may enhance this process further. On the other hand, many of these same genes are up regulated in OA-affected chondrocytes; yet their roles in the pathogenesis of OA have not been fully elucidated. Discerning whether 'chondrogenic' mechanisms are pathological or reparative is critical not only for understanding OA pathogenesis, but in the development of biological therapies for OA.

A particularly important consideration in studying the regulation of chondrogenesis in the context of OA is the distinction between articular and 'growth' chondrocytes. Much of what is known about chondrogenesis is based on studies of 'growth' chondrocytes of the developing limb and, to a lesser degree, the post-natal growth plate during endochondral ossification. While 'growth' chondrocytes and the adult articular chondrocytes likely arise from the same progenitors and share many features, they probably have undergone distinct differentiation pathways. In many instances, 'growth' and articular chondrocytes do not respond to regulatory cues in the same manner. In addition, articular chondrocytes display considerable heterogeneity. Studies from several laboratories, including our own, have confirmed that cells within 100 μm of the articular surface (superficial zone chondrocytes, SZC) are phenotypically distinct from those near the calcified zone and sub-chondral interface (deep zone chondrocytes, DZC) [1-9].

Here, we review many of the mechanisms that regulate chondrogenesis in the context of embryonic bone and joint formation and consider how they may relate to the normal physiology of adult articular cartilage, neo-cartilage tissue formation (cartilage tissue engineering), and pathologic changes in OA.

CHONDROGENESIS DURING LIMB AND JOINT DEVELOPMENT

The skeleton develops from the primitive, avascular, densely packed cellular mesenchyme, termed the skeletal blastema. Common precursor mesenchymal cells divide into chondrogenic, myogenic, and osteogenic lineages that determine the differentiation of cartilage centrally, muscle peripherally, and bone. The surrounding tissues, particularly epithelium, influence the differentiation of mesenchymal progenitor cells to chondrocytes in cartilage anlagen. The cartilaginous nodules appear in the middle of the blastema, and, simultaneously, cells at the periphery become flattened and elongated to form the perichondrium. In the vertebral column, cartilage disks arise from portions of the somites surrounding the notochord. In the limb, chondrocytes undergo maturation and hypertrophy, whereby the growth plate is calcified and is replaced by bone by a process termed endochondral ossification. The latter process requires extracellular matrix remodeling and vascularization, or angiogenesis. These events are controlled exquisitely by cellular interactions with the surrounding matrix, growth and differentiation factors and other environmental factors that initiate or suppress cellular signaling pathways and transcription of specific genes in a temporal-spatial manner. The organization of the growth plate allows for the directional growth of bone, where the least mature chondrocytes remain near the joint surface. The genesis of this organization may provide insights into the significance of chondrocyte derangement in OA. Furthermore, understanding the earliest events in chondrogenesis, such as condensation, may be important for optimizing techniques to generate neo-cartilage tissue from

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adult mesenchymal stem cells (MSCs) derived from bone marrow or other tissues (as addressed by Tuan in this issue).

CONDENSATION AND LIMB BUD FORMATION

Formation of the cartilage anlage occurs in four stages: (1) cell migration, (2) aggregation regulated by mesenchymal–epithelial cell interactions, (3) condensation, and (4) overt chondrocyte differentiation, or chondrification [10, 11]. Interactions with the epithelium determine mesenchymal cell recruitment and migration, proliferation, and condensation [11-13]. The aggregation of chondroprogenitor mesenchymal cells into pre-cartilage condensations was first described by Fell [14] and depends on signals initiated by cell-cell and cell-matrix interactions, the formation of gap junctions, and changes in the cytoskeletal architecture. Prior to condensation, the prechondrocytic mesenchymal cells produce extracellular matrix that is rich in hyaluronan and collagen type I, as well as type IIA collagen, which contains the exon-2-encoded aminopropeptide found in noncartilage collagens [15]. The initiation of condensation is associated with increased hyaluronidase activity and the appearance of the cell adhesion molecules, neural cadherin (N-cadherin) and neural cell adhesion molecule (N-CAM), which disappear in differentiating chondrocytes and are detectable later only in perichondrial cells. Prior to chondrocyte differentiation, the cell-matrix interactions are facilitated by fibronectin binding to syndecan, thereby downregulating N-CAM and setting the condensation boundaries. Increased cell proliferation and ECM remodeling, with the disappearance of type I collagen, fibronectin, and N-cadherin and the appearance of tenascins, matrilins, and thrombospondins, including cartilage oligomeric protein (COMP), initiate the transition from chondroprogenitor cells to a fully committed chondrocyte [13, 16-18].

Much of our current understanding of limb development is based on early studies in chicken and more recently in mice. Early patterning events are regulated by signals produced from the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA) [16, 17]. Important factors include FGF, hedgehog, bone morphogenetic protein (BMP), and Wnt pathways, each of which functions sequentially over time, and these continue to play roles later in chondrogenesis [19]. The Wnts are diffusible factors that signal through binding to frizzled (FRZ) receptors and other related co-receptors such as LRPs. Wnt signaling *via* β-catenin is required to induce FGFs, such as FGF-10 and FGF-8, which act in positive feedback loops [19, 20]. FGF-2, -4, and -8 (induced by Wnt-3A [21], from the specialized epithelial cells in the AER that are covering the limb-bud tip, control proximal-distal outgrowth [22]. The homeobox (Hox) transcription factors encoded by the HoxA and HoxD gene clusters, which are critical for the early events of limb patterning in the undifferentiated mesenchyme, are required for the expression of FGF-8 and Sonic hedgehog (Shh) [23], and they modulate the proliferation of cells within the condensations [11]. Among the Hox genes, Hoxa13 and Hoxd13 enhance and Hoxa11 and Hoxd11 suppress early events in the formation of the cartilage anlagen.

