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REVIEW

TGF-β signaling and the development of osteoarthritis

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Osteoarthritis (OA) is a common joint degenerative disease affecting the whole joint structure, including articular cartilage, subchondral bone and synovial tissue. Although extensive work has been done in recent years to explore the molecular mechanism underlying this disease, the pathogenesis of OA is still poorly understood and currently, there is no effective disease-modifying treatment for OA. Recently, both *in vitro* and *in vivo* studies suggest that confirmed (TGF-β)/SMAD pathway plays a critical role during OA development. This short review will focus on the function and signaling mechanisms of TGF-β/SMAD pathway in articular chondrocytes, mesenchymal progenitor cells of subchondral bone and synovial lining cells during OA development.

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INTRODUCTION

Osteoarthritis (OA) is a degenerative disease of articular cartilage that is projected to affect >50 million people in the United States by the year 2020.[1] The primary characteristic of OA includes the progressive loss of the articular cartilage tissue, synovial tissue inflammation, subchondral bone sclerosis and osteophyte formation at the margin of the joint, which will result in chronic pain, joint stiffness and eventually impaired mobility. Risk factors for OA development can be classified as aging, environment factors, joint dysplasia and injury, and inherent genetic alternations.[2] Despite extensive work over the past 20 years to delineate the pathogenic mechanism(s) of OA, a full understanding of the initiators of the disease and factors that accelerate it, is yet to be achieved. Thus, there is no clinical diagnostic for early OA and no effective disease-modifying treatment for late OA except pain relieving and replacement of damaged joints.[3–5] However, recent research findings provide substantial evidence that confirmed (TGF-β) signaling pathway contributes to OA development and progression. In this review, we will focus on the role of TGF-β pathway in articular cartilage, subchondral bone and synovium tissue during OA progression.

TGF-β SIGNALING AND OA IN PATIENTS

Recently, by the tremendous advances of genome-wide association analysis, the correlation between the genetic variants of TGF-β signaling pathway components, ranging from ligands to transcription factors, and OA is reported in patients. In Japanese and Chinese women populations, a polymorphism in TGF-β1 signal region (T29 to C) has been linked to the incidence of spinal osteophyte formation, an indication of OA development.[6–7] Mutations in TGF-β1 gene are also found in Camurati–Engelmann disease, leading to elevated TGF-β1 activity.[8] The patients have long bone osteosclerosis, which is thought to be related with OA development.[9–11] Another genetic variant in asporin (*ASPN*), an inhibitor of TGF-β pathway, was reported to be associated with higher susceptibility to OA in Asian and Spanish Caucasian populations.[12–15] The *ASPN* gene encodes a small leucine-rich extracellular matrix molecule, contains three repeat encoding for aspartic acid (D) within exon2.[12] Compared to common asporin D-13, the D-14 allele of *ASPN* is found to be highly expressed in OA cartilage tissue, inhibiting TGF-β signaling-mediated synthesis of cartilage specific extracellular matrix components, such as type II collagen and proteglycan in chondrocytes.[16] An asporin polymorphism (D-14), a strong inhibitor of TGF-β pathway than the common D-13 repeat, showed a significantly higher frequency in OA patients.[12] This indicates decreased TGF-β response might be correlated with increased susceptibility to OA.

In addition to TGF-β ligands and antagonists, polymorphism and mutations of the critical signaling molecule, Smad3, is reported to be involved as a risk factor of OA as

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well. A single-nucleotide polymorphism in the intron region of human *Smad3* gene has been linked to the incidence of hip and knee OA in a 527 European patient cohort.[17] Furthermore, mutations have been identified in human *Smad3* gene coding region in patients with aneurysm–osteoarthritis syndrome.[18–22] Similar to Marfan syndrome and Loeys–Dietz syndrome,[23–24] patients with aneurysm–osteoarthritis syndrome have connective tissue disorders, such as thoracic arotic aneurysms, dissections and tortuosity throughout the arterial tree. However, the major clinical diagnostic is that most of these affected individuals presented with early-onset osteoarthritis.[21] Genetic mapping reveals that aneurysm–osteoarthritis syndrome is caused by the mutations in the *Smad3* gene in chromosome 15q22.2–24.2 locus. One of the mutations is a deletion of two nucleotides (c. 741–742delAT), leading to a frameshift reading and premature termination of protein translation in exon 7. This deletion mutation is a truncating mutation, nearly removing the complete MH2 domain, which is critical for Smad3 phosphorylation and heteromer formation with co-Smad (Smad4). The other mutation is missense mutation, c. 782C>T, in MH2 protein interface domain. Substitution of threonine for isoleucine (p. Thr261Ile) disturbs the local charge status in this highly conserved protein interface region, leading to structural and conformational change and further forces the abnormal rearrangement of the residues and three-dimensional structure, which is essential for Smad3 interacting with other Smads to conduct the TGF-β signaling.[21] The findings, shown above, endorse the fact that changes in TGF-β itself or TGF-β signaling components are highly related to OA development.

