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Promising targets for therapy of osteoarthritis: a review on the Wnt and TGF-β signalling pathways

Chahrazad Cherifi, Silvia Monteagudo and Rik J. Lories

***Abstract*:** Osteoarthritis (OA) is the most common chronic joint disorder worldwide, with a high personal burden for the patients and an important socio-economic impact. Current therapies are largely limited to pain management and rehabilitation and exercise strategies. For advanced cases, joint replacement surgery may be the only option. Hence, there is an enormous need for the development of effective and safe disease-modifying anti-OA drugs. A strong focus in OA research has been on the identification and role of molecular signalling pathways that contribute to the balance between anabolism and catabolism in the articular cartilage. In this context, most insights have been gained in understanding the roles of the transforming growth factor-beta (TGF-β) and the Wingless-type (Wnt) signalling cascades. The emerging picture demonstrates a high degree of complexity with context-dependent events. TGF-β appears to protect cartilage under healthy conditions, but shifts in its receptor use and subsequent downstream signalling may be deleterious in aged individuals or in damaged cartilage. Likewise, low levels of Wnt activity appear important to sustain chondrocyte viability but excessive activation is associated with progressive joint damage. Emerging clinical data suggest some potential for the use of sprifermin, a recombinant forms of fibroblast growth factor 18, a distant TGF-β superfamily member, and for lorecivivint, a Wnt pathway modulator.

Keywords: cartilage, osteoarthritis, transforming growth factor-beta, wnt

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Introduction

Osteoarthritis (OA) is a disease leading to disability and reduced quality of life for patients. OA is the most common chronic joint disease worldwide, with estimates indicating that it will affect 78 million people by 2040.[1,2] Currently, OA is recognized as a skeletal disease of the whole joint, characterized by progressive degeneration of articular cartilage, associated with synovial inflammation, subchondral bone remodelling and bony outgrowths named osteophytes. OA is considered a complex disease to which both genetic and acquired factors contribute and can be considered as a clinical outcome of different processes and risk factors.[3,4] Current treatments for OA remain limited to pain relief and physiotherapy to support and improve the functional status.[5]

The specific roles of the articular cartilage and subchondral bone as primary sites for osteoarthritic processes have been long debated and may differ between patients, with inflammation and osteophyte formation commonly considered as secondary phenomena. In this review, we focus on molecular signalling pathways that have been associated with OA and that are well studied in cartilage. The articular cartilage is an avascular tissue that contains a unique cell population, the articular chondrocytes, embedded in their self-produced extracellular matrix (ECM) rich in proteoglycans, collagens and water. The homeostasis of the ECM depends largely on the articular chondrocytes. The complex structure defines a specific biomechanical environment, with a certain degree of adaptive capacity upon variation in loading and stress conferred by the articular

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chondrocytes. Likewise, the ECM has an important role in the regulation of chondrocyte function, thereby creating a specific macro- and microenvironment of molecules that regulate the activation of diverse signalling pathways.

In the physiological and healthy state, there is a balance between the anabolic and catabolic factors of the cartilage to maintain matrix turnover. Anabolic factors are typically growth factors such as members of the Transforming Growth Factor β (TGF-β) superfamily including both TGF-βs and Bone Morphogenetic Proteins (BMP).[6–8] Conversely, catabolic factors include tissue-destructive and remodelling enzymes such as matrix metalloproteases (MMP-1, MMP-3, MMP-13) and aggrecanases (ADAMTS-4 and ADAMTS-5) that are produced when the mechanical properties are altered and by the presence of soluble mediators of inflammation including inflammatory cytokines such as IL-1β and IL-6.[9] The catabolic reactions disrupt the collagen network and the structure of the ECM.[10,11] Other mechanisms that appear directly relevant for onset and progression of OA are the hypertrophic differentiation of articular chondrocytes that can lead to cell apoptosis and matrix calcification, rendering the ECM suboptimal from a biomechanical point of view. The latter process has been associated with excessive activation of the Wnt signalling cascade.[12]

The maintenance of homeostasis and the prevention of OA is therefore dependent on tight regulation of the balance between anabolism and catabolism. In this context the importance of the TGF-β superfamily, including BMPs, and the Wnt signalling cascades has been demonstrated in multiple *in vitro* and *in vivo* models, and agonists or antagonists of these cascades have been evaluated in preclinical models and early phase clinical trials.[13,14] Here, we summarize current views on the role of the TGF-β and Wnt signalling cascades in joint health and disease.

TGF-β signalling

The TGF-β superfamily of growth factors and cytokines includes over 30 secreted proteins that are involved in various biological processes such the immune response, growth control, cell differentiation, early development and, more specifically, skeletogenesis.[15,16] Based on their sequence homology, receptors affinities and activities *in vivo*, the TGF-β superfamily can be divided into two major subfamilies: the TGF-β/Activin/Nodal family and the Bone Morphogenetic Protein (Bmp) family. The TGF-β family itself comprises TGF-βs (TGF-β 1–3), Activins (Act A–D) and Nodal/Nodal-related proteins.

The TGF-βs (1–3) are ubiquitous multifunctional cytokines secreted in an inactive form. TGF-βs are involved in the regulation of a plethora of biological functions. First identified as major inhibitors of cell proliferation, one of their main functions is as a powerful immunosuppressant.[17] However, it has become clear that TGF-β has more complex effects in an inflammatory context, with both pro and anti-inflammatory actions, depending on the microenvironment where the cytokine acts. TGF-βs, as homodimers, are associated with a homodimer of LAP proteins (Latency Associated Protein) to form the Small Latent Complex (SLC) called latent TGF-β. This complex is secreted from the cell together with the LTBP1 protein (Latent TGF-β Binding Protein-1) to form the LLC complex (Large Latent Complex). LTBP1 can bind to ECM proteins such as fibronectin, and this plays an important role in the stabilization and the creation of local gradients of TGF-β in ECM-rich tissues such as cartilage.[18] TGF-β must be released from these complexes in order to bind to its receptors and activate downstream signalling cascades. This release is facilitated by the proteolytic activity of serine proteases such as cathepsins or MMPs.[19–21] All TGF-β ligands bind and signal through membrane-bound receptor complexes consisting of two type I (or Activin Receptor like-kinase, ALK) and type II receptors. Seven types of type I receptors and five type II receptors have been identified and diverse combinations of type I and type II receptors contribute to the complexity of the signalling system.[22,23]