Wnt7a is expressed early during limb bud development where it acts to maintain Shh expression. Shh, produced by a small group of cells in the posterior zone of the ZPA (in response to retinoic acid in the mesoderm [24] and FGF-4 in the AER [25] plays a key role in both directing anterior-posterior patterning [24, 26] and stimulating expression of BMP-2, -4, and -7, and Hox genes [27-29] Shh signaling, which is required for early limb patterning, though not for limb formation, is mediated by the Shh receptor Patched (Ptc1), which activates another transmembrane protein Smoothened (Smo) and inhibits processing of the Gli3 transcription factor to a transcriptional repressor [20, 30]. Dorsal-ventral patterning depends on the secretion of Wnt-7A [20, 31].

First identified by Urist [32] as the activity within the unmineralized bone matrix that is able to induce local ectopic bone, the BMPs act by binding to several activin-like kinase receptors (ALKs, also known as BMP receptors IA, B and II) [33-35]. BMP-2, -4, and 7 coordinately regulate the patterning of limb elements within the condensations depending upon the temporal and spatial expression of BMP receptors, involving SMAD-dependent and independent intracellular signaling and soluble BMP antagonists, such as Noggin and Chordin [19, 36-38]. *In vitro* and *in vivo* studies have shown that BMP signaling is required both for the formation of pre-cartilaginous condensations and for the differentiation of precursors into chondrocytes [39]. Growth of the condensation ceases when Noggin inhibits BMP signaling and permits overt differentiation to chondrocytes, which are often designated as "chondroblasts". The cartilage thus formed then serves as a template for formation of cartilage elements in the vertebrae, sternum, rib, etc. and for limb elongation or endochondral bone formation. The cartilage anlagen grow by cell division and deposition of the extracellular matrix and by apposition of proliferating cells from the inner chondrogenic layer of the perichondrium.

The nuclear transcription factor, Sox9, is one of earliest markers expressed in cells undergoing condensation and is required for the subsequent stage of chondrogenesis characterized by the deposition of matrix containing collagens II, IX and XI and aggrecan in the cartilage anlagen [40, 41]. Two additional Sox family members, L-Sox5 and Sox6, which are not present in early mesenchymal condensations, but are co-expressed late with Sox9 and required for chondrocyte differentiation [42], have a high degree of sequence identity with each other, but have no sequence homology with Sox9 except in the HMG box. They can form homo- or heterodimers, which bind more efficiently to pairs of HMG box sites than to single sites, and unlike Sox9, they contain no transcriptional activation domain. The expression of SOX proteins is dependent upon BMP signaling *via* BMPR1A and BMPR1B, which are functionally redundant and active in chondrocyte condensations but not in the perichondrium [39]. Recent studies indicate that p63, a transcription factor in the p53 family also controls Sox and Col2a1 expression [43]. The runt-domain transcription factor, Runx2 (also known as Core binding factor, Cbfa1), is also expressed in all condensations; its activity is particularly important later, during chondrocyte hypertrophy [44-46].

In contrast to BMP, Sox9 and Runx2, which are 'pro-chondrogenic' the Notch and related factors appear to be negative regulators of chondrogenesis which are none the less important from the earliest stages of limb development, including mesenchymal condensations [47]. Of the Notch family members, Notch-1, -2 and -3 are expressed at various

stages of chondrogenesis [48]. Condensation provides an ideal milieu for the cell-cell interactions required for activation of Notch, which are cell surface molecules with their ligands, Jagged, Serrate and Delta, which are also cell surface molecules [48]. Downstream activation of HES transcription factors are important in the modulatory action of Notch activation in chondrogenesis [48, 49], and activation through Jagged and Delta binding have been described at various steps of chondrogenesis [49, 50]. The Notch pathway has come to be of particular interest in the field since the identification of Notch-positive stem cells in post-natal articular cartilage [51]. However, it remains unproven whether Notch or its ligands are actual markers of such cells [52].

INTERZONE FORMATION AND JOINT SPECIFICATION

The morphology of the developing synovial joint and the process of joint cavitation have been described in many classic studies carried out on the limbs of mammalian and avian embryos [53]. Within developing condensations, a cartilagenous interzone first appears, specifying the future joint and segmenting a portion of the limb bud. This is followed by development of a three-layered interzone, which consists of two chondrogenic, perichondrium-like layers that cover the opposing surfaces of the cartilage anlagen and are separated by a narrow band of densely packed cellular blastema that remains and forms the interzone. It is within this central interzone that cavitation and development of the joint cavity eventually ensues. This process does not involve necrosis or apoptosis of interzone cells nor remodeling by metalloproteinases, but rather mechanospatial changes and cell migration facilitated by increasing levels of hyaluronan, synthesized by uridine diphosphoglucose dehydrogenase (UDPGD) and hyaluronan synthase. and its cell surface receptor, CD44 induced by Wnt14 [54-57].

