Wnt Signaling in

Cartilage Development and Degeneration

Bin Ma

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PhD thesis, Univeristy of Twente, Enschede, The Netherlands

ISBN: 978-90-365-3422-2

The research described in this thesis was supported by the Project P2.02 OAcontrol of the research program of the BioMedical Materials institute, co-funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation, and by a Career Establishment Award from the European Society of Calcified Tissue to Prof. Dr. M. Karperien.

Printed by Wöhrmann Print Service, Zutphen, The Netherlands.

Cover Design: Bin Ma. Illustration of protein-protein interaction.

Figures on front pages of each chapter: Oracle bone scripts for animals.

WNT SIGNALING IN
CARTILAGE DEVELOPMENT AND DEGENERATION

DISSERTATION

to obtain

the doctor's degree at the University of Twente,

on the authority of the rector magnificus,

Prof. Dr. H. Brinksma

on account of the decision of the graduation committee,

to be publicly defended

on Wednesday, September 12th 2012, at 12.45

by

Bin Ma

born on November 6th, 1983

in Zhengzhou, Henan, China

献给我的父母

To My Parents

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Chapter 1

Introduction

Cartilage is a flexible connective tissue consisting of a dense matrix predominantly consisting of collagens and proteoglycans, which provide compressive and tensile strength to the cartilage tissue. Articular cartilage provides mechanical support for joints and is responsible for smooth and pain free joint movement. It is the primary tissue affected in joint diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA) which are the leading causes of mobility-associated disability. Chondrocytes are sparsely embedded in cartilage matrix and perform matrix-generation and maintenance functions. Investigation of mechanisms underlying cartilage development and degeneration will provide important implications for treatment of joint diseases. Multiple signaling pathways have been shown to be involved, of which Wnt signaling is one of the most crucial pathways. This thesis aims to elucidate the role of Wnt signaling in cartilage development and degeneration. After introduction of the general outline of this thesis in chapter 1, chapter 2 provides a comprehensive review of the current thinking of the role of Wnt signaling in cartilage development and disease.

In chapter 3 we describe an inducible conditional knockout approach to manipulate Wnt signaling in cartilage after birth. Cartilage development is a complicated process. The best way to study Wnt signaling in cartilage development is using in vivo animal models. A large number of knockout mouse models have been generated to study Wnt signaling in development and disease. These models target at different players spread over the pathway including ligands, receptors, intracellular intermediates and transcription factors (1). Many of these knockout mice show early embryonic lethality which precludes functional studies at later stage. To overcome this problem, the advent of Cre-lox technique and additional systems tools have come available allowing both spatial and temporal control over gene expression in the organism. Therefore, we exploited a Col2a1-CreER^{T} mouse line in which the CreER^{T} fusion protein is specifically expressed in Col2a1 expressing tissue and is activated only in the presence of the estrogen receptor (ER) ligand tamoxifen (2). We have chosen this mouse model with the aim to induce conditionally Cre-mediated recombination of the Adenomatous Polyposis Coli (APC) gene in articular cartilage after birth. We wished to inactivate the APC gene, since APC is the intracellular gatekeeper of β-catenin, the main effector protein in the canonical Wnt signaling pathway (3). We started by testing the specificity and efficiency of Cre-induced recombination. For

this purpose, we used a ROSA26 reporter (ROSA26R) mouse line, in which LacZ gene expression is induced after Cre-mediated recombination (4). To manipulate canonical Wnt pathway in cartilage, we crossed Col2a1-CreER^{T} with *APC*^{15lox} (5) and *APC*1638N (6) mice to generate Col2a1-CreER^{T};*APC*$^{15lox/15lox}$, Col2a1-CreER^{T};*APC*$^{15lox/1638N}$, and other control genotypes. Upon tamoxifen injection and activation of recombination, it is expected that exon 15 of the APC gene will be deleted which results in complete inactivation of the APC gene. APC1638N is a truncated form of APC and retains partial activity of wild-type APC. These mutant mice may exhibit differential APC activities, thus different β-catenin levels. This enables the study of dose-dependent effect of Wnt signaling on cartilage development.

In chapter 4 and 5, we aimed at elucidating the role of canonical Wnt signaling in human cartilage in contrast to its well studied role in animal cartilage models. In chapter 4 we have focused on the role of β-catenin in cytokine-induced matrix metalloproteinases (MMPs) expression. In chapter 5 we have focused on the role of TCF/LEF transcription factors as the downstream targets of β-catenin. Compared to the large body of knowledge regarding the role of Wnt signaling in chondrogenesis and cartilage development, less is understood concerning the role of Wnt signaling in the maintenance and degeneration of cartilage. A role for Wnt/β-catenin signaling in OA has been proposed predominantly based on observations in animal models: i) in postnatal mouse models, conditional activation of β-catenin signaling in cartilage results in increased articular cartilage degeneration by stimulating cartilage degradation, endochondral ossification and other phenotypes resembling OA (7); ii) activation of Wnt/β-catenin signaling in rabbit and mouse chondrocytes stimulates the expression of cartilage matrix degrading (MMPs) (8, 9); iii) in a spontaneous guinea pig OA model, development of OA is associated with increased β-catenin expression in cartilage (8); and iv) pro-catabolic factors like IL-1 implicated in OA development induce expression of various Wnt proteins resulting in the activation of β-catenin (9, 10). This evidence leads to the hypothesis that the canonical is a pathogenic factor in cartilage degeneration. In contrast, inhibition of β-catenin signaling in articular chondrocytes also causes OA-like cartilage degradation in a Col2a1-ICAT transgenic mouse model (11). It suggests that Wnt's role in the regulation of cartilage might be complicated. In addition, the exact function of Wnt signaling in human cartilage is largely unknown,

although differential expression of Wnt proteins in human OA and RA cartilage has been documented (12, 13). To study the function of Wnt signaling in human cartilage in a straightforward way, we used human chondrocytes isolated from OA, RA and healthy donors. Bovine and mouse cells were used as comparison. Cell proliferation, expression of cartilage marker genes such as COL2A1 and SOX9 and catabolic genes such as MMPs were evaluated. A variety of strategies, such as ligand stimulation, gene overexpression and knockdown, were used to manipulate Wnt signaling at different levels. These findings provide important implications for development of specific and effective therapeutic strategies for OA and RA by targeting at Wnt signaling.