The receptors have transmembrane domains with intrinsic serine/threonine phosphorylation kinase activity. The binding of a ligand on its receptor induces the formation of a heteromeric complex and phosphorylation of the TβR-I receptor by the TβR-II receptor. The main signal transduction cascade initiated by TGF-β is dependent on intracellular SMAD proteins. Activated TβR-I (also known as ALK5) phosphorylates SMAD2 and SMAD3. These SMADS associate with Co-SMAD4 to translocate into the nucleus acting as transcription factors that enhance or repress the expression of many different target genes. Intracellular negative regulation of this signalling

cascade is dependent on inhibitor (I)-SMADS. I-SMAD7 competes with R-SMADS for binding to TGFβR-I or recruits Smurf proteins (a ubiquitin ligase for SMAD proteins) leading to the degradation of TGFβRI.[24] Other pathways, independent of SMAD proteins, are also involved in the TGF-β signalling, such as MAPK, PI3K-Akt, PP2A, Rho and Par6 activation,[25] adding to the overall biological complexity.

TGF-β signalling and osteoarthritis

TGF-β signalling in chondrocytes and its role in cartilage homeostasis

The role of TGF-β in OA has been investigated for several decades.[26] From a genetic point of view, one interesting study highlighted that a non-functional mutation in SMAD3 leads to a familial Marfan-like syndrome with early onset OA.[27] Common genetic variation in the *SMAD3* gene was subsequently also associated with the risk of both non-syndromic hip OA and knee OA.[28]

TGF-β can be produced and secreted by different types of cells within the joint that include the articular chondrocytes, the synovial fibroblasts and macrophages. The mean level of TGF-β in synovial fluid was found to be at a 4 ng/ml concentration, a concentration that is within the range of those commonly used for *in vitro* experiments in cell culture settings.[29] In cartilage ECM, TGF-β precursor molecules represent a reserve of 300 ng/g.[30] Hypertrophic chondrocytes appear to produce more TGF-β (5 ng/10^6 cells) compared with articular ones (2, 3 ng/10^6 cells).[31] Not surprisingly, experimental work indicates that changes in TGF-β signalling or TGF-β components may have an impact on cartilage homeostasis and can lead to the development of OA.

Differential activation of TGF-β receptors and the ensuing downstream events control the molecular characteristics of articular chondrocytes. Shifts from homeostatic ALK5-mediated signalling towards ALK1-mediated signalling have been extensively investigated. Canonical TGF signalling is dependent on ALK5 and phosphorylated SMAD2/3 translocation to the nucleus. This mechanism contributes to the expression of anabolic genes such as type 2 collagen. In contrast, when TGF-β signals through the ALK1 receptor, phosphorylated SMADs 1/5/8 translocate to the nucleus and increase MMP13 expression. Likewise, overexpression of ALK5 increased aggrecan in mouse chondrocytes, as opposed to overexpression of ALK1 that resulted in an increase in MMP13. Conversely, ALK5 knockdown in chondrocytes promoted chondrocyte terminal differentiation of which MMP13 expression is characteristic. Similar data have been reported from human samples, where ALK5 expression was correlated with type 2 collagen and aggrecan expression.[32] A clear switch from ALK5/SMAD3/2 signalization towards ALK1/SMAD1/5/8 was also observed in aged mice[32] (Figure 1).

Serra and collaborators investigated overexpression of a truncated dominant-negative TGF-βRII, and described a severe skeletal phenotype with OA-like characteristics.[33] In another recent study, different mouse models were used to demonstrate the role of the TGF-βRII receptor and the linked downstream effects. Interestingly, the OA phenotype observed in the absence of TGF-βRII receptor signalling was counteracted by MMP13 or ADAMTS5 deletion, identifying these destructive enzymes as effectors for the negative effects of absence of TGF-β signalling. The importance of TGF-β was also highlighted in SMAD3 full knockout mice or its conditional deletion in cartilage both leading to a degenerative joint disorder resembling human OA. Genetic modulation of TGF-β inhibitors highlighted the intrinsic protective effect of the cytokine as overexpression of Smurf2 or SMAD7 and mLAP1 in mice leads to OA feature.[34–36] Thus, the emerging picture suggests that the ALK5/SMAD2,3 cascade activation is an important factor in cartilage health, but that upon aging or under other unknown circumstances a switch towards the ALK5/SMAD1, 5, 8 cascade represents a deleterious event that contributes to the development of OA.

This complex concept of TGF-β signalling, in which its downstream effects and outcomes may be defined by the available receptor repertoire, complicates clinical questions on its value as a therapeutic target or strategy. Active TGF-β is detected in the synovial fluid of OA patients.[37,38] OA-like changes of the cartilage have been described after exposure of knee joints to exogenous TGF-β, a feature that appears to be dose and time dependent.[39,40] TGF-β activation in naïve mice stimulated proteoglycan cartilage synthesis, whereas in a second study repeated intra-articular administration of TGF-β and prolonged exposure induced changes in articular cartilage with focal loss of proteoglycans in the deep cartilage layer and micro cracks.[39–41] In line with this, systemic administration of an