A recent study has shown that the cartilaginous interzone consists of committed chondrocytes that have already begun to express COL2A1, but which cease to express matrilin-1 (Mat1) [58]. While it is unclear what the function of Mat1 may be in defining differentiated phenotypes, the sequence of gene expression observed in this study suggests that a divergence in differentiation between articular and 'growth' chondrocytes may occur after initial chondrocyte commitment, but at the initial stage of joint specification, when the interzone first appears.

These earliest events in joint formation establish the joint as a center of chondrogenic modulation that provides inhibitory signals at the ends of future bones to balance the vascular in growth and ossification that will later occur in the middle of those bones. For example, Wnt 14 whose expression is required for the earliest steps of joint specification acts through a non-canonical (non-β-catenin) pathway, negatively regulating chondrogenesis at the site of future joints [59]. The BMP family member growth and differentiation factor-5 (GDF5, also called cartilage-derived morphogenetic factor -1 or CDMP1) is a critical downstream activator of Wnt 14 during joint formation. In contrast to Wnt 14, GDF5, on its own, has relatively 'pro-chondrogenic' activities such as mesenchymal cell recruitment and inducing their chondrogenic differentiation [60-63]. Exogenous application of GDF5 to developing joints causes chondrocyte overgrowth and joint fusions, however, underscoring the necessity for inhibition of chondrogenesis in the developing joint [61, 62]. In that light, BMP antagonists, such as Chordin, which is also downstream of Wnt14, and Noggin, whose deficiency results in joint fusion and overgrowth not unlike the application or over expression of GDF5 or BMPs around the developing joint [62, 64], are required for normal joint development. Expression of FGFs-2 and -9 are also essential for joint formation, and these factors may also balance BMP activity. The ETS factor ERG/C-1-1 is also an 'anti-maturational' factor for chondrocytes, directly antagonizing the activity of Runx2 [65]. Clearly, early chondrogenesis requires a balance of pro- and anti-chondrogenic signals.

CHONDROCYTE PROLIFERATION AND MATURATION

Proliferation of chondrocytes in the embryonic and post-natal growth plate is regulated by multiple mitogenic stimuli, which converge on the cyclin D1 gene [66]. As in earlier stages of development the FGF/BMP axis remains important. Hedgehog signaling also plays an important role, although at this stage, Indian hedgehog (Ihh) and its interaction with parathyroid hormone related peptide (PTHrP) comes to prominence. The temporal and spatial balance between BMP and FGF ligands and receptors determines the rate of chondrocyte proliferation during chondrogenesis, thereby adjusting the pace of the differentiation [67, 68]. BMP-2, -3, -4, -5, and -7 are expressed primarily in the perichondrium and only BMP-7 is expressed in the proliferating chondrocytes [67]. BMP-6 is found exclusively later in hypertrophic chondrocytes along with BMP-2 [68]. FGFR2 is upregulated early in condensing mesenchyme and is present later in the periphery of the condensation along with FGFR1, which is expressed in surrounding loose mesenchyme. FGFR3 is associated with proliferation of chondrocytes in the central core of the mesenchymal condensation and may overlap with FGFR2 [69].

During the transition of proliferating chondrocytes to the prehypertrophic stage in the growth plate, FGFR3 serves as a master inhibitor of chondrocyte proliferation *via* phosphorylation of the Stat1 transcription factor, which increases the expression of the cell cycle inhibitor p21 [70]. FGF-18 is the preferred ligand of FGFR3, since Fgf18-deficient mice have an expanded zone of proliferating chondrocytes similar to that in Fgfr3-deficient mice [71]. FGF18 and FGF9 are expressed in the perichondrium and periosteum and form a functional gradient in the proximal zone of proliferating chondrocytes, where FGF18 acts *via* FGFR3 to downregulate proliferation, promoting subsequent maturation [71, 72]. As the epiphyseal growth plate develops, FGFR3 disappears and FGFR1 expression is upregulated in prehypertrophic and hypertrophic chondrocytes, suggesting a role for FGFR1 in the regulation of cell survival and differentiation, and possibly cell death [69]. In the prehypertrophic and hypertrophic zones, both FGF18 and FGF9 interact with FGFR1 and regulate vascular invasion by inducing the expression of VEGF and VEGFR1.

The proliferation of growth plate chondrocytes is also under the control of a local negative feedback loop involving signaling by PTHrP and its inhibition of Ihh. This feedback loop is not only important in regulating the pace of chondrocyte differentiation, but it is also critical for maintaining the

organization of the developing growth plate, as demonstrated in knock out studies which result in the severe disruption of growth cartilage morphology [73-77]. The organization of the growth plate, which allows for the directional growth of bone, places the least differentiated chondrocytes, responsible for modulating chondrocyte differentiation through expression of PTHrP in the peri-articular perichondrium, in the future joint surface.