In chapter 6, we have studied the molecular mechanisms involved in chondrocyte dedifferentiation, an undesired side-effect of expansion of primary chondrocytes in monolayer culture. Cartilage has limited regenerative capacity once damaged. The golden standard for repair of focal cartilage defects is autologous chondrocyte implantation (ACI) (14, 15). This procedure relies on the isolation of chondrocytes out of a biopsy taken from a non-weight bearing site of the affected joint and their subsequent expansion by in vitro cell culture. When cultured in monolayer for long term, chondrocytes rapidly lose their phenotype and ability to produce cartilage-specific matrix, a process designated as dedifferentiation (16, 17). Dedifferentiation of chondrocytes during in vitro culture is a major obstacle in the ACI procedure. The mechanism underlying human chondrocyte dedifferentiation is still unclear. Therefore we investigated gene expression changes during human chondrocyte expansion during monolayer expansion using whole genome microarray. Relevance of significantly changed pathways such as Wnt signaling was further explored. These results provide useful knowledge for modifying culture conditions of human articular chondrocyte and eventually improve the outcome of cell therapy to repair cartilage lesions.

In the general discussion (chapter 7), the main findings of this thesis are summarized and a new model for the role of Wnt signaling in human cartilage is presented.

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Chapter 2

Wnt Signaling and Cartilage:

of Mice and Men

Wnt signaling and cartilage: of mice and men

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Abstract

In adult articular cartilage, the extracellular matrix is maintained by a balance between the degradation and synthesis of matrix components. Chondrocytes that sparsely reside in the matrix and rarely proliferate are the key cellular mediators for cartilage homeostasis. Indications have been found for the involvement of the Wnt signaling pathway in maintaining articular cartilage and deregulation of Wnt signaling was observed in cartilage degeneration. Furthermore, several Wnts have been found to be involved in the subsequent stages of chondrocyte differentiation during development. Even though gene expression and protein synthesis can be activated upon injury, articular cartilage has a limited ability of self-repair and efforts to regenerate articular cartilage are so far not sufficient. Since Wnt signaling was found to be involved in the development and maintenance of cartilage as well as in the degeneration of cartilage, interfering with this pathway might contribute to improving cartilage regeneration. However, most of the studies on elucidating the role of Wnt signaling in these processes were conducted using *in vitro* or *in vivo* animal models. Since discrepancies have been found in the role of Wnt signaling in chondrocytes between mouse and human, extrapolation of results from mouse models to the human situation remains a challenge. Elucidation of detailed Wnt signaling functions will provide knowledge to improve cartilage regeneration.

Adult articular cartilage is an avascular tissue composed of a dense matrix predominantly composed of collagens, proteoglycans and glycosaminoglycans, which provide compressive and tensile strength to the cartilage tissue. The chondrocytes that reside in this matrix maintain the extracellular matrix by remodeling of the extracellular matrix. Since the metabolic activity of chondrocytes is relatively low and cartilage is scarcely populated with cells, cartilage remodeling is a slow process. Articular cartilage is composed of four distinct regions, the superficial zone which faces the synovial cavity, the middle zone, the deep zone and the calcified cartilage zone adjacent to the subchondral bone plate, which differ in cell density and matrix composition. Also the chondrocyte morphology differs between zones, as chondrocytes in the superficial zone are small and flattened, middle zone chondrocytes are more rounded and in the deep zone chondrocytes are grouped in columns or clusters. Since chondrocytes rarely divide and are separated from each other by the matrix, it is unclear how they are regulated to maintain the extracellular matrix (ECM) under homeostatic conditions. It is known though, that gene expression and protein synthesis can be activated upon injury.

During aging and joint disease, the equilibrium within the cartilage tissue is disrupted and the synthesis of new matrix components is exceeded by the loss of collagens and proteoglycans from the cartilage matrix (1). This disbalance between anabolic and catabolic processes results in progressive cartilage degeneration. Even though the exact etiology of cartilage degenerative diseases is largely unknown and likely multiple onsets exist, cartilage destruction is considered to be the result of increased expression and activity of proteolytic enzymes, for example as a response to abnormal mechanical loading, genetic predisposition, trauma or inflammation. Matrix metalloproteinases (MMPs) and aggrecanases are the major proteinases that degrade collagens and proteoglycans in joint disease.

Articular cartilage has a limited ability for self-repair. Even though steps have been made towards the regeneration of articular cartilage, at present, no curative treatment is available to regenerate or restore native articular cartilage. Research efforts are made to improve treatment options for cartilage degeneration.

In order to be able to reverse degeneration and induce regeneration of articular cartilage, detailed understanding of chondrogenic differentiation during skeletal development and

the maintenance of articular cartilage at adult age, is of crucial importance. Using this knowledge, it might be possible to induce chondrogenic differentiation and shifting the balance from catabolism to anabolism. In this review, we describe the processes and molecular components that are involved in skeletal development with emphasis on articular cartilage formation and homeostasis. We focus on the role of the Wnt signaling pathway and its components in skeletal development and the maintenance of cartilage homeostasis, since this pathway was found to play an important role in embryonic development and indications of deregulated Wnt signaling have been found in degenerative cartilage disease. We also touch upon the role of Wnt signaling in cartilage degeneration. The different components of the Wnt signaling pathway that are involved in cartilage development and disease are shown in Table 1.

Table 1. Involvement of players of the Wnt signaling pathway in cartilage development and disease.