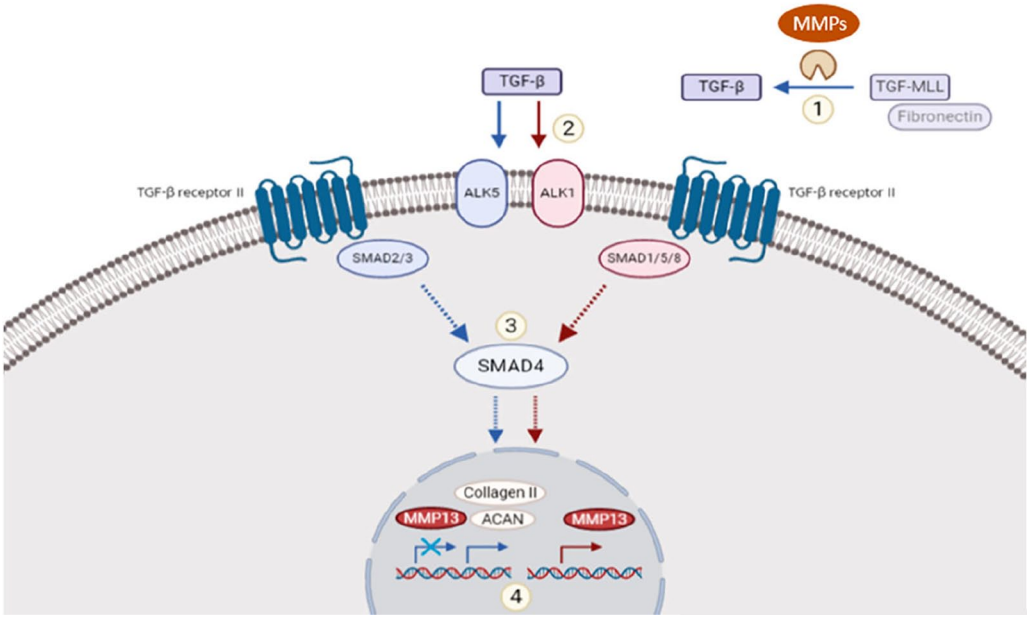

Figure 1. TGF-β signalling in chondrocytes: Latent TGF-β in the extracellular matrix must be released by the proteolytic activity of enzymes like MMPs (1) to bind to its receptors and activate downstream signalling cascades (2). Phosphorylated SMADs after ALK receptor activation by TGF-β, interact with SMAD4 (3) and translocate to the nucleus to modify gene expression programmes (4). When TGF-β interacts with ALK5, it induces SMAD 2/3 signalling and an anabolic response. Conversely, during osteoarthritis and aging, a switch from TGF-β signalling to SMAD1/5/8 is observed with a decrease in the SMAD2/3 to SMAD 1/5/8 ratio and the activation of a catabolic response. Created in BioRender.com.

anti-TGF-β antibody prevented cartilage damage in the ACLT OA model. *In vitro* experiments also demonstrated that short and long TGF-β stimulation show opposite effects on cartilage health.[42] Short treatment of chondrocytes with TGF-β leads to a decrease in MMP13 while a longer stimulation increases MMP13 in which SMAD3 and Runx2 have been demonstrated as a key central mechanism. Yet, TGF-β treatment of chondrocytes counteracts the catabolic effect of IL1-β, with IL1-β leading to a decrease in TGF-βRII levels.[6,43,44] In human cartilage explants, only the chondrocytes in the deep layers stained positive for MMP13 after TGF-β stimulation.[45] In addition, inhibition of endogenous TGF-β enhances loss of proteoglycans in experimental OA.[34] Altogether, these combined and seemingly conflicting data suggest a complex dual role of TGF-β in OA pathology in which both the lack and high activation status may cause joint pathology.

A further level of complexity is found in the effects of TGF-β beyond cartilage homeostasis on synovial inflammation and osteophyte formation. TGF-β is a pleiotropic cytokine and growth factor that also influences many aspects of inflammation and immunity. Direct injections of TGF-β into the mouse knee joint induced mild synovitis.[39] When OA is triggered by collagenase injection TGF-β injection leads to synovial thickening,[40] while inhibition of TGF-β in the papain model reduced synovitis.[35] The inflammatory reaction in human OA also appears diverse and remains to be further defined. Hence, it is difficult to extrapolate this type of mouse model data. High levels of TGF-β activation resulted in moderate fibrosis in the joint and in extensive osteophyte formation, while lower but long-lasting activation leads to more pronounced fibrosis and moderate osteophyte formation.[41,46] Notably, only the endogenous inhibition of TGF-β suppressed osteophyte formation while unluckily leading to more cartilage damage.[34] Some of these data may also be related to differences in preclinical models: in the papain cartilage damage model TGF-β isoform staining was increased in articular cartilage,[34] whereas in spontaneous and instability-associated OA model expression was very low compared with normal mice.[47] An overview of the main studies in given in Table 1.

Table 1. An overview of genetic and pharmacological manipulation of TGF-β signalling in osteoarthritic disease.

OA model	Modulation	Knee joint tissues damage	Publication
naïve	Injection of TGF-β1	Stimulation of proteoglycan cartilage synthesis, osteophyte formation and synovitis.	Van Beuningen *et al.*[39]
Collagenase injection	TGF-beta injection	PG increase in the superficial cartilage. Severe focal PG loss in deep layer of cartilage, osteophyte formation and thickening of synovium.	Van Beuningen *et al.*[40]
ACLT	Systemic administration of TGF-β antibody	Prevention of cartilage damage, osteophyte volume and subchondral bone sclerosis.	Xie *et al.*[48]
Papain	Systemic treatment with soluble TGF-βRII (solRII)	Reduced osteophyte formation, loss of proteoglycan and reduce of articular cartilage thickness.	Scharstuhl *et al.*[34]
	TGF-βRII$^{-/-}$ TGF-βRII$^{-/-}$ and MMP13$^{-/-}$ TGF-βRII$^{-/-}$ and ADAMTS5$^{-/-}$	TGF-βRII$^{-/-}$ induced cartilage damage, osteophyte formation and no synovial fibrosis. Both TGF-βRII$^{-/-}$ and MMP13$^{-/-}$ or TGF-βRII$^{-/-}$ and ADAMTS5$^{-/-}$ prevented joint tissues damage.	Shen *et al.*[49]
	Overexpression of truncated TGF-βRII	Severe skeletal defect phenotype or hypertrophic chondrocytes. Synovium excess, osteophyte formation.	Serra *et al.*[33]
	SMAD3 KO	SMAD3 mutant mice developed degenerative joint disease resembling human OA. OA lesions severity, loss of proteoglycan, osteophyte formation, abnormal skeletal development and growth plate increase.	Yang *et al.*[50]
	SMAD3 conditional knockout	More cartilage damage. Increased severity of OA lesions, loss of proteoglycan, hypertrophy differentiation. Abnormal skeletal development.	Chen *et al.*[51]
Papain	Adenovirus over-expressing SMAD7 and mLAP-1	Cartilage proteoglycan loss. Reduction of synovitis and osteophyte formation.	Scharstuhl *et al.*[35]
	Overexpression of Smurf2	Decreased articular cartilage area, subchondral bone sclerosis and osteophyte formation.	Wu *et al.*[36]
	Ltbp-3 null mice	Degenerative changes were observed in the articular cartilage.	Dabovic *et al.*[52]

OA, osteoarthritis.