TGF-β SIGNALING IN OSTEOBLAST LINEAGE CELLS, CHONDROCYTES AND SYNOVIAL FIBROBLASTS

TGF-β is a large family of growth factors, which plays a critical role in early embryonic development and postnatal growth and regulates cell proliferation, differentiation, apoptosis and migration in different tissues or cell populations. Even for the same cell population, with different microenvironment, TGF-β can exert different functions.[25] In mammalian cells, TGF-β is secreted in a biologically inactive form as a propeptide precursor with a latency-associated peptide domain. TGF-β has to be activated by cleaving this latency-associated peptide domain to release the mature domain. There are three isotypes of TGF-β, TGF-βs 1, 2 and 3, which shared a highly conserved homology of around 90%. These three isoforms are differentially expressed in different tissues or cell populations, controlled by different promoter sequences.[26]

The canonical TGF-β signaling pathway is initiated by three isoforms of TGF-β binding to the type II receptor, followed by phosphorylation of type I transmembrane serine-threonine kinase receptors (ALKs). The phosphorylated type I receptors, usually ALK5, then transduct TGF-β signal intracellularly by phosphorylating R-Smads, including Smads 2 and 3 at conserved C-terminus SSXS motif. The activated R-Smads form heteromeric complexes with co-Smad (Smad4) and then enter the nucleus, associating with other DNA binding proteins to regulate downstream gene transcription.[27] Besides Smad-dependent TGF-β signaling pathway, TGF-β can activate a non-Smad route as well through TGF-β-activated kinase 1, a MAP kinase kinase kinase, to initiate JNK/p38 MAP kinase pathway. In chondrocytes, TGF-β has been shown to signal via ALK1 to activate bone morphogenetic protein (BMP) signaling.[28–30] BMP signaling is mainly routed through ALK1, 2, 3 and 6 to phosphorylate Smad1/5/8, which act as opposite cellular function than TGF-β in several different cell types such as chondrocytes and osteoblasts.[28,31]

TGF-β signaling pathway is tightly regulated in several cellular levels to ensure its proper physiological function, including ligand, receptor and R-Smad levels. Both chrodin and noggin are endogenous TGF-β antagonists, inhibiting TGF-β binding to its receptor to activate the signaling pathway.[32] Exogenous addition of latency-associated peptide in a high concentration can bind to the mature TGF-β factors, and function as a TGF-β inhibitor.[33] Besides extracellular ligand regulation, TGF-β signaling can also be turned off through receptor internalization.[34] TGF-β receptors are endocytosed in a clathrin-coated vesicle dependent manner.[35] TGF-β receptor activation can be further regulated by inhibitory Smad (I-Smad), including Smads 6 and 7. It has been well documented that I-Smads form complex with TGF-β type I receptor, blocking R-Smads' binding to its receptor, thus R-Smads cannot be phosphorylated to form R-Smad/co-Smad complex to activate TGF-β signaling in the nucleus.[27] I-Smad can recruit E3 ubiquitin ligase as well, such as Smurfs 1 and 2, as an endogenous negative regulator of TGF-β pathway, because Smurf1/2 can trigger the ubiquitination of R-Smads and co-Smad, leading to proteasome-dependent degradation.[36–38]

Both TGF-β isoforms and TGF-β receptors are broadly expressed in cartilage, bone and synovial tissues. However, TGF-β signaling plays quite different role in these tissues. During chondrogenesis, TGF-β is the main initiator of mesenchymal stem cell (MSC) condensation.[39–42] After aggregation, TGF-β signaling further stimulates chondrocyte proliferation while inhibiting chondrocyte hypertrophy and maturation. During this process, TGF-β signaling promotes chondrocytes to express cartilage-specific extracellular matrix molecules, *Col2* and *Agc1*, to form cartilage tissue. Alternatively, TGF-β signaling promotes osteoblast terminal maturation, depositing two main extracellular matrix components, *Col1* and *osteocalcin*, in bone tissue.[43]

In synovial fibroblasts, TGF-β signaling is an inducer of synovial tissue fibrosis characterized as fibroblast proliferation and types I and III collagen accumulation.[44] With this in mind, we will distinguish the roles of TGF-β signaling in these cell types during OA development.