	Development		**Disease**	
Factor	**Expression**	**Action**	**Expression**	**Action**
Wnt-1	No endogenous expression (34).	Inhibition of cartilage formation (34, 35).	Expressed in RA synovial tissues(79).	Enhances fibronectin and MMP-3 expression in fibroblast-like synoviocytes (79).
Wnt-3A	Expressed in early stages of chondrogenesis, decreased when chondrogenic differentiation proceeds (38).	Increases self-renewal and decreases apoptosis of MSCs (36, 37); Blocks collagen type II expression and proteoglycan deposition (38).		Promotes chondrocyte proliferation (11) and induces dedifferentiation (38).
Wnt-4	Expressed in periphery of joint interzone and hypertrophic chondrocytes (39, 40).	Accelerates chondrocyte maturation (39-41).		
Wnt-5A	Expression in perichondrium surrounding condensations (39, 40).	Recruitment of mesenchymal cells (42); Delays chondrocyte differentiation (39-41).	Expressed at high level in chondrocytes (72); Overexpressed in RA synovial tissues (79).	Upregulates MMP expression (90) and induces chondrocyte dedifferentiation (73) in rabbit.
Wnt-5B	Expressed in pre-hypertrophic chondrocytes and in the perichondrium (38).	Promotes initial steps of chondrogenesis in micromass cultures and delays terminal differentiation *in vivo* (39).		

Wnt-7A	Expressed in dorsal ectoderm in developing limb bud (44).	Inhibition of chondrogenic differentiation *in vitro* and *in vivo* (34).	Expressed in rabbit chondrocytes and increased in response to IL-1 (71).	Induces rabbit chondrocyte dedifferentiation (71).
Wnt-7B			Upregulated in OA cartilage and RA synovium (80); Expressed in human chondrocytes and increased in response to IL-1 (72).	Downregulates MMP expression in human chondrocytes (72).
Wnt-14	Expressed in developing joint interzone (46).	Arrests or reverses chondrogenic differentiation (40).		
β-catenin	Expressed at low levels in chondrogenic mesenchymal condensations (25).	Induces expression of Sox9 and promotes chondrogenic differentiation at low levels (25, 26).	Expression elevated in osteoarthritic cartilage (32, 33) and dedifferentiated chondrocytes (69).	Induces OA phenotype in mouse model expressing constitutively active β-catenin (32, 33); Inhibits NF-κB activity and MMP expression in human chondrocytes (72).
FRZB	Expressed in pre-chondrogenic mesenchymal condensations and in epiphyseal pre-articular chondrocytes (46, 48).	Blocks chondrocyte maturation and prevents endochondral ossification *in vivo* (47, 48); Promotes glycosaminoglycan synthesis and expression of Sox9 and Collagen II *in vitro* (49).	Multiple variants are expressed in human (74-77).	Risk factor for OA (74-77); Frzb knockout mice are more sensitive to OA-inducing factors (83, 84).
DKK-1	Expressed at sites of programmed cell death in apical ectodermal ridge (93); Expressed at higher level in articular cartilage than growth plate cartilage (50).	Promotes glycosaminoglycan synthesis and expression of Sox9 and Collagen II *in vitro* (93); Inhibits chondrocyte hypertrophy (50).	Expression is increased in RA and decreased in OA (94).	Overexpression ameliorates cartilage destruction in animal models (95 ,96).
WIF-1	Expressed in mesenchyme surrounding cartilage elements and articular cartilage (51).	Promotes chondrogenic differentiation (51).		

Wnt signaling

The Wnt family of secreted glycoproteins, which are characterized by several conserved cysteine residues, consists of 19 members, of which several encode distinct isoforms arising by differential splicing (2). The Wnt signaling pathway is composed of several conserved components and plays a fundamental role in controlling cell proliferation, cell fate determination, and differentiation by inducing changes in gene expression during embryonic development and in adult cartilage (2, 3). At least three distinct intracellular signaling pathways that are activated by distinct sets of Wnts and Frizzled (Fzd) receptors and that lead to unique cellular responses are known (4). The canonical Wnt/β-catenin pathway is the best described pathway for Wnt signal transduction (Fig. 1). In an inactive state, in the absence of a Wnt ligand, β-catenin is phosphorylated at the NH_2 terminus by glycogen synthase kinase (GSK) 3β and casein kinase (CK) I in a so called destruction complex, which is brought together by the two scaffolding proteins axin and adenomatous polyposis coli (APC). This phosphorylation targets β-catenin for subsequent ubiquitylation and proteasomal degradation. When Wnts bind to the seven transmembrane frizzled receptor in combination with a coreceptor of the LDL related proteins (LRP) 5 or 6, disheveled (Dsh) is activated, resulting in suppression of GSK3β activity. As a result, β-catenin will not undergo phosphorylation and is stabilized in the cytoplasm. Upon reaching a certain level, β-catenin translocates to the nucleus, where it interacts with transcription factors of the T cell specific transcription factor/lymphoid enhancer-binding factor (TCF/LEF) family to initiate the transcription of target genes (5, 6).

Another intracellular Wnt pathway was first identified in Drosophila. This pathway is involved in regulating planar cell polarity by inducing cytoskeletal organization relative to the plane of the tissue in which the cells reside (4, 7). However, even though Fzd and Dsh were shown to play a role in this pathway, no involvement of LRP, β-catenin or TCF was found and current evidence suggests that no Wnt ligands are involved in the regulation of planar cell polarity (4). Fzd is also involved in the Wnt/Ca^{2+} pathway, which is the third pathway activated by subsets of Wnt ligands (4, 6, 8). In this pathway, a Wnt ligand induces activation of a heterotrimeric G-protein resulting in an increase in intracellular

levels of Ca^{2+}. This activates Ca^{2+}-dependent effector molecules such as the transcription factor Nuclear Factor Associated with T-cells (NFAT).

