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β-catenin, cartilage, and osteoarthritis

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Abstract

The early cellular events during the development of osteoarthritis (OA) are accelerated articular chondrocyte maturation and extracellular matrix degradation, which are usually seen in the weight-bearing region of articular cartilage. The results of our recent studies from transgenic OA mouse models indicate that upregulation of β-catenin signaling in articular chondrocytes is most likely responsible for the conversion of normal articular chondrocytes into maturing (arthritic) chondrocytes, which is associated with activation of chondrocyte maturational genes and matrix degradation. Conditional activation of the β-catenin gene in articular chondrocytes leads to an OA-like phenotype. Overexpression of Smurf2, an E3 ubiquitin ligase, also induces an OA-like phenotype through upregulation of β-catenin signaling. In addition, β-catenin upregulation was also found in articular cartilage tissues in patients with OA. These findings indicate that β-catenin plays a central role in articular cartilage function and that activation of β-catenin signaling may represent a pathologic mechanism for OA development.

Keywords

conditional activation; β-catenin; articular chondrocyte; osteoarthritis

Endochondral ossification and regulation

Endochondral ossification is the primary process responsible for the formation of the vertebrate skeleton. This process starts with the condensation of chondrogenic mesenchymal cells, which differentiate into chondrocytes and form cartilage rudiments. Chondrocytes express cartilage-specific proteins, including type II collagen (Col2) and proteoglycans, and deposit these proteins around the cells to form extracellular matrix. Chondrocytes then undergo unidirectional proliferation to form orderly parallel columns. These proliferating chondrocytes are continuously expressing and depositing cartilage matrix. After these cells exit the cell cycle, they undergo maturation and become terminally differentiated hypertrophic chondrocytes, expressing type X collagen (ColX) and matrix metalloproteinase (MMP) 13, respectively. These chondrocytes with different features are organized into growth plates, consisting of resting zones, proliferating zones, and hypertrophic zones. Terminally differentiated hypertrophic chondrocytes then undergo apoptosis, and hypertrophic matrix is remodeled and calcified. Meanwhile, blood vessels and osteoblasts invade this region and the primary ossification center is formed around embryonic E15.5.[1]

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Conflicts of interest
The authors declare no conflicts of interest.

Chondrocyte differentiation and maturation during endochondral ossification is tightly regulated by several key growth factors and transcription factors. Members of the TGF-β superfamily, such as TGF-β isoforms and BMPs, play important roles in the regulation of chondrocyte differentiation and maturation during this process. For example, TGF-β induces chondrogenesis including chondrocyte differentiation and cartilage matrix synthesis in developing limb buds.[2] On the other hand, TGF-β signaling inhibits chondrocyte maturation and *ColX* expression during long bone lengthening, evidenced by the loss of TGF-β signaling in the growth plate resulting in increased thickness of the hypertrophic zone and upregulation of *ColX* expression.[3,4] Similar to the stimulatory effect of TGF-β signaling in chondrogenesis, BMP signaling is critical for mesenchymal condensation and chondrocyte differentiation during limb bud development.[5] However, in contrast to the inhibitory effect of TGF-β signaling on chondrocyte maturation, BMP signaling promotes this process.[5] TGF-β/BMP signaling is initiated by binding to their receptors, which induces the phosphorylation of downstream signaling molecules, including Smad1, Smad5, and Smad8 (BMP signaling), as well as Smad2 and Smad3 (TGF-β signaling).

Recent evidence suggests that canonical Wnt signaling, which is transduced via β-catenin, is implicated in multiple steps of endochondral bone formation. For example, ectopic β-catenin in the cells of the chondrogenic lineage inhibits chondrocyte differentiation but stimulates chondrocyte maturation and ossification during embryonic development.[1,6] In general, β-catenin levels are upregulated by Wnt ligands. In the absence of Wnt ligands, β-catenin is degraded through proteasome-mediated degradation in a phosphorylation-dependent manner, which is catalyzed by the enzyme GSK-3β in a "destruction complex" brought together by Axin/Axin2 and APC. Upon Wnt ligand binding to its receptors, the complex is disassociated, and the kinase activity of GSK-3β is suppressed, and hence the nonphosphorylated β-catenin accumulates in the cytoplasm and then translocates into the nucleus to activate expression of Wnt target genes. The β-catenin protein levels are therefore tightly regulated by the Wnt-mediated activity of the "destruction complex," and any mechanism by which normal β-catenin regulation is uncoupled from Wnt signaling control could result in dysregulation of osteoblast/chondrocyte function, leading to alterations in bone mass or degenerative joints. However, our understanding of the abnormal regulation of β-catenin in these diseases and animal models remains incomplete.