THE ROLE OF TGF-β SIGNALING IN ARTICULAR CHONDROCYTES AND OA DEVELOPMENT

It has been well established that the articular chondrocyte is the cell responsible for maintenance of articular cartilage homeostasis.[45] As such, the dysregulation of this cell is directly linked to the pathological process of cartilage degeneration in OA.[46–50] Most recently, several lines of evidence suggest that TGF-β/Smad pathway plays a critical role in the regulation of articular chondrocyte hypertrophy and maturation during OA development.[51–55]

Mice deficient for genes encoding any TGF-β isoform show embryonic lethality and loss of TGF-β2 or TGF-β3 genes leads to numerous bone defects affecting the forelimbs, hindlimbs and craniofacial bones, suggesting that TGF-β plays an important role in skeletogenesis.[56] Consistent with genome-wide association studies in human patients, genetic manipulation of TGF-β pathway components also demonstrated that TGF-β signaling plays a critical role during OA development. Transgenic mice that overexpress the dominant-negative type II TGF-β receptor (*dnTgfbr2*) in skeletal tissue exhibit progressive skeletal degeneration.[47] The articular chondrocytes in the superficial zone of cartilage tissue become hypertrophy with increased type X collagen expression. Loss of proteoglycan and progressive degradation of cartilage tissue have been observed in 6-month-old mice which strongly resemble human OA.[52] This observation is supported by studies in mice with global knockout of the *Smad3* gene. *Smad3* knockout mice developed spontaneous joint degeneration resembling human OA as characterized by chondrocyte hypertrophy with expression of type X collagen in superficial zone, progressive loss of articular cartilage tissue and formation of osteophytes.[53] Supportive of these findings, Smurf2-transgenic mice under control of the *Col2a1* promoter exhibit destruction of cartilage tissue, articular cartilage fibrillation, clefting, eburnation, subchondral bone sclerosis and osteophyte formation. Increased expression of type X collagen and MMP-13 were also detected in articular cartilage from these transgenic mice. All of these changes of osteoarthritic hallmark coincided with reduced TGF-β signaling as well as reduced pSmad3 levels. This finding was further strengthened by the fact that human OA cartilage strongly expressed Smurf2 as compared to healthy human cartilage.[38]

The observations described demonstrate that inhibition of TGF-β signaling in chondrocytes leads to chondrocyte terminal differentiation and the development of OA. Using *Col2-CreER* transgenic mice,[57–58] we have recently generated chondrocyte-specific *Tgfbr2* conditional knockout mice ($Tgfbr2^{Col2ER}$) in which deletion of the *Tgfbr2* gene is mediated by Cre recombinase driven by the chondrocyte-specific *Col2a1* promoter in a tamoxifen-inducible manner. This is the first time to study the role of TGF-β signaling in postnatal stage, specifically in chondrocytes, which can recapitulate OA initiation and progression in patients. Deletion of the *Tgfbr2* gene in adult mice resulted in upregulation of *Runx2*, *Mmp13*, *Adamts5* and *Col10* expression in articular chondrocytes. Histological analysis showed articular cartilage degradation, increased hypertrophic chondrocyte numbers, early osteophyte formation and increased subchondral bone mass in 3-month-old $Tgfbr2^{Col2ER}$ mice. Loss of entire articular cartilage, formation of extensive osteophytes, and substantially increased subchondral bone mass were observed in 6-month-old $Tgfbr2^{Col2ER}$ mice.[51] Our data support the earlier findings that TGF-β receptor expression was reduced in aged mice which are prone to OA development.[28–54] To determine if up-regulation of *Mmp13* and *Adamts5* expression is responsible for $Tgfbr2^{Col2ER}$-induced OA development, we generated *Tgfbr2*/*Mmp13* and *Tgfbr2*/*Adamts5* double knockout mice. Deletion of the *Mmp13* gene significantly alleviates OA-like pathological changes observed in 3-and 6-month-old $Tgfbr2^{Col2ER}$ mice. In contrast, deletion of the *Adamts5* gene only prevented OA-like phenotype in 3-month-old $Tgfbr2^{Col2ER}$ mice. Treatment of $Tgfbr2^{Col2ER}$ mice with MMP-13 inhibitor CL82198 (10 mg/kg) for 2 months decelerated OA progression in 3-month-old $Tgfbr2^{Col2ER}$ mice.[51] These observations were consistent with the fact that deletion of the *Mmp13* gene attenuated articular cartilage degeneration observed in destabilization of medial meniscus mouse model.[59–60] In this study, we demonstrate that inhibition of TGF-β signaling in articular chondrocytes leads to a progressive OA-like phenotype in mice. *Mmp13* and *Adamts5* are critical downstream target genes of TGF-β signaling during OA development.