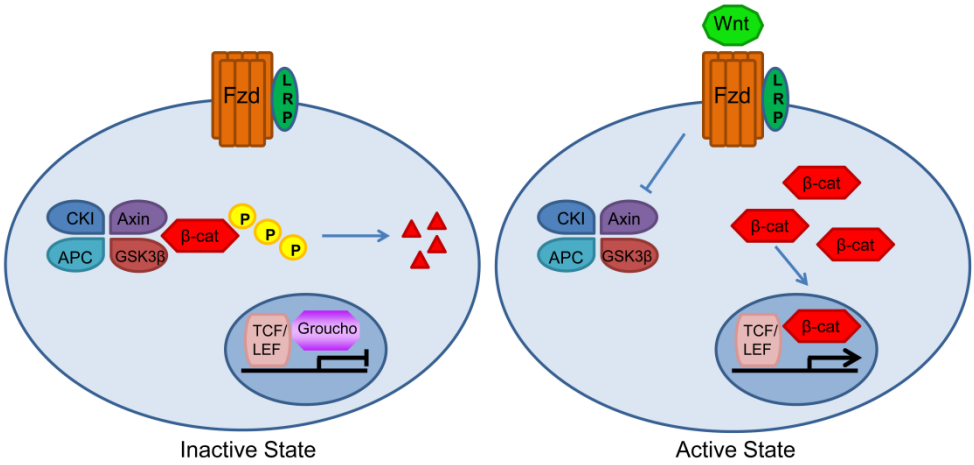

Figure 1. Wnt/β-catenin signaling pathway. In the inactive state, β-catenin is phosphorylated by a degradation consisting of GSK3β, APC, Axin and CKI, targeting β-catenin for ubiquitilation and subsequent proteosomal degradation. When a Wnt ligand binds to the Fzd receptor and coreceptors LRP5/6 the degradation complex is disrupted, resulting in stabilization of β-catenin in the cytoplasm. Subsequent translocation to the nucleus and binding to transcription factors TCF/LEF to initiate gene transcription.

The specificity of activation downstream of Wnt is determined by selective receptor activation, receptor-mediated endocytosis and the presence of cofactors such as heparin and sulfate proteoglycans (9). Accumulating evidence has suggested that Wnt protein may activate multiple pathways depending on the engagement of distinct receptors (4). For example, Wnt-5A, which is most often associated with non-canonical pathway, also able activates the canonical β-catenin signaling (10); the "canonical" Wnt-3A is also able to activate the non-canonical Ca^{2+} pathway (11).

Wnt signals can be modulated extracellularly by secreted proteins, including members of the secreted Frizzled-related protein (sFRP) family, Wnt-inhibitory factor (WIF) 1, Cerberus, SOST and Dickkopf (DKK) 1. sFRPs, WIF-1 and Cerberus can bind to Wnts directly, thereby preventing their interaction with Fzd receptors, whereas sFRPs can also bind Fzd receptors to form non-functional complexes (2, 12). Both SOST and DKK-1 antagonize Wnt signaling by binding to LRP5/6, co-receptors, albeit to distinct regions, and thereby preventing binding of Wnts (13). Intracellularly, Wnt/β-catenin signaling can be

modulated by Inhibitor of β-catenin and T-cell factor (ICAT), which disturbs the interaction between β-catenin and transcription factors TCF/LEF, thereby inhibiting β-catenin-mediated transcription (14, 15).

Skeletal development

The formation of most of the vertebral skeleton occurs via endochondral bone formation, a process starting with the aggregation, proliferation and condensation of mesenchymal stem cells (MSCs) at specific locations within the embryo. These MSCs originate from the neural crest (forming craniofacial bones), the sclerotome of the paraxial mesoderm (forming the axial skeleton), or the lateral plate mesoderm (forming the appendicular skeleton) (16). MSCs commit to the skeletal lineage once they have differentiated into skeletal precursor cells (SPCs), from which chondrocytes and osteoblasts can derive. Cellular condensations form as the result of altered mitotic activity, failure of cells to move away from a center or aggregation of cells towards a center, as occurs in limb formation. This process leads to increased mesenchymal cell density, without an increase in cell proliferation. Consequently, cellular condensation is associated with an increase in cell-cell contacts through cell-cell adhesion molecules and gap junctions that facilitate intercellular communication (17). Prior to condensation, MSCs secrete an ECM rich in hyaluronan and collagen type I, preventing intimate cell-cell interaction. As condensation begins, an increase in hyaluronidase activity is observed, so the ECM hyaluronan content decreases, allowing cell migration. The increased cellular interaction as observed during condensation is thought to be involved in triggering signal transduction pathways that initiate chondrogenic differentiation. Adhesion molecules like neural cadherin (N-cadherin) and neural cell adhesion molecule (N-CAM) are expressed in condensing mesenchyme, while they disappear in differentiating chondrocytes. Furthermore, cell-matrix interactions, involving fibronectin, play an important role in mesenchymal condensation. Fibronectin expression was found to be upregulated in cellular condensation, and its expression decreased when chondrogenic differentiation continues. At the periphery of these condensations, SPCs form a perichondrial layer, while in the core they differentiate into chondrocytes that produce cartilage specific extracellular matrix (ECM) proteins and continue to proliferate.

The differentiation of chondroprogenitor cells is characterized by the deposition of a cartilaginous extracellular matrix containing collagen types II, IX and XI and aggrecan. One of the earliest markers expressed in cells undergoing condensation is Sox9, which is required for the expression of the collagen type II α1 (COL2A1) gene and other cartilage specific extracellular matrix proteins (18, 19). Continuous proliferation of chondrocytes and secretion of ECM contribute to the elongation of the cartilage template, which prefigures the shape of the future bone.

After formation of the cartilaginous template, the core chondrocytes mature and become hypertrophic, secreting a progressively calcified ECM. Simultaneously, perichondrial SPCs differentiate into osteoblasts, forming the future periosteum, which modulates the final shape and size of the cartilage template. After mineralization of the cartilage ECM, vascular invasion and apoptosis of terminal hypertrophic chondrocytes initiate the formation of the primary ossification center. This complex differentiation program radiates centrifugally, leading to the development of trabecular bone (16, 20-24).