The expression of Ihh is restricted to the prehypertrophic zone and the adjacent, surrounding perichondrial cells express the Hedgehog receptor patched (ptc). Signaling is similar to that of Shh, involving intracellular Smo and Gli transcription factors [76, 78-80]. Early work indicated that Ihh induces expression of PTHrP in the peri-articular perichondrium [76], *via* transforming growth factor-β (TGF-β) and/or other secondary signals [81, 82] and that PTHrP signaling then stimulates cell proliferation *via* its receptor [75]. Under the proliferative influence of PTHrP, chondrocytes are inhibited from maturation to the prehypertrophic stage, thus delaying further the expression of Ihh and completing the feedback loop [83]. Recent studies have uncovered additional new mechanisms through which PTHrP may modulate chondrocyte maturation. These include transcriptional regulation by the zinc the finger protein Zfp521, which interacts with Runx2 to inhibit its 'pro-maturational' activities, as well as ECM1, directly downstream of PTHrP, whose effect is to negatively regulate chondrocyte differentiation [43]. Evidence indicates that Ihh also acts independently of PTHrP on periarticular chondrocytes to stimulate differentiation of columnar chondrocytes in the proliferative zone [83]. Furthermore, FGF-18 signaling *via* FGFR3 can inhibit Ihh expression [71] whereas BMP signaling can upregulate Ihh expression in cells that are beyond the range of the PTHrP-induced signal [67] providing integration between the FGF/BMP and PTHrP/Ihh axes. Thus, Ihh and PTHrP, by transiently inducing proliferation markers and repressing differentiation markers, function in a temporo-spatial manner to determine the number of cells that remain in the chondrogenic lineage versus those that enter the endochondral ossification pathway. They also allow for directional bone growth by maintaining an organization where the least differentiated, PTHrP-expressing cells are at the ends of the bones, near the joint surface, whereas Ihh is in a remote, deeper zone in the prehypertrophic chondrocytes.

MECHANICAL SIGNALS

Joint formation and the organization of the growth plate are both dependent on mechanical signals that regulate chondrocyte behaviors through a number of different mechanisms. In addition to inducing PTHrP, Ihh signaling can also induce another mitogenic factor, BMP4, and this occurs through mechanical stimulation [84]. That cilia may be important in mechano-regulation is suggested by the growth plate defects that occur in the absence of Kif3a [85]. However, comparisons of Ihh and Kif3a-deficient chondrogenesis differ significantly indicating that Ihh actions may not depend solely on their association with cilia [86]. Expression of integrin β1 through which chondrocytes interact with ECM molecules such as collagen, is also necessary for the normal organization of the growth plate, and may also participate in the formation of joints [87, 88].

HYPERTROPHY AND ENDOCHONDRAL OSSIFICATION

Endochondral ossification involves terminal differentiation of chondrocytes to the hypertrophic phenotype, cartilage matrix calcification, vascular invasion, or angiogenesis, and ossification [89-92]. During chondrocyte hypertrophy, cellular fluid volume can increase by almost 20 times. Ihh, which synchronizes skeletal angiogenesis with perichondrial maturation, is expressed in prehypertrophic chondrocytes as they exit the proliferative phase, enter the hypertrophic phase and begin to express the hypertrophic chondrocyte markers, type X collagen (COL10a1) and alkaline phosphatase [93]. The runt-domain transcription factor, Runx2, serves as a positive regulatory factor in chondrocyte maturation to the hypertrophic phenotype and subsequent osteogenesis [45, 89, 94-96] particularly through interactions with BMP-induced Smad1 [94, 97, 98].

Runx2, which is required for terminal differentiation [45, 95] positively, regulates not only COL10a1, but also matrix metalloproteinase-13 (MMP13). The activities of MMP-13, and other MMPs such as MMP-9 are required for the ECM remodeling that is the rate-limiting step in chondrocyte hypertrophy and angiogenesis [99].

During angiogenesis, the perichondrium and hypertrophic zone are invaded by blood vessels [96, 100], a process regulated by vascular endothelial growth factor (VEGF) isoforms VEGF188, VEGF120 or VEGFA and VEGF164 [101, 102], and the VEGF receptors, Flk expressed in endothelial cells and perichondrium, neuropilin (Npn) 1 expressed in late hypertrophic chondrocytes, and Npn2 expressed exclusively in the perichondrium [90]. Release of VEGF from the ECM by MMP-9 is an important step in activation of the matrix-bound forms of VEGF [99]. Vascular invasion and ECM remodeling are prerequisites to migration and differentiation of osteoclasts and osteoblasts, which remove mineralized cartilage matrix and replace it with bone.

Chondrocyte hypertrophy and endochondral ossification are coordinated by transcriptional regulatators that inhibit or enhance the function of Runx2 [103]. Since there is no SMAD site on the Runx2 promoter, it has been proposed that homeobox genes of the Dlx family such as Dlx3 could activate Runx2 signaling in response to BMP-2 during endochondral ossification, whereas Dlx5 and Msx2 are known to inhibit Runx2-mediated activation of genes such as osteocalcin at later stages [104, 105]. In a recent study, we identified GADD45β, which has been implicated in the stress response and cell survival during terminal differentiation of different cell types, as a prominent early response gene induced by BMP-2 through a Smad1/Runx2-dependent pathway that acts as a survival factor in hypertrophic chondrocytes and maintains *Mmp13* and *Col10a1* expression [106]. The homeodomain protein Nkx3.2, which is an early BMP-induced signal required at the onset of chondrogenesis, is a direct transcriptional repressor of Runx2 promoter activity [107]. The bHLH factor Twist transiently inhibits Runx2 function and prevents premature osteoblast differentiation [108], whereas cooperation of the Groucho homologue Grg5 or the leucine zipper protein ATF4 with Runx2 promotes chondrocyte maturation [109] or osteoblast differentiation [110], respectively. Histone deacetylase 4 (HDAC4), which is expressed later in prehypertrophic chondrocytes, prevents

premature chondrocyte hypertrophy by interacting with Runx2 and inhibiting its activity [111].