THE ROLE OF TGF-β SIGNALING IN SUBCHONDRAL BONE CELLS AND OA DEVELOPMENT

TGF-β signaling plays a critical role not only in the regulation of chondrocyte homeostasis during cartilage destruction, but also in the manipulation of subchondral bone cell behavior during osteophyte formation, another feature of OA.[43] Osteophyte is a fibrocartilage-capped bony outgrowth at the margins of diarthodial joints. Studies from murine experimental OA models clarified that osteophyte originated from MSC-like periosteal lining cells at the bone–cartilage junction, but not synovial lining cells.[61–62] Those condensed progenitor cells inside the

developing osteophyte differentiates into chondrocytes and undergo chondrogenesis to produce matrix molecules, such as type II collagen and aggrecan. Following early chondrocyte differentiation, the cells rapidly proliferate, enlarging the cartilage templates that contribute to the growth of osteophyte. The most central cells eventually withdraw from the cell cycle and initiate the process of hypertrophic differentiation and endochondral ossification, depositing type X collagen and mineral. The terminally differentiatied chondrocytes ultimately undergo apoptosis and are replaced by osteoblast and osteoclast to establish bone marrow and angiogenesis, which uniform the osteophyte as a part of subchondral bone.[63]

In murine models, triple injection of TGF-β isoforms or BMP2 or 9 can lead to osteophyte formation in the knee joint.[64–66] The osteophytes induced by TGF-βs originate from the periosteal lining cells, located at the margin of subchondral bone. However, BMP injection stimulates osteophyte formation adjacent to growth plate cartilage. Thus, based on the localization and the pattern of development, TGF-β induced osteophyte formation is more similar to OA-related osteophytes.[61,64–65] This observation is further strengthened by the finding that the cells in the outer layer of osteophytes strongly express TGF-β1 to activate the TGF-β signaling pathway in the experimental OA murine models.[61] The role of TGF-β signaling pathway in osteophyte formation is further explored by blocking studies using specific TGF-β inhibitors. Several groups demonstrate that ablation of endogenous TGF-β activity, by intra-articular overexpression soluble TGF-β type II receptor extracellular domain or Smad7, suppresses osteophyte formation in experimental murine OA models.[67] These observations clearly demonstrate that TGF-β plays a dominant role in the induction of osteophytes, at least in murine OA models.

Cao group recently further clarified the role of TGF-β pathway in the subchondral bone at the onset of OA by using an anterior cruciate ligament transaction (ACLT) model of OA.[68] In the ACLT mice, elevated TGF-β activity was observed in the subchondral bone, followed by alterations of subchondral bone structure and proteoglycan loss in articular cartilage tissue. High concentrations of TGF-βs, released and activated from damaged joint tissues during OA development, induces the migration and formation of nestin-positive MSC clusters, leading to formation of marrow osteoid islets accompanied by high levels of angiogenesis.[69] These observations were further confirmed by the clinic report that TGF-β1 concentrations were high in subchondral bone as well from OA patients. Moreover, transgenic mice, overexpressing of active TGF-β1 in osteoblastic cells, spontaneously developed OA with subchondral bone sclerosis and cartilage destruction, whereas inhibition of TGF-β activity in subchondral bone, by injection of TGF-βRI inhibitor or TGF-β neutralizing antibody, stabilized the subchondral bone microarchitecture and attenuated the degeneration of articular cartilage by decreasing the uncoupled bone formation and angiogenesis in osteoid islets of ACLT mice. In particular, inducible knockout of the TGF-β type II receptor (TβRII) in nestin-positive MSCs led to less changes in the subchondral bone and articular cartilage degeneration relative to wild-type mice after ACLT. Thus, in response to abnormal mechanical loading, TGF-βs were released, activated and accumulated in subchondral bone to stimulate aberrant bone formation and angiogenesis through recruitment of nestin-positive MSCs or osteoprogenitor cells during the pathological changes of osteoarthritis; and inhibition of this process could be a potential therapeutic approach to treat OA.