Involvement of Wnt signaling in skeletal development

In skeletal development, the fate of MSCs to differentiate into either chondrocytes or osteoblasts depends on the expression of the transcription factors Sox9 or Runx2 respectively. Skeletal precursor cells express both transcription factors, and the intracellular expression levels of β-catenin determine the fate of these cells. High levels of β-catenin inhibit Sox9 expression and activity, while potentiating Runx2, which results in osteoblast differentiation. In contrast low β-catenin levels induce Sox9 expression and thereby chondrocyte differentiation (25, 26).

An increasing amount of evidence indicates the important role of Wnt/β-catenin signaling in essentially all aspects of skeletal development and maintenance. The role of canonical Wnt/β-catenin signaling at subsequent stages of skeletogenesis has been suggested based on the expression patterns of many Wnt pathway members, as well as Wnt reporter expression in mice (25-31). Involvement of Wnt signaling in chondrogenic differentiation is depicted in Figure 2.

Since β-catenin is a key molecule in the canonical Wnt signaling pathway, it is the most studied molecule involved in this pathway. *In vitro* and *in vivo* data suggest that β-catenin

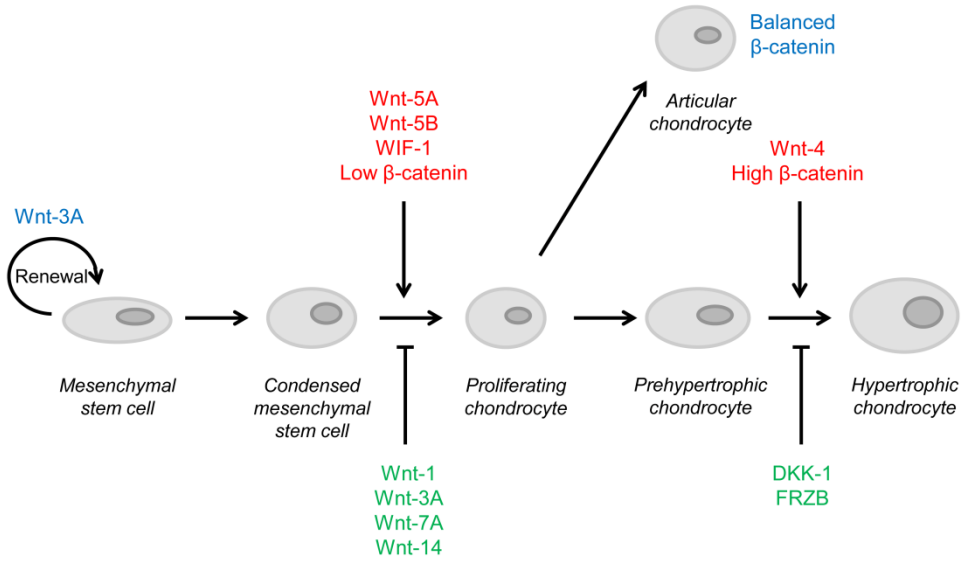

Figure 2. Involvement of Wnt signaling in consecutive stages of chondrogenic differentiation. Wnt-3A induces proliferation and self-renewal of MSCs, which form mesenchymal condensations at the initial stage of differentiation. Subsequently, chondrocyte differentiation is induced by low levels of β-catenin, Wnt-5A, Wnt-5B and WIF-1, whereas Wnt-1, Wnt-3A, Wnt-7A and Wnt-14 block chondrocyte differentiation. In adult articular cartilage, a fine balance of β-catenin levels is involved in maintaining the chondrocyte phenotype. Hypertrophic differentiation of chondrocytes is induced by Wnt-4 and high levels of β-catenin and blocked by Wnt antagonists DKK-1 and FRZB.

plays an essential role in cell fate determination in skeletal development, as it acts as a molecular switch between chondrocyte and osteoblast differentiation in SPCs. Upregulation of β-catenin in mesenchymal condensations was found before expression of the osteoblast-specific transcription factors Runx2 and Osx was detected, indicating that high levels of β-catenin precede osteoblast differentiation. In contrast, β-catenin expression was downregulated in chondrogenic mesenchymal condensations (25). Since conditional deletion of β-catenin is prenatally lethal, an essential role for β-catenin in early skeletal development was indicated (25). Deletion of β-catenin in early embryonic development results in the arrest of osteoblast differentiation at the level of osteo-chondrogenic progenitor cells, which instead differentiate into chondrocytes (26). Furthermore, inactivation of β-catenin in micromass cultures enhances chondrocyte differentiation *in vitro* (25). Mice in which ablation of β-catenin is induced in cartilage after birth, develop an osteoarthritis-like phenotype. These studies indicate that β-catenin

might be involved in maintaining cartilage in adult indicating that a fine balance of β-catenin is required both for normal chondrogenic differentiation, as well as for the maintenance of cartilage tissue after formation (32, 33).

A number of Wnt ligands have been implicated in the regulation of various aspects of endochondral ossification. Even though Wnt-1 is not endogenously expressed during limb development, overexpression of Wnt-1 in chick embryos *in vivo* was found to cause skeletal abnormalities such as truncation or deletion of skeletal elements (34, 35). Since retroviral expression of Wnt-1 in limb bud micromass cultures resulted in severely inhibited cartilage formation, this might be the underlying mechanism for these skeletal abnormalities *in vivo* (35). Wnt-3A regulates the expansion of the MSC population, through increasing self-renewal and decreasing apoptosis (36, 37). Expression of Wnt-3A is decreased when chondrogenic differentiation progresses. Furthermore, addition of exogenous Wnt-3A blocked the collagen type II expression and suppressed the deposition of sulfated proteoglycans, indicating that downregulation of Wnt-3A is required for chondrogenesis (38). Wnt-4 was found to be involved in joint formation and cartilage development, as it is expressed in developing joints, in the periphery of the joint interzone and in a subset of hypertrophic chondrocytes (39, 40). Different effects of Wnt-4 were found during different stages of skeletal development. Misexpression of Wnt-4 resulted in the shortening of long bones and histological examination of developing limbs revealed that cartilage elements in these limbs showed an expanded hypertrophic zone and a thicker osteoid layer of the bone collar, compared to the contralateral control limb (41). These findings indicate that Wnt-4 accelerates chondrocyte maturation, which results in an accumulation of terminal differentiated hypertrophic chondrocytes at the expense of immature round chondrocytes at the ends of the cartilage elements (40). During skeletal development, Wnt-5A is initially expressed in the mesenchyme around the developing condensations, indicating that Wnt-5A might be involved in the recruitment of mesenchymal cells into the chondrogenic lineage. At later stages, Wnt-5A expression was found in the perichondrium, indicating the involvement of Wnt-5A in the formation of bone. Expression of Wnt-5B was restricted to pre-hypertrophic chondrocytes, as well as cells in the outer layer of the perichondrium (39, 40, 42). Wnt-5A and Wnt-5B both promote the first steps of chondrogenesis in micromass cultures, whereas cartilage