The hypoxia-inducible factor (HIF) 1α is required for chondrocyte survival during hypertrophic differentiation, partly due to its regulation of VEGF expression [92, 112]. A related factor HIF-2α has also recently been shown to regulate positively Col10a1 gene expression [43]. A long form of c-Maf interacts with Sox9 at early stages to upregulate Col2a1 expression [113]; and recently, the p63 transcription factor (related to the p53 family) has also been shown to regulate not only Sox9 but also other key chondrogenic genes such as Sox6 and Col2a1[43]. On the other hand, C/EBPβ and γ and AP-2α may inhibit chondrocyte differentiation by blocking transcription of Col2a1, aggrecan, and other cartilage-specific genes by direct or indirect mechanisms [114-116]. The transcriptional repressor TRPS1, which is associated with human tricho-rhino-phalangeal syndrome, may also delay maturation [43]. Activation of the TCF/Lef transcription factors by Wnt/β-catenin function in a cell autonomous manner to induce osteoblast differentiation and suppress chondrocyte differentiation in early progenitors [117, 118]. Later during chondrogenesis, Wnt/β-catenin may promote chondroprogenitor differentiation or chondrocyte hypertrophic differentiation depending upon the Wnt signal and the level of activaton [117, 119].

POST-NATAL JOINT DEVELOPMENT

In humans and mice, the articular cartilage of many joints is not fully developed at birth. Growth plate cartilage remains at the ends of many bones. Around many joints, such as the knees and hips, a secondary center of ossification (SCO) appears sometime after birth. As it grows, the cartilage at the ends of the bones is divided into the metaphyseal growth plate proximally, and growth cartilage that is contiguous with the joint surface distally. Formation of the SOC depends on the matrix degrading activity of MT-MMP1 (MMP14) [120]. The process of bone formation in the SOC likely involves the same mechanisms that occur in the metaphyseal growth plate, where local chondrocyte regulation through factors such as PTHrP, Ihh, FGF and BMPs persists, but where they may be secondary to influences from the endocrine system. In the post-natal (metaphyseal) growth plate, hormones such as thyroid hormone and estrogen are key regulators of chondrocyte maturation and endochondral ossification [121]. The process that occurs in the sub-chondral region around joints however, is likely to be significantly different from that in the growth plate, however, for around the joint, ossification must be halted in a manner to leave cartilage at the joint surfaces. Appearance of the histologic tidemark, an indication of the formation of the zone of calcified cartilage occurs independently of growth plate closure, suggesting that these processes are under the control of divergent mechanisms. Our recent studies indicate that the PTHrP/Ihh axis is still active post-natally, regulating mineralization by chondrocytes at the osteochondral interface of immature (bovine) joints [122]. Further investigations will be required to elucidate other mechanisms that may control the growth of articular cartilage and formation of the mature osteochondral interface.

Whereas cartilage appears simply to persist at the joint surface as formation of the SCO is completed, a recent study suggests that this is not the case. Measuring rates of bone growth proximal to developing joints in the rabbit, Hunziker *et al*. [123] have found evidence that most, if not all of the cartilage present at the ends of the bone at birth is replaced by bone and that the cartilage of the articular surfaces is formed anew over the first 3 months or so of post-natal life. In agreement with this study, Hayes *et al.* have proposed that post-natal cartilage growth occurs by apposition at the joint surface rather than interstitially, as previously believed [124]. This concept is supported by recent findings by several investigators of the presence of stem cells at the articular surface of neonatal joints [51, 125, 126]. Investigating these post-natal developmental processes is critical if we are to understand fully the genesis of articular cartilage.

THE ADULT ARTICULAR CHONDROCYTE

The chondrocyte in normal articular cartilage exists in a quiescent state in an avascular extracellular matrix (ECM) nourished by diffusion from the synovial fluid and the vasculature of the subchondral bone. The specialized ECM consists primarily of type II collagen and aggrecan., Additional components include cartilage-specific collagens type IX and XI, small proteoglycans biglycan and decorin, as well as perlecan, COMP, matrilins and tenascin, which contribute not only to cartilage structure, but also to the interaction of chondrocytes and growth factors [127-131].