Articular cartilage: development and characteristics

Articular cartilage covering the ends of long bone emerges postnatally and its maturation is completed by adulthood in the mouse. Appearance of articular cartilage occurs at postnatal week 2, when a small domain of secondary ossification occurs in the center of the epiphysis, which separates the articular cartilage from the growth plate cartilage.[7] At this stage, the deep zone of the newly formed articular cartilage is a growth plate-like tissue that contains a thin layer of enlarged chondrocytes expressing *ColX*, which is essential for formation of the calcified zone seen in adult articular cartilage. ColX assembly into a lattice-like network around hypertrophic chondrocytes is required for retaining or trapping the necessary types and amounts of matrix components, including matrix vesicles and proteoglycans promoting initiation of normal mineralization.[8–11] By 1 month of age, the secondary ossification center is expanded, and the calcified zone starts to occur.[8]

Articular cartilage has mechanical characteristics necessary for frictionless movement of joints. Its ability to undergo reversible deformation depends on its unique architecture of extracellular matrix and zonal articular chondrocytes. The matrix is a highly hydrated network of collagen fibrils composed of major collagen type II and minor collagen types IX, and XI embedded in a gel of negatively charged proteoglycans.[12] This collagen/proteoglycan composition of the matrix endows the cartilage with tensile strength and resistance to compressive loads. Articular

chondrocytes, which are sparsely distributed in the matrix, are small and flattened in the superficial zone and are rounded in the middle and deep zones. Articular chondrocytes build matrix by synthesizing and depositing these matrix components around the cells during articular cartilage development. As articular cartilage matures, the synthesis of matrix components (Col2 and proteoglycans) by articular chondrocytes is decreased. In fact, Col2 mRNA cannot be detected in 3-month-old murine articular cartilage.[7] In spite of the decrease/cessation of matrix synthesis in early adult articular cartilage, the existing collagen network has been cross-linked, and articular cartilage has been matured into a highly specialized tissue with the ability to absorb and respond to mechanical stresses. Since the half-life of collagen fibers is decades,[13] the collagen network is stable for an entire life span.

Osteoarthritis: development and progression

Osteoarthritis (OA) is a degenerative joint disease, which is characterized by articular chondrocyte maturation, extracellular matrix degradation, articular cartilage loss, and osteophyte formation. Unlike the cartilage in the growth plate, in which chondrocytes mature and matrix turns over rapidly, the articular cartilage is a permanent tissue with minimal turnover of its cells and matrix. Under normal conditions, articular chondrocytes persist and function in a stable state throughout postnatal life.[14] As mentioned above, articular chondrocytes do not mature into the same hypertrophic state seen in growth plate chondrocytes and are responsible for the maintenance of the matrix. However, during OA development, articular chondrocytes undergo cloning to form clusters and mature into a hypertrophic state like that seen in the growth plate. Consistent with this, they express maturational marker genes, such as *ColX* and *MMP-13*.[15–17] In addition, other types of MMPs and aggrecanases (ADAMTS-4, and -5) are also expressed in OA articular cartilage.[18] These enzymes in turn degrade matrix, leading to articular cartilage degeneration. During OA development, articular chondrocytes in the remaining arthritic cartilage are usually metabolically active and increase their gene expression of proteoglycans and collagens, which is generally appreciated as an attempt to repair the damaged extracellular matrix. As OA progresses, subchondral bone beneath the calcified zone is exposed and osteophytes, which bridge the joint cavity, are formed in the margin of the joint.

Genetic contribution to osteoarthritis

While OA is a leading cause of disability in older people and the associated health care burden has been growing,[19,20] the underlying molecular mechanisms are largely unknown. OA is generally considered to be a multifactorial disorder involving genetic factors, age, and environmental/lifestyle risk factors.[12,21] Mutations in *collagen types II, IX, and XI* (*Col2, Col9, Col11*) result in osteochondrodysplasia and/or OA. However, these diseases are very rare.[12,21,22] Most environmental factors such as obesity, repetitive injury of joints, and joint malalignment can result in abnormal loading on joints, leading to the development of OA. These situations, along with related genetic OA models, including *Col9* and *Col11* knockout mice and *MMP-13* transgenic mice, provide the evidence that abnormal mechanical stresses on matrix resulting from either normal loading on weakened matrix or overloading of joints are sensed by the articular chondrocytes embedded within the matrix and initiate changes in cell behavior leading to the development of OA.[12,21,23] In addition, OA progression is also affected by proinflammatory factors such as prostaglandins, TNF-α, interleukin-1, interleukin-6, and nitric oxide. However, accumulating evidence using experimental OA models in knockout mice has not produced supporting evidence that these factors may play a key role in the pathogenesis of OA.[24] Overall, since these factors explain only a small part of the pathogenesis of OA, the causes for most OA cases are still not clear. Thus, to pursue the underlying molecular mechanisms of OA, several OA models have been established. For example, constitutive expression of dominant-negative *TGF-β type II receptor* in chondrocytes or knockout of the *Smad3* gene in mice results in an OA-like phenotype.[3,4] On the other hand,

local administration or overexpression of TGF-β in the knee joints induces osteophyte formation.[25–27] Similar to TGF-β, BMP-2 overexpression in knee joints also induces osteophyte formation.[27] However, these events do not occur in humans with OA.