elements in which Wnt-5A was misexpressed are smaller in size and show a delay in the maturation of hypertrophic chondrocytes histologically and molecularly (39, 41). Expression of Wnt-7A was found in the dorsal ectoderm in the developing limb (43). Chondrogenesis was blocked by Wnt-7A in micromass cultures *in vitro* as well as after *in vivo* overexpression in chick embryos (34). Furthermore, Wnt-7A induces a chondro-inhibitory effect, which is mediated by MAP kinase and AP1 signaling (44). Wnt-14 expression was found in the developing joint interzone, like Wnt-4 (45). It is implicated in the initial steps of joint development and it was found to arrest and even reverse chondrogenic differentiation. Ectopic expression of Wnt-14 in the prechondrogenic region prevents prechondrogenic cells from further differentiating into chondrocytes, with downregulated expression of chondrogenic markers Collagen II and Sox9 (40). In the mature joint, Wnt-14 continues to be expressed in the synoviocytes and the joint capsule.

The regulation and specificity of Wnt signaling is not only dependent on the presence of specific ligands and receptors, but also on the action of endogenous antagonists of Wnt signaling. Frzb-1 expression was specifically found in mesenchymal prechondrogenic condensations and at later stages in epiphyseal pre-articular chondrocytes (46, 47). Overexpression of Frzb-1 *in vivo* blocks chondrocyte maturation at an early hypertrophic stage and prevents endochondral ossification (47). In addition, Frzb knockout mice, in which a mild activation of Wnt/β-catenin signaling was observed, exhibit accelerated hypertrophic chondrocyte maturation (48). Addition of Frzb to micromass cultures of MSCs promoted glycosaminoglycan synthesis, as well as gene expression and protein expression of Sox9 and collagen type II. DKK-1 was found to have a similar effect, although to a lesser extent (49). A recent study provided evidence that the Wnt antagonists DKK-1 and FRZB in combination with the bone morphogenetic protein (BMP) antagonist GREM1 are significantly higher expressed in articular cartilage compared to growth plate cartilage (50). The authors provided evidence that these antagonists are natural breaks of hypertrophic differentiation and subsequent endochondral ossification of articular cartilage. WIF-1 expression was found in the mesenchyme surrounding cartilage elements forming in the limb during early skeletal development. In late embryonic and postnatal development, WIF-1 expression was observed in articular cartilage. Moreover, WIF-1 interferes with Wnt-3A mediated inhibition of chondrogenesis in micromass cultures (51).

Cartilage degeneration in arthritis

Arthritis is a joint disorder that is usually limited to involvement of one or more joints. Osteoarthritis (OA), also known as degenerative arthritis, is the most common form of arthritis. It is a heterogeneous disease characterized by progressive degradation of joint cartilage, typical bone changes and signs of mild synovitis particularly in more advanced stages of the disease. In contrast, rheumatoid arthritis (RA) is a chronic, systemic inflammatory disorder that may affect many tissues and organs, but principally involves synovial joints. In both OA and RA, articular cartilage is the primary target for damage due to loss of cartilage homeostasis. In healthy joints, cartilage homeostasis is maintained by the balance of synthesis and degradation of extracellular matrix (ECM). Cartilage undergoes destruction when the balance is lost.

In the adult, articular chondrocytes are fully differentiated cells that play a critical role in the pathogenesis of OA by responding to adverse environmental stimuli by promoting matrix degradation and downregulating processes essential for cartilage repair. Multiple risk factors have been implicated in the initiation and progression of OA, including mechanical injury, genetics and aging (52). For instance, in response to traumatic injury, chondrocytes activate general gene expression, which results in increased expression of inflammatory mediators, cartilage-degrading proteinases, and stress response factors (53, 54).

Synovial inflammation likely contributes to deregulation of chondrocyte function, amongst others by secreting cytokines that impact chondrocyte activity (55). Chondrocytes can respond to a number of cytokines and chemokines in the joint tissue and joint fluid. These cytokines and chemokines can be produced by other cell sources such as fibroblast-like synoviocytes (FLS) which play an important role in the pathogenesis of RA (56). IL-1β and TNF-α are able to induce the synthesis of ECM degrading enzymes such as MMPs as well as the production of other pro-inflammatory mediators such as prostaglandin E2 (PGE2) and nitric oxide (NO) (57, 58). In addition, the association of the increased levels of catabolic enzymes and inflammatory mediators such as prostaglandins and NO and the levels of cytokines like IL-1β and TNF-α in synovial fluid and joint tissue has been established (59). Pro-inflammatory cytokines induce loss of the chondrocytic phenotype of chondrocytes in the matrix and can induce chondrocyte apoptosis (60, 61).

These findings indicate that the pro-inflammatory mediators are crucial mediators of cartilage degeneration.