The chondrocytes are distributed within the articular cartilage in four distinct regions: (1) the superficial tangential (or gliding) zone, (2) the middle (or transitional) zone, (3) the deep (or radial) zone, and (4) the calcified cartilage zone, which is located immediately below the tidemark and above the subchondral bone [132, 133]. In the superficial zone, the chondrocytes are small and flattened, and the matrix is composed of thin collagen fibrils in tangential array, associated with a high concentration of decorin and a low concentration of aggrecan. The middle zone, comprising 40 to 60 percent of the cartilage weight, consists of rounded chondrocytes surrounded by radial bundles of thick collagen fibrils. In the deep zone, the chondrocytes are frequently grouped in columns or clusters with thick collagen bundles arranged in a radial fashion. From the surface to the deep zone, the cell density progressively decreases, the cell volume increases, and the proportion of proteoglycan increases relative to collagen increases. The calcified zone persists after growth plate closure as the "tidemark" [134] and serves as an important mechanical buffer between the uncalcified articular cartilage and the subchondral bone. Differences in the sub-populations of superficial, middle and deep zone chondrocytes (SZC, MZC and DZC) have largely been thought to arise as a consequence of their differing mechanical milieu [133, 135]. *In vitro* studies with isolated SZC and DZC indicate that differences in the metabolic activity of SZC and DZC maintain the zonal difference in matrix composition [1-9, 136].

Chondrocytes maintain active membrane transport systems for exchange of cations, including Na^+, K^+, $Ca^{2+,}$ and H^+, whose intracellular concentrations fluctuate with load and changes in the composition of the cartilage matrix [137]. Because articular cartilage is not vascularized, the chondrocyte must rely upon diffusion from the articular surface or subchondral bone for the exchange of nutrients and metabolites. Glucose serves both as the major energy source for the

chondrocytes and as an essential precursor for glycosaminoglycan synthesis [138, 139]. Facilitated glucose transport in chondrocytes is mediated by several distinct glucose transporter proteins (GLUTs) that are either constitutively expressed (GLUT3 and GLUT8) or cytokine-inducible (GLUT1 and GLUT6) [139, 140]. Chondrocyte metabolism operates at low oxygen tension within the cartilage matrix, ranging from 10% at the surface to less than 1% in the deep zones. *In vitro*, chondrocytes adapt to low oxygen tensions by upregulating hypoxia inducible factor-1α (HIF-1α), which can stimulate expression of GLUTs [140] and angiogenic factors such as VEGF [141, 142], as well as a number of genes associated with cartilage anabolism and chondrocyte differentiation, including Sox9, TGF-β, and connective tissue growth factor [143, 144]. Thus, by modulating the intracellular expression of survival factors such as HIF-1α, chondrocytes have a high capacity to survive in the avascular cartilage matrix and to respond to environmental changes. Expression of anti-angiogenic factors such as endostatin by articular chondrocytes may inhibit angiogenesis despite up regulation of HIF-1α and/or VEGF [145].

In contrast to 'growth' chondrocytes, articular chondrocytes express factors that modulate the activities of SMAD1 and Runx2, preventing the progression towards endochondral ossification. For example, articular chondrocytes (but not 'growth' chondrocytes) express a transcription factor of the ETS family, C-1-1 that directly inhibits the pro-hypertrophic activity of Runx2 [65]. Recently, Sox9 has also been shown to modulate the osteoblastic activity of Runx2 [146]. However, that study did not clarify whether Sox9 expression in articular chondrocytes functions in that way. Ubiquitination and turn over of Smads *via* Smurfs may be another mechanism whereby chondrocytes maintain a less Runx2 activity /immaturity [147]. The calcified cartilage at the osteo-chondral interface of articular cartilage is also a barrier to vascular invasion. The capacity of mineralization is a specific feature of DZC that is not shared by other subpopulations of articular chondrocytes [7]. We have recently shown that the PTHrP/IHH axis controls articular chondrocyte mineralization *in vitro* [122].

Several other factors important for chondrogenesis also play critical roles in articular cartilage homeostasis, particularly in supporting matrix metabolism. Of these IGF1, FGF, TGFβ and BMPs have been most widely investigated. IGF-1 is considered an essential mediator of cartilage homeostasis through its capacity to stimulate proteoglycan synthesis, promote chondrocyte survival and oppose the activities of catabolic cytokines [148-150]. Chondrocytes at different stages of differentiation express IGF-I and IGF receptors, as well as different arrays of IGF-binding proteins (IGFBPs), thus providing a unique system by which IGF-I can exert different regulatory effects on these cells [151].

Of members of the FGF family the most extensively studied is FGF-2, or basic FGF, which is a potent mitogen for adult articular chondrocytes [152]. Early studies suggested that low concentrations of FGF-2 could stimulate chondrocyte mitogenesis and proteoglycan synthesis whereas high concentrations might have opposite effects [153]. Recent studies showing that FGF-2 stored in the adult cartilage matrix is released with mechanical injury or with loading suggest a mechanism for modulating chondrocyte proliferation and anabolic activity [154, 155]. FGF-2 can inhibit the anabolic activities of IGF-1 and OP-1 *in vitro* [156]. Both FGF-9 and FGF-18 increase matrix synthesis by mature chondrocytes [157-160]. A recent study demonstrated that FGF-18 promotes cartilage repair in a rat meniscal tear model of OA [161].

TGF-β was named based on its discovery as a factor that could transform cells to grow in soft agar. However, it is not a potent inducer of chondrocyte proliferation. Knockout studies resulting in defects in TGFβ signaling result in premature joint degeneration suggesting that endogenous activity is necessary for normal matrix homeostasis [162, 163]. Findings that IL-1 differentially regulates inhibitory Smads [164] and decreases TGFβ signaling also supports a protective effect of endogenous TGFβ during OA progressio [165]. Furthermore, administration of agents that block TGFβ activity, such as the soluble form of TGFβRII, inhibitory SMADs, or the physiological antagonist, latency-associated peptide-1 (LAP-1), increases proteoglycan loss and cartilage damage in an experimental model of OA [166]. However, a recent finding that TGFβ induces expression of ADAMTS-4 in primary human chondrocytes and promotes the degradation of aggrecan suggests that it may be involved in normal turnover of proteoglycans in mature cartilage [167]. Indeed, effects of exogenous TGFβ on chondrocyte matrix metabolism are variable.