TGF-β signalling role in subchondral bone

In vivo studies with different murine models of OA induced by joint instability show an increase in subchondral bone remodelling in the early stages of the disease and osteo-condensation of subchondral plate at the late stage.[53] In addition, mice with increased bone remodelling (induced by ovariectomy or RUNX2 overexpression) develop more severe OA after joint instability compared with normal mice. Inhibition of bone remodelling by bisphosphonates, estrogen or OPG (osteoprotegerin), a soluble RANKL receptor, decreases cartilage damage in preclinical models.[54] These results show the importance of bone remodelling and factors released by bone cells (osteoblasts, osteoclasts and osteocytes) of the subchondral bone in the pathogenesis of OA. This hypothesis may be specifically linked to

TGF-β in this process. The expression of TGF-β1 is increased in osteoarthritic subchondral bone in humans and in murine models.[55]

In mice, expression of TGF-β1 is correlated with the number of osteoclasts in bone, and blockade of osteoclasts with alendronate in the rat anterior cruciate ligament transection model (ACLT) is chondro-protective and associated with a reduced release of active TGF-β.[56] Overexpression of TGF-β1 in osteoblasts of subchondral bone induces cartilage damage, whereas its inhibition in subchondral bone slowed cruciate ligament transection-induced cartilage degradation. TGF-β1 produced by osteoblasts of the subchondral bone stimulates clustering of mesenchymal bone marrow cells and promotes neo-angiogenesis.[55] The mesenchymal cell clusters differentiate into osteoblasts and stimulate the formation of osteoid. Thus, knockout of the TGF-β type II receptor (TβRII) in nestin+ mesenchymal stem cells (MSCs) in the subchondral bone, suppressing TGF-β mesenchymal cell clusters, reduced subchondral angiogenesis, calcification and cartilage damage.[55] These findings were confirmed in another study where a decrease in the number of nestin+MSCs was found, and reduced bone formation and hence 'osteoid islets' in the ACLT mouse model after treatment with TGF-β antibody, demonstrating the potential role of TGF-β targeting in subchondral bone during OA pathogenesis.[57] As above for its role in the articular cartilage, the impact of TGF-β signalling activation in the subchondral bone may move from a homeostatic signalling effect towards a pathological one depending on levels, context and receptor repertoire.

TGF-β and loss of articular chondrocyte identify: the case for hypertrophy

TGF-β treatment blocks the transition of mature chondrocytes to hypertrophic chondrocytes, a developmental differentiation step that is erroneously recapitulated in the context of post-natal OA. TGF-β acts as an inhibitor of chondrocyte hypertrophic differentiation, and reduces the expression of collagen type X of cultured chondrocytes and metatarsal bones.[58,59] During endochondral ossification, Ihh stimulates TGF-β signalling *via* ALK5-SMAD2/3 to induce PTHrP expression and inhibit hypertrophic differentiation of chondrocytes.[60] Notably, lack of TβRII signalling or knockout of SMAD3 in transgenic mice leads to abnormal hypertrophic chondrocyte accumulation and progressive degeneration of the articular cartilage.[49,50] As highlighted above, SMAD3 likely plays an important role in this. SMAD3 contributes to the maintenance of the cartilage ECM by inducing collagen II expression and repressing RUNX2-inducible MMP-13 expression. An additional level of complexity comes from the observation that the TGF-β effect on hypertrophy appears to be dependent on multiple downstream signalling cascades: the early response on MMP13 suppression is SMAD3 dependent and the later response on MMP13 induction may be mediated by P38 signalling.[51] In addition, SMAD3 inhibits RUNX2 during chondrocyte differentiation according to a mechanism independent of PTHrP by forming a SMAD3–RUNX2 complex and recruiting histone deacetylase HDAC4.[61] Conversely, TGF-β can interact with ALK1, and induce SMAD 1/5/8 signalling and MMP13 expression *via* RUNX2.[61]

In this context it is worth noting that SMAD 1/5/8 signalling is also triggered by the BMP family members, a group of growth factors that belong to the TGF-β superfamily. Largely but not exclusively dependent on ALK1, -2, -3 and -6 receptors, BMPs lead to the formation of SMAD4-SMAD1/5/8 complexes. BMPs and TGF-β both play roles in cartilage homeostasis as extensively discussed recently by Thielen *et al.*[62] Therefore, the role of BMPs in triggering the expression of SOX9 and RUNX2 could also be important in disease. BMP signalling is potentially contributing to the hypertrophic phenotype and – in a disease context – to progression of OA.[62] The specific contribution of distinct BMPs to OA remains to be further explored, as cellular models and developmental phenotypes in mice are insufficient to put together a good view on post-natal pathology. Whereas the TGF-β–ALK1 activation in the context of OA has been clearly demonstrated,[32,63,64] more research is needed to pinpoint the roles of BMPs in this context.

TGF-β and aging

Upon aging, chondrocytes are evolving towards decreased anabolic and proliferative responses after stimulation by growth factors compared with young cells. Thus, isolated aged chondrocytes showed reduced proliferation after stimulation with serum, TGF-β, bFGF (fibroblast growth factor), PDGF (platelet-derived growth factor) or IGF-1 (insulin) compared with chondrocytes isolated from young donors.[26,65] Similarly, the synthesis of proteoglycans and

collagens induced by IGF-1 or TGF-β decreases with age. As highlighted above, some of the aging-associated changes could be explained by differences in activation of signalling pathways. This consists of decrease in ALK5 and an increase in ALK1 expression[32,63,64] resulting in predominant stimulation of the ALK1-SMAD1/5/8 pathway to the detriment of the ALK5-SMAD3/2 pathway.[32,66] This catabolic response is also identified for other stimuli. Thus, chondrocytes isolated from old patients showed high MMP13 expression after stimulation with of fibronectin or IL-1β compared with chondrocytes isolated from young patients.[67] Whether aging also affects other aspects of OA such as increased osteophyte formation can be debated, as causality is difficult to demonstrate in patients.