Cartilage homeostasis is achieved when catabolic and anabolic activities of the chondrocytes are in balance. When the balance is disturbed and catabolic activity of the chondrocyte prevails, the cartilage ECM undergoes remodeling and degradation. Such disequilibrium may be induced by abnormal mechanical loading and synovial inflammation. The relation between increased production of proteinases, including MMPs, MMP-1, MMP-3, MMP-8, MMP-13, and the aggrecanases, particularly ADAMTS-5, with cartilage damage has been documented (62, 63). FLS in the synovium also produce pro-MMP-3 (precursor form of MMP-3 or stromelysin 1), which in its mature form enhances cartilage degradation (56). Production and activities of these proteinases are regulated by various mediators such as cytokines, growth factors, prostaglandins, matrix breakdown products, and oxygen species (64, 65). It has also been shown that expression of the COL2A1 gene is suppressed in upper zones of OA cartilage with progressing matrix destruction, whereas global COL2A1 gene expression is increased in late-stage OA cartilage compared to normal and early degenerative cartilage suggesting a compensatory mechanism (66). Cessation of cartilage ECM molecule synthesis can be caused by a number of factors such as pro-inflammatory cytokines (60) and NO (61). Anabolic factors such as BMP-2, activin A and tumor necrosis factor-β (TGF-β) superfamily members might be responsive for the compensatory increase in COL2A1 expression (67, 68). Importantly, once the cartilage is severely degraded the chondrocyte is unable to replicate the complex arrangement of collagen laid down during development. Therefore the imbalance between catabolic and anabolic activities of the chondrocytes is a key contributor to cartilage degeneration.

Phenotypic modulation of chondrocyte function by Wnt signaling

In addition to its function in chondrogenesis and chondrocyte maturation, Wnt signaling is also involved in the maintenance of fully differentiated chondrocyte phenotypes and may therefore play a crucial role in cartilage homeostasis throughout adult life. When differentiated chondrocytes are exposed to inflammatory factors such as IL-1 and retinoic acid or cultured in monolayer, their phenotype is rapidly lost and cells become fibroblast-like. This process is known as dedifferentiation and it is accompanied

by increased β-catenin protein expression (69). Accumulation of β-catenin by ectopic expression or inhibition of its degradation results in a decrease of cartilage-specific ECM molecule synthesis through activation of TCF/LEF transcriptional activity in rabbit chondrocytes (69). Conditional deletion of the APC gene, which results in upregulation of β-catenin in mature chondrocytes also results in a complete loss of the chondrocyte phenotype *in vivo* (70). In addition, Wnt-3A and Wnt-7A caused loss of type II collagen synthesis via stimulation of β-catenin-TCF/LEF transcriptional activity (38). Moreover, Wnt-3A induced the expression of c-Jun and its phosphorylation by c-Jun N-terminal kinase (JNK), resulting in activation of AP-1. AP-1 could suppress the expression of Sox9, a major transcription factor regulating COL2A1 expression (38). In contrast, Wnt3a inhibited chondrogenesis of mesenchymal cells by stabilizing cell-cell adhesion in a manner independent of β-catenin's transcriptional activity (38). It has also been shown that Wnt-7A inhibited NO-induced apoptosis by activating cell survival signaling, such as phosphatidylinositol 3-kinase and Akt, independent of β-catenin's transcriptional activity (71). Together, these results suggest that Wnt proteins regulate chondrocyte functions via different mechanisms.

However, all these studies were performed in animal chondrocytes, whereas the function of Wnt signaling in human chondrocytes is up to date not well studied. A recent study reported that Wnt-3A promoted human articular chondrocyte proliferation through the β-catenin-dependent canonical pathway while simultaneously inducing loss of expression of chondrocyte marker genes via a β-catenin-independent non-canonical pathway (Fig. 3) (11). Dedifferentiation of human chondrocytes in vitro could not be reversed by inhibition of the canonical Wnt pathway either by knockdown of β-catenin or addition of a TCF/β-catenin inhibitor (Ma et al., submitted). Remarkably during human chondrocyte dedifferentiation the non-canonical Wnt-5A is strongly upregulated which coincided with a downregulation of COL2A1 expression. Knockdown of Wnt-5A reversed COL2A1 expression again suggesting that dedifferentiation in human chondrocytes appears independent of β-catenin. These latter findings contrast observations in rabbit chondrocytes, in which β-catenin-TCF/LEF transcriptional activity contributed to chondrocyte dedifferentiation. The controversial findings in human and animal chondrocytes suggest that the exact function of the canonical Wnt pathway in articular

cartilage may be species-dependent. Such species difference was also observed in the regulation of MMP expression in human and animal chondrocytes (72).

In contrast to Wnt-3A and Wnt-7A, Wnt-5A and Wnt-11 primarily regulate cartilage-specific ECM molecule synthesis through the non-canonical pathway (73). Stimulation of rabbit chondrocytes with IL-1β upregulated Wnt-5A and downregulated Wnt-11 expression. Wnt-5A inhibited COL2A1 expression via the JNK pathway, whereas Wnt-11 stimulated COL2A1 expression via the PKC pathway, indicating that Wnt-5A and Wnt-11 have opposing effects on COL2A1 expression by signaling through distinct non-canonical Wnt pathways in rabbit chondrocytes (73). In human chondrocytes, Wnt-5A was also found to block COL2A1 expression, in agreement with its effects in rabbit chondrocytes (Ma et al., submitted). Interestingly, Wnt-3A is also able to downregulate COL2A1 and SOX9 expression through the non-canonical Ca^{2+}/CaMKII pathway (Fig. 3) (11). All these findings substantiate the role of non-canonical cascade in the deregulation of chondrocyte function. Collectively, a direct role of the β-catenin-dependent canonical pathway has not been suggested in human chondrocyte dedifferentiation.