Several BMPs. including BMP-2, 4, 6, 7, 9, 13 (also known as CDMP-2) and 15 (CDMP1) can enhance the synthesis of type II collagen and aggrecan by articular chondrocytes *in vitro* [168, 169]. In addition, BMP-2, -7and -9 counter many of the catabolic responses induced by IL-1β including induction of MMP-1 and MMP-13, down-regulation of TIMP expression, and down-regulation of proteoglycan synthesis in primary human articular chondrocytes [168]. BMP-7 (also known as osteogenic protein-1 or OP-1) is expressed in mature articular cartilage and is possibly the strongest anabolic stimulus for adult chondrocytes *in vitro*, because it increases aggrecan and type II collagen synthesis more strongly than IGF-I [168]. A requirement for signaling of BMPs and related factors such as GDFs has been shown in studies resulting in premature joint degeneration as a result of BMPR1A deletion in the joint [170]. The necessity for BMP signaling for normal cartilage matrix homeostasis has also been corroborated in studies in human cartilage explants [171, 172]. However, BMPs have pleiotropic effects *in vivo*, acting in a concentration-dependent manner. While initiating chondrogenesis in the limb bud, they generally set the stage for bone morphogenesis

THE AGING CHONDROCYTE

Aging is a major risk factor for OA, and this may be, in part, due to age related changes in cartilage and chondrocytes. Chondrocyte function, including mitotic and synthetic activity, deteriorates with age. Changes in matrix synthesis lead to small and irregular proteoglycan aggregates due to decreased size of aggrecan molecules and less functional link proteins. There is also a stiffening of the collagen network due to non-enzyme-mediated pentosidine cross-linking. Degradative changes are generally due to the actions of proteinases and are, at least partially, the cumulative consequences of adverse conditions, such as mechanic insults or inflamma-

tion, to which the chondrocyte is exposed throughout life. Deficiencies in cartilage matrix proteins may also disrupt chondrocyte-matrix interactions that are important to cell survival. Although programmed cell death, or apoptosis, increases with age in adult rats and mice, this may be due to skeletal growth that occurs throughout life in these animals. However, in human adult cartilage, apoptotic cell removal does not appear to be a widespread phenomenon [173]. Because the proliferative potential of adult articular chondrocytes decreases with age, replicative senescence, detected as β-galactosidase activity and decreased telomere length, has been proposed to contribute to the age-related changes in normal chondrocyte function [174, 175]. 'Chondrogenic' factors such as TGF-β, BMPs, FGFs and IGF-1 that support cartilage matrix biosynthesis are expressed at declining levels with aging or their activities are downregulated [176]. For example, the capacity of BMP-6 to stimulate proteoglycan synthesis and the production of BMP-7 (OP-1) decline with age. Chondrocytes also show an age-related decline in the anabolic response to IGF-I, possibly due to increased synthesis of IGFBP-3, which is itself antiproliferative. Indeed, chondrocytes from elderly donors depend strongly upon IGF-I and IGF-II for survival [177]. It has been proposed that the reduction in TGF-β signaling in aging chondrocytes may be a factor in their reduced capacity to repair cartilage [178].

THE CHONDROCYTE IN OSTEOARTHRITIS

Despite age-related compromises in cellular metabolism, chondrocytes *in vivo* respond to structural changes in the surrounding cartilage matrix as occurs during the initial stages of OA with increased chondrocyte proliferation and synthesis of matrix proteins, although increased activity and/or expression of proteinases, and cytokines are also observed. The early changes in synthetic activity are viewed as an attempt to regenerate the matrix with cartilage-specific components, but the resident chondrocytes also undergo phenotypic modulation by synthesizing type I collagen, increased pericellular type VI collagen and collagen type IIA, which is the splice variant of the normal cartilage-specific type IIB collagen (COL2A1) associated with chondroprogenitors [179-182]. A general upregulation of collagen and proteoglycan synthesis has been observed in an animal model of OA [183]. In early OA increased levels of type II collagen mRNA levels [184, 185], which may be associated with the increased levels of anabolic factors such as BMP-2 and inhibin. A/activin have been reported [184-186]. With disease progression, however, the pathognomic loss of matrix becomes evident-early surface cartilage fibrillation may eventually progress to complete loss of the articular cartilage. Matrix loss has been attributed to the activity of MMPs and ADAMTSs and other enzymes produced both by chondrocytes and synovial cells in response to inflammatory mediators, cytokines and aberrant mechanical stresses. A recapitulation of embryonic skeletal development also occurs in the deep and calcified zones where the hypertrophic chondrocyte- specific type X collagen is expressed, and in the upper middle zone where type III collagen expression is detected [187]. Although OA pathogenesis is complex and involves many different pathways, here we will focus on the ones that relate to the regulation of chondrogenesis.