THE ROLE OF TGF-β SIGNALING IN SYNOVIAL FIBROBLASTS AND OA DEVELOPMENT

Besides cartilage tissue and pericartilage (subchondral) bone tissue, there is increasing recognition that synovium tissue contribute to OA development as well.[70] Synovial lining cells are the major components in the synovial membrane, including macrophages, fibroblasts and MSCs.[71] Since articular cartilage is a non-vascular tissue, subchondral bone and synovial tissue are the two major nutrient sources to support normal cartilage function.[1] Besides oxygen and nutrients, the cellular elements of the synovium tissue are the unique source of synovial fluid, which contains lubricin and hyaluronic acid. These two important molecules produced by synovial lining cells, functioning as a lubricant, contribute to protect and maintain the integrity and function of articular cartilage surfaces in diarthrodial joints. Besides reducing friction, synovial fluid is also the important reservoir to remove chondrocyte metabolism products and articular matrix turnover debris.[72–73]

Synovial lining hyperplasia, infiltration of macrophages and fibrosis can be often observed during OA progression in patients.[74] In the past, OA was categorized as a non-inflammatory form of arthritis. In past decades, however, compared to rheumatoid osteoarthritis, synovial inflammation was not thought to play a critical role in the setting of OA. However, recent research efforts provide substantial evidence that low-grade inflammation in earlier-stage OA promotes cartilage degeneration during OA progression.[70,75] The inflammatory pattern is equally the same, low grade in comparison to high grade in RA, although the pattern of synovial change is diverse, varying from the stage of disease.[70] Recent clinical reports have pointed out that TGF-β signaling pathway plays a critical role in synovitis during OA progression, since elevated TGF-β activity has been detected in synovial fluid of OA

patients.[44] Murine models with intra-articular injection of activated recombinant TGF-β, or adenovirus overexpression of active TGF-β, further demonstrated substantial synovial fibrosis characterized by progressive synovial hyperplasia and fibroblast proliferation and extracellular matrix deposition.[62,64] In this experimental OA model, TGF-β promotes synovial lining cells proliferation, disrupts the apoptotic process and deposits extracellular matrix, leading to synovial tissue expansion and hyperplasia. TGF-β can also function as a chemotatic factor to recruit fibroblasts into synovial tissue to make it fibrotic.[62,64] Moreover, TGF-β induces synovial lining cells to produce inflammatory factors, such as IL-1 and TNF-α, which can further stimulate articular chondrocytes terminal hypertrophy, depositing type X collagen instead of type II collagen and aggrecan.[70] Further investigation demonstrated that blocking of TGF-β itself or of TGF-β signaling by overexpression of Smad7, significantly attenuated TGF-β-induced synovial fibrosis in murine experimental OA models.[76–77] These findings indicate that TGF-β is an important driving force for synovial fibrosis in OA and contributes to the articular cartilage pathology.

CONCLUSION

OA is a degenerative joint disease, affecting the whole joint structure, including articular cartilage tissue, pericartilage (subchondral) bone tissue and synovium tissue. Recent confirmed and transgenic mice studies indicate that TGF-β signaling pathway plays a critical and unique role in chondrocytes, MSCs and synovial lining cells during OA development and progression, by driving chondrocytes toward hypertrophy, promoting osteoprogenitor cell differentiation into osteoblasts and angiogenesis in subchondral bone, and stimulating synovial lining cells expansion and fibrosis. TGF-β signaling, especially the critical downstream target genes, such as *Runx2*, *Mmp13* and *Adamts5*, could serve as a potential key target for therapeutic intervention for the treatment of OA disease. In addition to TGF-β signaling, modulating of Wnt/β-catenin, Notch, and Indian Hedgehog pathways contribute to OA progression as well. The interaction of these signaling pathways with TGF-β signaling needs to be further explored. *Mmp13* and *Adamts5* are two common target genes involved in the signaling networks, promoting chondrocyte hypertrophy and leading to cartilage degradation.[78–80] Epigenetic and microRNA regulation of *Mmp13* and *Adamts5* was also observed during OA development and progression, indicating epigenetic factors and microRNAs may also play a role in the pathophysiology of OA.[81–82]

Conflict of Interest

The authors declare no conflict of interest.

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