Mechanical loading and TGF-β

The cartilage–bone unit in the joints is continuously exposed to mechanical loading in daily activities. Within its physiological range, mechanical loading is a critical factor involved in the maintenance of cartilage homeostasis. Excessive loading may contribute to joint damage, and the biomechanical properties of cartilage and bone are altered in the damaged joint as present in OA patients. Indeed, non-OA chondrocytes showed an increase in anabolic and a decrease in catabolic responses under mechanical stimulation while OA chondrocytes had an opposite profile.[68] The processes of mechano-transduction and mechano-responses are far from completely understood, but interactions with TGF-β signalling have been demonstrated. Mechanical forces impact TGF-β signalling through increases in TGF-β maturation and expression or through receptor activation.[30,69,70] In cartilage explants, SMAD2/3 signalling is activated under mechanical stress[69] whereas unloading resulted in a suppressive effect.[30] In another study, aging was associated with changes in the effect of loading on TGF-β signalling in chondrocytes. Young and aged cartilage explants that undergo loading were compared with decreased SMAD2/3 activation in aged explants compared with the young ones. In addition, the anabolic response assessed by collagen II and BMP2 expression was lower in aged cartilage.[70]

Therapeutic application

TGF-β is an excellent theoretical target for the treatment of OA but the complexity and context-dependent effects complicate the situation. TGF-β has the capacity to stimulate proteoglycan synthesis and inhibit hypertrophic chondrocyte differentiation and repression of the negative IL1-β effect. Yet effects on osteophyte formation and synovitis and fibrosis appear to be a strong limitation. Moreover, the molecular changes in receptor repertoire associated with aging and OA are a major conceptual challenge.

TissueGene-C is an original approach to try to treat OA using a cell therapy product of chondrocytes, partially transduced to express TGF-β. Phase II and phase III clinical trials have recently been reported[71–73] and suggest statistically significant improved clinical scores and suggested trends towards better outcomes compared with placebo in structural damage parameters. However, a major limitation for the understanding of these data is the use of a saline-control group rather than a cell therapy control group, which makes it difficult to address the specific effect of TGF-β in this setting.

The Wnt signalling pathway and osteoarthritis

Wingless-like (Wnt) glycoproteins are involved in the regulation of multiple cellular activities.[74] These factors induce a complex network of different downstream intracellular signalling cascades when they bind to their Frizzled receptors on the surface of the cell. Initially discovered in the fly model, this family comprises 19 secreted glycoproteins that are largely conserved in mammalian species. They have an important role in several cancers, development and skeletal formation, for instance by promoting the differentiation of the mesenchymal cells towards osteoblasts. The Wnt signalling cascade has created great scientific interest but is considered difficult to study experimentally. The glycoproteins carry a lipid tail thereby severely compromising their solubility and the production of recombinant molecules. Their affinity for cell surface and ECM molecules also contributes to their specific characteristics. Wnts appear to act largely as autocrine and paracrine rather than systemic factors. Therefore, in connective tissues with substantial amounts of ECM such as cartilage and bone, they can have strong effects through the formation of concentration gradients, a concept typical for signalling systems during development but also highly relevant for joint health and disease such as OA.

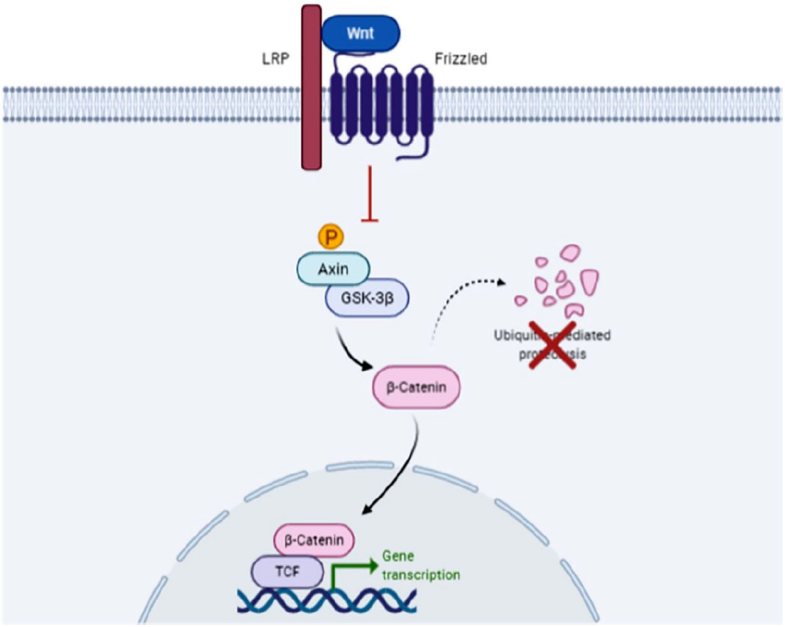

Figure 2. Canonical Wnt signalling in chondrocyte: Wnts glycoproteins bind to their receptors (frizzled) and co-receptors LRP5/6. As a result, the polyprotein complex Axin/APC/GSK3β is inhibited which prevents the non-ubiquitinylation of β-catenin, thus its translocation to the nucleus. Finally, the β-catenin binds to Wnt response element to induce Wnt target gene expression. Adapted from BioRender.com.

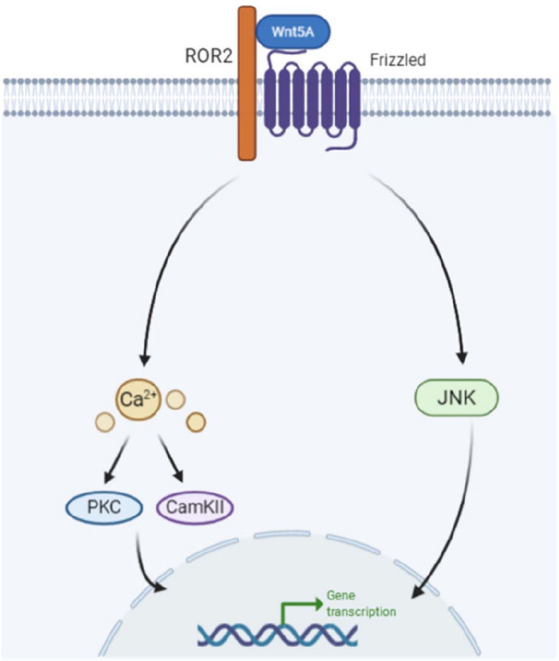

Figure 3. Schematic representation of Wnt non-canonical signalling in chondrocyte. Wnt ligands bind to receptor (frizzled) and co-receptor ROR2. This binding can induce calcium intracellular release to activate sensitive calcium proteins PKC and CamKII or mitogen-activated kinases JNK. These last factors move to the nucleus to influence gene expression programmes.