In contrast to the anabolic aspects, analogies between cartilage catabolism in OA and matrix remodeling during endochondral ossification are more difficult to identify. Proinflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), major 'players' in OA pathogenesis do not seem to have critical roles during chondrogenesis in the embryonic or postnatal growth plate [188]. MMPs do play an important role in matrix remodeling in both processes, however. The localization of MMP-13 expression in the deep zone of OA cartilage has lead to speculation that the chondrocyte hypertrophy and the associated tidemark advancement and subchondral bone thickening are major features of progression of the disease. The recent studies identifying OA-associated polymorphisms in the gene encoding asporin, which inhibits cartilage anabolism by binding to TGF-β, and in FRZB, which encodes secreted frizzled-related protein 3 (sFRP3) [189, 190] lend support to this concept. Members of the sFRP family, including sFRP3, are glycoproteins that antagonize the signaling of Wnt ligands through frizzled membrane-bound receptors [191]. In OA cartilage, sFRP may have a role in chondrocyte apoptosis [192]. Since activation of β-catenin in mature cartilage cells stimulates hypertrophy, matrix mineralization, and expression of VEGF, ADAMTS5, MMP-13 and several other MMPs [119], defective inhibition of Wnt signaling due to FRZB polymorphisms may disrupt normal homeostasis resulting in abnormal cartilage and bone metabolism. Future investigations will be required to determine whether these polymorphisms associate with OA because of the roles of Wnts, TGF-β or other chondrogenic regulators in the initial formation of joints or because of potential roles in articular cartilage homeostasis or aging.

The upregulation of genes normally involved in chondrogenesis and chondrocyte maturation is a puzzling phenomenon. The overlapping pattern of gene expression that is observed in OA-affected chondrocytes contrasts the highly organized pattern that underlies bone formation during embryogenesis. However, this pattern itself may not be pathologic, as chondrocytes in the post-natal growth plate also express factors such as PTHrP and Ihh in the same zone [121, 193]. One possibility is that articular chondrocytes are normally suspended in an 'immature' state, but that OA-related mechanisms such as aging and mechanical injury can 'turn on' a maturational program that induces previously 'quiescent' articular chondrocytes not only to activate reparative functions such as proliferation or increased matrix metabolism, but also to hypertrophy. This view is supported by studies that show induction of the hypertrophy in articular chondrocytes by use of de-methylating agents [194]. However, these studies do not clarify whether there are a subpopulation of resident stem cells within articular cartilage that can respond in this way or whether the general population of articular chondrocytes as a whole is composed of 'immature' cells that are susceptible to such induction.

Although not necessarily 'immature', isolated DZC hypertrophy (enlarge), mineralize and express markers of maturation such as Runx2 in culture [4, 7]. The presence of such cells in the deep zone is not surprising, and may indicate a normal, albeit slow, process of mineralization at the border between the deep/radial and calcified zones of articular cartilage. Recently, we have shown that DZC mineralization is modulated by interactions with SZC [122]. One possibility is that loss of SZC or other modulating factors during aging or

in early OA, allows the hypertrophy of DZC and resulting in the phenotypic derangements observed in OA.

The phenotypic changes that occur in OA-affected chondrocytes are not, of course, the only pathology that is apparent in the disease. However, understanding their role in disease pathogenesis will be important in identifying whether and how sub-populations of cells of a different maturational state may contribute to progression or modulation of disease.

CONCLUSION

Regulation of chondrogenesis in the context of embryonic limb formation and endochondral ossification provides a wealth of information about how chondrocyte behaviors are regulated. Much is already known and novel regulators continue to be discovered, including those that bear direct relevance to adult articular chondrocytes in normal and OA-affected cartilage. The initiation of joint formation requires the negative regulation of 'pro-chondrogenic' signals, and joint development also requires a balance of negative and positive chondrogenic mediators. A better understanding of this modulation will be required if successful neo-cartilage tissue formation is to be achieved using MSCs from bone marrow or other sources. Bony overgrowth is often seen in humans and animal models after cartilage repair, and to inhibit that may require the formation of cartilage not destined for endochondral ossification. While the use of cartilage-derived stem cells offers promise, it is likely that appropriate developmental signals will also have to be provided to induce articular cartilage tissue formation. For that, a better understanding of how a thin layer of cartilage stem cells at the articular surface gives rise to the complex, highly organized adult articular cartilage through early post-natal events will likely be required. In the near term, it is clear that chondrogenic factors may enhance tissue formation and repair by differentiated articular chondrocytes. In so far as autologous chondrocyte implantation is already in clinical use, judicious addition of growth factors to this process *via* gene transfer or other techniques may be a next step in enhancing cartilage tissue formation in a clinical setting. Indeed, previous studies by our group are only a small sample of investigations showing the enhancement of cell-based cartilage repair through genetic modification of the implanted cells to overexpress a variety of chondrogenic signals [195, 196]. In the context of OA, the upregulation of chondrogenic signals remains an intriguing phenomenon. Identification of the sub-population that undergoes these changes-whether resident stem cells or DZC - may be a revealing avenue of investigation in this regard. Clearly, discerning whether the re-expression of chondrogenic genes represent pathology or attempted repair will be critical for developing biological approaches to OA prevention and/or treatment.

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