A genetic contribution to OA has been suggested in several epidemiologic studies.[28] Genome-wide scans, fine-scale mapping, and candidate gene association analyses have identified several loci that may be associated with hip OA.[28,29] One such locus was identified by two separate genome-wide scans for familial OA susceptibility and finer mapping suggests a peak linkage signal at D2S2284 (2q31.1).[28,30,31] Further, single nucleotide polymorphism (SNP) analysis of eight candidate genes in this region demonstrates an association of hip OA with a functional SNP resulting in an Arg324Gly substitution at the residue of frizzled-related protein.[32] In addition, haplotype coding for substitutions of two highly conserved arginine residues (Arg200Trp and Arg324Gly) in *Frzb* was a strong risk factor for primary hip OA, with an odds ratio (OR) of 4.1.[32] A role for the same alleles/haplotypes in generalized radiographically observed OA[33] and in hip OA[34] has also been reported in other studies in Caucasian populations. *Frzb* encodes for secreted frizzled-related protein 3 (sFRP3), an antagonist of Wnt signaling. It has been reported that sFRP3 and Wnt/β-catenin signaling regulates chondrocyte formation and maturation.[35,36] This observation was further confirmed by the phenotype of *Frzb* KO mice, which are more susceptible to chemically induced OA.[37] It has been also reported that a panel of Wnt and Wnt signaling-related genes, including WISP-1 (Wnt-induced signaling protein 1), was significantly upregulated in murine OA and in human OA tissues, and their expression patterns were mirrored at histologic levels.[38] Furthermore, over-expression of WISP-1 in knee joints induces cartilage damage in mice.

Development of a transgenic mouse model targeting articular chondrocytes

Although human genetic association studies suggest that genetic factors make significant contributions to the pathogenesis of OA,[32–34,39] the direct genetic evidence and molecular mechanisms for OA pathogenesis are still not understood. Since OA normally occurs in adult patients and animals, the generation of a proper tool to specifically target adult articular chondrocytes is extremely important. Type II collagen is a chondrocyte-specific protein and its expression is detected in growth plate and articular chondrocytes in long bones and other cartilage tissues in the body. The Col2a1 promoter has been used in a variety of animal models to achieve tissue-specific gene expression in chondrocytes.[40–43] In recent studies, we generated transgenic mouse lines in which the Cre recombinase was fused to a mutated ligand binding domain of the human estrogen receptor (ER)[44,45] driven by the Col2a1 promoter (*Col2a1-CreER^{T2}*). The fusion protein has been reported to be translocated into the nuclei in response to the estrogen antagonist tamoxifen or 4-OH-tamoxifen, an active metabolite of tamoxifen, *in vivo*.[45,46] This transgenic mouse model can serve as a valuable tool for gene targeting in a chondrocyte-specific and temporally controlled manner.[47,48]

The *Col2a1-CreER^{T2}* transgene consists of the 1.0-kb mouse Col2a1 promoter linked to *CreER^{T2}* with mutations of G400V, M543A, and L544A in the ligand-binding domain of human estrogen receptor (ER).[44,45] To assess recombination *in vivo*, *Col2a1-CreER^{T2}* transgenic mice were crossed with *Rosa26* reporter mice (*R26R strain*). The *Rosa26* reporter mice have the *lacZ* gene inserted into the ubiquitously expressed Rosa locus that is preceded by a transcriptional stop cassette flanked by loxP sites.[49,50] In *Col2a1-CreER^{T2};R26R* transgenic mice, β-Gal activity was observed only in chondrocytes in mice treated with tamoxifen. To determine the specificity and efficiency of the Cre-recombination in articular cartilage of postnatal and adult mice, 2-week-old *Col2a1-CreER^{T2};R26R* mice were treated with tamoxifen (1 mg/10 g body weight, i.p. injection, daily for 5 days). Mice were sacrificed 1 and 6 months after tamoxifen induction. Strong X-Gal staining was observed in articular cartilage at both time points.[48] We further sought to determine whether tamoxifen-induced Cre