Wnt signalling can act along two main intracellular signalling pathways, defined as canonical and non-canonical.[75] The canonical pathway engages the binding of Wnts to their receptors (frizzled) and co-receptors low density receptor related protein 5 or 6 (LRP5/6). This binding recruits a polyprotein complex composed, among others, of Axin/APC/GSK3β to the receptor complex thereby counteracting the activity of the complex on the key signalling molecule β-catenin. Wnt receptor–Axin complex interaction inhibits phosphorylation and subsequent ubiquitinylation of β-catenin and its proteasomal degradation. This allows β-catenin to accumulate within the cell and to translocate from the cytoplasmic compartment into the nucleus, where it associates with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors and induces expression of different Wnt target genes[75] (Figure 2). The non-canonical pathways are independent of β-catenin signalling and the co-receptor LRP5/6 and remain far from understood. These pathways involve activation of PKC (Protein kinase C), calmodulin-dependent Kinase II, and c-Jun N-terminal Kinase (JNK), and small G proteins (ROA)[75,76] (Figure 3). Different extracellular Wnt ligand or receptor binding proteins like sFRP-1 (soluble factor related inhibitory), WIF-1 (Wnt inhibitory factor), Dkk1 (Dickkopf), CTGF (connective tissue growth factor) and SOST (sclerostin) are critical to fine-tune the activation of the Wnt pathways. Other intracellular modulators are also described, among them IWP, IWR and the beta catenin interacting protein (ICAT). Mutations with gain or loss of functions in the Wnt pathway have been identified in human diseases. Prominent among these are skeletal disorders and malignancies.

Wnt signalling has been strongly linked with skeletal development and growth. Whereas Wnts are critical for the differentiation and maturation of osteoblasts, a role that is sustained in adult life, their effects on early stages on skeletogenesis are more complex.[76] The early stages of limb skeletal development are characterized by the formation of a cartilage template that is subsequently replaced by bone. In those initial stages, progenitor cells undergo a multistep differentiation process, first becoming chondrocytes and later

hypertrophic chondrocytes. Canonical Wnt signalling inhibits early differentiation of the progenitor cells, whereas in the later stages the pathway drives chondrocytes towards hypertrophy. Whereas in skeletal development these processes are part of the predefined plan, similar events in mature articular cartilage can be considered deleterious: inhibition of the typical cartilage molecular programme could disrupt normal tissue homeostasis and stimulation of chondrocyte hypertrophy is a feature seen in OA. The hypertrophy and the associated changes in ECM impact biomechanical loading in the joint and are hypothesized to accelerate the progress of OA.

Excessive Wnt signalling leads to osteoarthritis

Genetic and functional studies with specific attention towards *Frzb*, a secreted Wnt antagonist, were among the first efforts to link Wnt biology and OA. FRZB or Frizzled-related protein is a secreted Wnt modulator first identified from a chondrogenic extract of articular cartilage.[77] Studies showed that polymorphisms in the *Frzb* gene may predispose patients to hip OA, suggesting that the Wnt/β-catenin signalling may be a key pathway to the development of OA.[78] Direct experimental evidence of Wnt involvement in OA quickly accumulated. *Frzb* knockout mice exhibited more cartilage damage in different models of OA and were associated with increased Wnt signalling in cartilage and increased cortical bone thickness.[77] This study suggested that excessive Wnt activation can contribute to OA. Similarly, Zhu and collaborators demonstrated that increasing Wnt signalling by stabilizing β-catenin also leads to OA development in mice. Indeed, histological analysis of the joints of those mice revealed more cartilage damage in 5- and 8-month-old mice with signs of excessive hypertrophy.[79] In addition, an increase of β-catenin staining was found in human OA cartilage samples.

However, the effects of Wnt signalling on OA are complex, and additional data emerged that show that both excessive signalling as well as absence of Wnt activation can contribute to joint damage. Indeed, strong inhibition of active Wnt/β-catenin signalling in cartilage leads to OA with complete cartilage destruction and cell death in 15-month-old mice. The inhibition of Wnt/β-catenin signalling was carried out by the overexpression of ICAT, a molecule that prevents the binding of β-catenin to TCF-1.[80] In the same manner, WNT16, induced after destabilisation of the medial meniscus (DMM) surgery in superficial zone of adult cartilage, was found to play a pivotal role in cartilage homeostasis. Indeed, $WNT16^{-/-}$ mice are more susceptible to instability-induced OA.[81] WNT16 seems to protect against cartilage breakdown.

The previous studies highlighted evidence for Wnt signalling involvement in cartilage homeostasis, and other studies explored the effects of Wnt in the different tissues of the joint. Blom *et al.*[82] found that WNT-16 and WISP-1 are drastically increased in cartilage and synovium in two experimental models of OA. The up-regulation of WISP-1 expression was also confirmed in human OA samples and the overexpression of WISP-1 in murine knee joints resulted in MMP and aggrecanase expression and cartilage damage.

Two other interesting studies investigated the role of Wnt antagonists. First, cohort studies identified a correlation between high levels of circulating DKK-1 and reduced progression of radiographic hip OA.[83,84] The role of the DKK family was further explored; blocking Wnt signalling either with recombinant Dkk-1 injection or its overexpression specifically in cartilage exerted a protective effect in joint tissues.[85] In line with this, knockout of DKK-1 induced more OA in rat.[86] Targeting DKK-1 in subchondral bone by overexpression was also beneficial for cartilage integrity.[87] *In vitro*/*ex vivo* data demonstrated that DKK1 is upregulated by IL1-β and is associated with chondrocyte apoptosis.[88] In an *in vivo* context of inflammation by tumor necrosis factor overexpression in mice, DKK1 inhibition reduced erosive bone destruction but increased osteophyte formation. Combined, these observations point to the duality of Wnt signalling in a health and disease context, low levels required for cell survival but hyperactivation leading to deleterious phenotypes including osteophyte formation.[89]

Investigations into another potent antagonist of Wnt signalling sclerostin (Sost) in chondrocytes demonstrated protective effects by restoring anabolic markers and down-regulation of the catabolic reactions induced by both IL-1β and WNT-3a stimulation.[90–93] Moving towards *in vivo* validation, one study showed no difference in cartilage damage with Wnt antagonist sclerostin gene deletion and systemic administration of sclerostin-neutralizing monoclonal antibody in rat.[94] However, in another study, the lack of sclerostin in mice subjected to DMM aggravated cartilage damage and subchondral bone remodelling.[92] A recent publication provided data on both

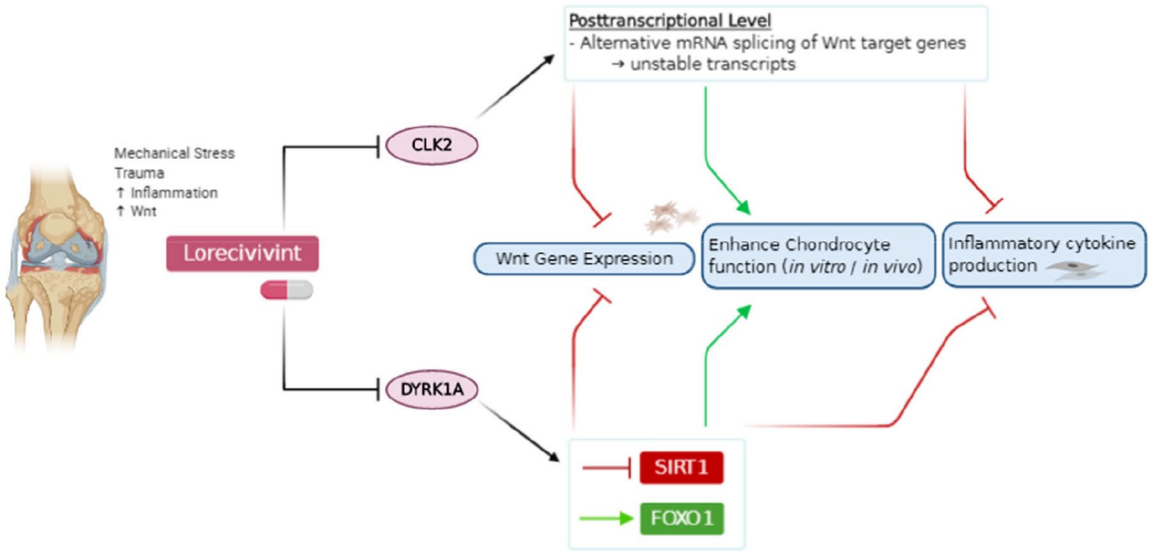

Figure 4. Proposed dual mechanism of action of lorecivivint. CLK2 inhibition causes alternative mRNA splicing at a posttranscriptional level, which leads to reduced expression of Wnt target genes and inflammatory cytokines. DYRK1 inhibition blocks SIRT1 (positive regulator of Wnt pathway) and promotes activation of FOXO1 (stimulates cartilage homeostasis), leading to negative regulation of Wnt signalling and direct chondroprotective effects. Green arrows indicate positive effects; closed red lines indicate inhibitory effects.

overexpression and deletion of sclerostin in a post-traumatic OA model (ACL) and showed that the overexpression protected against OA compared with wild-type mice and knockout mice. Taken together, these data identify sclerostin as a protective molecule reducing cartilage catabolism *via* a reduction in MMP2/3, a rescue that is TNFα and NFkB dependent manner.[95]

With the overall picture indicating that homeostasis of different joint tissues is dependent on fine-tuning of Wnt signalling, new modulators were discovered and may provide specific opportunities for future management of OA. Genetic evidence identified histone methyltransferase DOT1L as another important factor in OA: polymorphisms in this gene were associated with hip cartilage thickness acting as a proxy for OA and subsequently also with OA.[96] DOT1L is the main enzyme responsible for histone 3 lysine 79 (H3K79) methylation. Knockdown of DOT1L in an *in vitro* chondrogenesis model inhibited cartilage differentiation. Translational studies demonstrated that the levels of H3K79 methylation are reduced in both mouse models of the disease and in cartilage samples from OA patients. Experimental evidence supports a key role for DOT1L in modulating Wnt signalling. DOT1L protects against hyperactivation of the signalling cascade and hence is an important factor in preserving cartilage health. Chemical or genetic interference with DOT1L function leads to strongly increased Wnt signalling and cartilage damage both *in vitro* and *in vivo*.[97,98] Interactions with another epigenetic modulator, SIRT1, play a role in the Wnt hyperactivation process. While adding another level of complexity in the regulation of the Wnt pathway, the presence of these multiple regulators of pathway activity underlines its importance in joint biology and opens up different ways to target the deleterious hyperactivation status.

Lorecivivint is a small molecule currently in clinical trials for the treatment of knee OA.[99] The compound was discovered in drug-screening approaches to modulate Wnt signalling. *In vivo* animal experiments provide evidence of a cartilage-protective effect.[100] The underlying mechanism was recently revealed, with lorecivivint being a potent and fairly specific modulator of kinase CLK1 and DYRK1A.[100] The CLK enzymes are involved in splicing events and their inhibition likely results in abnormal transcripts that block Wnt signalling at the transcriptional level. In addition, the DYRK1A inhibitory activity may protect against inflammation[100] (Figure 4).

Lorecivivint has already been through phase I and phase II clinical trials[101–103] (Table 2). These trials were randomized and placebo controlled. Several dosages by a single intra-articular

Table 2. An overview of the current molecules with a high therapeutic potential in osteoarthritic disease.