recombination can be achieved when the growth plate cartilage is fully developed at 2 months of age. If this is the case, we may be able to separate the role of a specific gene in articular chondrocytes from its potential effect on the growth plate cartilage, which may indirectly affect the function of articular cartilage. Tamoxifen induction (1 mg/10 g body weight ×5 days) was performed in 3-month-old *Col2a1-CreER^{T2};R26R* mice. Mice were then sacrificed 2 months after tamoxifen induction at the age of 5 months and Cre-recombination efficiency was evaluated by X-Gal staining. When tamoxifen was administered to 3-month-old mice, high Cre-recombination efficiency (>80%) was achieved in articular chondrocytes, and this was evaluated 2 months after tamoxifen induction.[16] In contrast, around 20% Cre-recombination efficiency was observed in growth plate chondrocytes in these mice. These findings suggest that *Col2a1-CreER^{T2}* transgenic mice could be used to target the floxed genes in articular chondrocytes specifically at the adult stage. No positive X-Gal staining was detected in noncartilage tissues such as heart, kidney, and liver. To further confirm the specificity of tamoxifen-induced Cre expression, mRNA expression of Cre recombinase was examined by RT-PCR in multiple tissues including liver, heart, kidney, lung, spleen, skin, spine, tibia, and trachea. The Cre expression was detected in spine, tibia, and trachea, but not in liver, heart, kidney, lung, spleen, and skin tissues.[47]

Development of osteoarthritis in β-catenin conditional activation mice

We have bred *Col2a1-CreER^{T2}* transgenic mice with β-*catenin$^{fx(Ex3)/fx(Ex3)}$* mice and generated *Col2a1-CreER^{T2}*;β-*catenin$^{fx(Ex3)/wt}$* (β-catenin cAct) mice. Since amino acids encoded by exon 3 contain critical GSK-3β phosphorylation sites, deletion of exon 3 of the β-catenin gene results in the production of a stabilized fusion protein which is resistant to phosphorylation by GSK-3β.[51] The articular cartilage phenotype of β-catenin cAct mice was analyzed by histologic study, showing that progressive loss of the smooth surface of articular cartilage occurs in 5- and 8-month-old β-catenin cAct mice. At the age of 5 months, mild cartilage degeneration was observed as was reduced Safranin O and Alcian blue staining. Furthermore, articular chondrocytes were missing in the weight-bearing area of the articular surface in β-catenin cAct mice. At 8 months of age, severe destruction of articular cartilage was observed in β-catenin cAct mice. Cell cloning, surface fibrillation, and vertical clefts as well as the formation of chondrophytes and osteophytes were observed. Complete loss of articular cartilage and an increase in subchondral bone mass were also found in β-catenin cAct mice. Finally, MMP-13 mRNA and protein expression were significantly increased in β-catenin cAct mice.[16] Overall, these phenotypic changes resemble the clinical features commonly observed in OA patients. Correlating all of this to human disease, we also found that β-catenin protein expression was upregulated in knee joint samples from patients with OA.[16]

Smurf2-induced osteoarthritis is mediated by β-catenin

Smurf2, a Hect domain E3 ubiquitin ligase, is highly expressed in human OA tissue and overexpression of Smurf2 in *Col2a1-Smurf2* transgenic mice induces an OA-like phenotype in aged mice.[17] We first characterized the phenotypic changes in embryonic limb development since the process of OA recapitulates many cellular events that occur during development and growth facilitated by growth plate chondrocytes. We found that chondrocyte maturation and endochondral ossification is accelerated in *Col2a1-Smurf2* transgenic limbs, in which the expression patterns of maturational genes (*ColX*, *MMP-13*, etc.) and ossification are quite similar to that in *Col2a1-Wnt14* transgenic limbs.[6,52] Indeed, a dramatic increase in β-catenin protein levels in chondrocytes was detected in developing *Col2a1-Smurf2* transgenic limbs, which is due to Smurf2-induced ubiquitination and proteasomal degradation of GSK-3β.[52,53] Similar phenotypic changes are recapitulated in transgenic articular cartilage during postnatal stages, that is, the *ColX*-expressing domain in deep articular cartilage is expanded at

2 weeks of age, and calcification of this zone is accelerated at 1 month of age. Similar to the expression pattern of β-catenin in embryonic *Col2a1-Smurf2* transgenic limbs, a dramatic increase in β-catenin was detected in transgenic articular cartilage.[52,53] This is consistent with the known effects of β-catenin on the maturational progression of the articular chondrocyte observed in these mice that was discussed above: cloning of chondrocytes in the superficial and middle zones followed by maturation of these cells into enlarged hypertrophic chondrocytes, similar to those seen in the growth plate. Notably, upregulation of β-catenin protein levels are is detected in both postnatal and adult *Col2a1-Smurf2* transgenic articular cartilage.[52] Thus, β-catenin is continuously upregulated in *Col2a1-Smurf2* transgenic articular chondrocytes throughout adult life, consistent with the characteristics of the *Col2a1* promoter used for generation of *Col2a1-Smurf2* transgenic mice.[17] Since both Smurf2 and β-catenin are highly expressed in human OA, the Smurf2-GSK-3β-β-catenin pathway may represent an important pathologic mechanism underlying the development of OA in humans.

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