Molecule	Target	Therapeutic potential	Clinical trial	Reference
Sprifermin	FGF18	Improvement in cartilage thickness (stimulation of TGF-β signaling)	Phase II	Hochberg *et al.*[104]
Lorecivivint (SM04690)	CLK1 and DYRK1A	Wnt pathway blockage	Phase III	Yazici *et al.*[99]

injection in knee joints had a good safety profile and appeared not to lead to detectable systemic exposure. The first phase II trial was performed over 52 week time period, using a range of concentrations of lorecivivint – 0.03 mg, 0.07 mg, 0.23 mg – or phosphate-buffered saline as placebo. Subjects were patients with moderately to severely symptomatic knee OA. The results propose that the 0.07 mg dose of the drug showed symptom improvement and potential slowing of joint narrowing, which indicated better structural progression. However, these outcomes mostly presented in the group of patients with unilateral symptomatic OA. A second phase II trial over 24 weeks was then set up to refine outcome measures, target population and dosage, while further evaluating safety. Single intra-articular injection over a wider dosage range (0.03, 0.07, 0.15 and 0.23 mg) was compared with placebo or sham-injection in patients with dominant unilateral knee OA (high pain score in target knee, low pain score in contra-lateral knee). The study showed statistically significant differences for the 0.07 and 0.23 mg dosages and pain and physical function, as well as for patient global assessment. In both phase II trials there were no apparent safety issues. The data convinced the company to continue the clinical development programme and currently phase III trials are ongoing.

Taken together, the different levels of regulation and modulation that characterize the Wnt signalling pathway highlight not only its importance in joint health and disease like OA but also provide ample opportunities for specific fine-tuning and modulation of these cascades and therefore therapeutic interventions.

Wnt non-canonical pathway

The Wnt canonical pathway has been extensively linked to joint homeostasis and disease, but more recently the non-canonical pathways have gathered more attention, with accumulating evidence showing that these are also involved in the pathogenesis of OA. Although Wnt ligands were originally classified as canonical or non-canonical, current evidence suggests that pathway activation by specific ligands also depends on the cellular context and receptor availability. WNT1, WNT3a and WNT8 have been clearly linked with canonical signalling, while Wnt-5a and Wnt-11 are often considered to be involved in non-canonical signalling.[105]

Huang *et al.*[106] showed that WNT5a, a non-canonical Wnt activator, is expressed in human OA cartilage and is able to induce catabolic responses through activation of non-canonical Wnt signalling. Similarly, a positive correlation between Wnt5a expression and cartilage erosion was found in knee cartilage of OA patients.[107] Surprisingly, chondrocytes stimulated with WNT5a presented a decrease in Col2, while silencing of Wnt5a rescued the latter's decrease under IL1-β stimulation.[108] WNT11 was reported to increase COL2 expression.[108] Of note, the canonical and non-canonical ligands are not exclusive to one pathway, as Nalesso *et al.*[91] showed that WNT3a, originally considered a canonical Wnt ligand, is able to simultaneously activate both canonical and non-canonical pathways in human articular chondrocytes. A crosstalk between these two major signalling pathways exists and the balance between canonical and non-canonical activation likely affects cartilage homeostasis.

ROR2 (receptor tyrosine kinase-like orphan receptor 2) is a transmembrane protein, crucial for skeletal development as its loss causes Robinow syndrome characterized by some skeletal dysplasia.[109] A similar syndrome was also observed with loss of Wnt5a.[110] ROR2 was reported to act as receptor and co-receptor for WNT5a. It has been also demonstrated that ROR2 activates Wnt5a signalling through JNK and inhibits β-catenin/TCF/LEF pathways.

More recently, ROR2 was also identified as an important regulator of cartilage integrity and to alleviate pain in OA disease models. ROR2 and

WNT5A were both shown to be upregulated in human OA cartilage. Genetic manipulation of ROR2 by an overexpression approach impaired chondrocyte differentiation. In contrast, ROR2 silencing positively affected chondrogenesis. An underlying mechanism of action was proposed in which ROR2 regulates YAP through G proteins and Rho, and by which YAP inhibition is required for chondrogenic differentiation mediated by ROR2 blockade.[111]

Wnt and TGF-β signalling crosstalk

Crosstalk between TGF-β and Wnt signalling was reported in several tissues.[112–115] A study in chondrocytes revealed that TGF-β activates β-catenin signalling *via* SMAD3 and β-catenin interaction.[116] Later, the mechanism of action was further detailed to show that both SMAD3 and SMAD4 were required for the interaction with β-catenin, thereby providing protection from proteasomal degradation. Additionally, the formation of the protein complex SMAD3/4/β-catenin is also involved in the nuclear translocation of β-catenin.[116,117] BMP-2 increased active β-catenin and LRP5 levels; an effect mediated through SMAD1/5/8 binding on LRP-5 promoter. This suggested that BMP-2 activates Wnt/β-catenin signalling.[118] An interaction between WISP1 and TGF-β3 was found in human bone marrow stromal cells (hBMSCs). The absence of WISP1 repressed SMAD2/3 activation by TGF-β3. These data were further confirmed in WISP1 KO primary chondrocytes as there was also a reduced effect of TGF-β3 on SMAD 2/3 activation.[114] Furthermore, canonical Wnt signalling activation *via* Wnt3a decreased the level of SMAD 2/3 phosphorylation signalling in chondrocytes and increased, as well as WISP1, SMAD 1/5/8 signalling.[113]

Conclusion

Our understanding of key growth factor signalling cascades in OA has greatly improved, yet the translation of this knowledge into therapeutic approaches remains an important challenge. Increased activation of signalling pathways such as TGF-β and Wnt signalling can be detected in OA samples. Changes in the dynamics and regulation of these cascades are strongly context dependent, indicating the need to better understand the different clinical presentations of OA and suggesting the importance of personalized medicine approaches. For clinical trials the context-dependent effects of these pathways on different cells and tissues within the joint is a challenge. The cardinal signs and symptoms of OA such as pain and loss of function likely represent the outcomes of a group of distinct disorders with common and diverging characteristics and mechanisms, but a similar ending. Further effort towards phenotypical, molecular, genetic and metabolic characterization of disease presentations in specific patient subgroups appears to be essential to make real progress in therapy.

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