Wnt signaling in cartilage degeneration

In the light of the involvement of Wnt signaling in cartilage development and the maintenance of adult chondrocyte phenotype and cartilage homeostasis, dysfunction of the Wnt pathway may lead to cartilage tissue disease. Indeed, differential expression of Wnt pathway components has been documented in joint disorders such as osteoarthritis (OA) and rheumatoid arthritis (RA). In several whole genome studies, the Wnt antagonist FRZB has emerged as a candidate gene associated with an increased risk for OA (74-77). A single-nucleotide polymorphism in FRZB resulting in an Arg324Gly substitution at the carboxyl terminus, which shows diminished ability to antagonize wnt signaling in vitro, was associated with hip OA in the female (75). The correlation of elevated circulating levels of DKK-1, another Wnt antagonist, with reduced progression of radiographic hip osteoarthritis (RHOA) in elderly women has also been suggested (78). This is in line with the proposed role of DKK-1 together with FRZB and GREM1 as natural brakes of chondrocyte hypertrophy. Derailed hypertrophic differentiation in articular cartilage has been implemented in the pathogenesis of OA at least in a subset of patients (50). Likewise,

differential expression of various Wnt proteins and their receptors has been reported in human joint disorders (79). For example, overexpression of Wnt-5A and Fzd5 has been found in RA synovial tissues in comparison to a panel of normal adult tissues (79) while the canonical Wnt-7B is upregulated in OA cartilage and RA synovium (80). In addition, increased expression of the Wnt target gene (WISP-1) was found in both mouse OA models and in human OA cartilage (81). A systematic analysis of the Wnt signaling pathway revealed up-regulation of Wnt-16, down-regulation of FRZB, up-regulation of Wnt target genes, and nuclear localization of β-catenin in injured cartilage (82). In addition, in OA, Wnt-16 and β-catenin were barely detectable in preserved cartilage areas, but were dramatically up-regulated in areas of the same joint with moderate to severe OA damage (82). These findings were subsequently corroborated by observation of increased nuclear β-catenin staining in human OA cartilage compared to control (32). Therefore, these studies indicate that cartilage degeneration is associated with increased Wnt signaling.

To explore the exact function and underlying mechanism of Wnt signaling in joint biology and disease, a variety of studies have been conducted using animal models. Although not developing a noteworthy developmental phenotype, $Frzb^{-/-}$ mice display greater cartilage loss in comparison to wild-type controls when exposed to factors known to induce OA, like enzymatic treatment (papain-induced OA), accelerated instability (collagenase-induced ligament and meniscal damage) or inflammation (mBSA induced monoarthritis) (83, 84). The mild phenotype might be explained by partial compensation by other antagonists like DKK-1 and GREM1 (50). Cartilage degradation in the $Frzb^{-/-}$ mice is associated with up-regulation of β-catenin and MMP-9. Interestingly, it was also shown *in vitro* that cartilage injury results in increased Wnt activity and decreased expression of FRZB (85). In postnatal mouse models, inducible conditional activation of β-catenin signaling in cartilage results in increased articular cartilage degeneration by stimulating endochondral ossification and other phenotypes resembling OA (32). Activation of Wnt/β-catenin signaling in rabbit and mouse chondrocytes stimulates the expression of cartilage matrix degrading MMPs (86, 87). In a spontaneous guinea pig OA model, development of OA is associated with increased β-catenin expression in cartilage (86). Interestingly, inhibition of β-catenin signaling in articular chondrocytes also causes OA-like cartilage degradation in a Col2a1-ICAT transgenic mouse model (14). Since ICAT may have other

cellular targets than β-catenin, it is unclear whether the OA-like phenotype in the latter mice can solely be attributed to inhibition of β-catenin. Taken together, all these findings have led to the hypothesis that low levels of Wnt/β-catenin signaling are required for maintenance of normal cartilage function and that deregulation of this pathway may contribute to the development and progression of cartilage degeneration. This hypothesis is supported by strong experimental evidence in animal models. The support for such a role in human OA is however less strong and predominantly based on circumstantial evidence showing associations between increased β-catenin levels and an OA phenotype in cartilage specimens. Evidence for a causal relationship in human is currently lacking.

In contrast to its pro-catabolic role in animal cartilage by inducing ECM degrading enzymes such as MMPs, Wnt activation was found to inhibit MMP expression in a TCF/LEF-independent pathway in human articular chondrocytes (72). In animal cells, the Wnt pathway regulates MMP expression through β-catenin-TCF/LEF transcriptional activity (72, 88). In human chondrocytes, activation of canonical Wnt blocks MMP expression through an inhibitory interaction of β-catenin with NF-κB (Fig. 3) (72). This species difference in the regulation of pro-catabolic MMP expression gives rise to the question whether canonical Wnt signaling is a pathogenic factor in human cartilage degeneration. However, similar to findings in animal models, in fibroblast-like synoviocytes from RA patients, activation of canonical Wnt signaling by Wnt-1 transfection increases expression of MMP-3, while interference with Wnt signaling using anti-Wnt-1 blocking antibody or the Wnt antagonist sFRP-1 decreases MMP-3 expression (89). Wnt signaling may exhibit complicated functions in joint disease by activating multiple cascades and interacting with other pathways, and this might also be tissue-dependent. Therefore, the role of Wnt signaling in human cartilage degeneration remains elusive and more studies should be focused on extrapolation of knowledge obtained from animal models to the human situation. Our recent study also reveals that TCF4 induces MMP expression and apoptosis probably through potentiating NF-κB signaling (Fig. 3) and its expression is upregulated in OA cartilage compared to normal human cartilage (Ma et al., submitted). Thus TCF4 may serve as a potential therapeutic target for OA.

A role of non-canonical Wnt pathway in cartilage degeneration is suggested by the induction of MMP expression by Wnt-5A in rabbit chondrocytes (90) and repression of

chondrocyte marker gene expression by Wnt-5A or Wnt-3A-mediated Ca^{2+} pathway (11). It has been suggested that canonical and non-canonical pathways reciprocally inhibit each other (11). Therefore blockade of the canonical/β-catenin pathway will also cause articular chondrocyte dedifferentiation through de-repression of the Ca^{2+} pathways. Stimuli such as IL-1 (91) and biomechanics (92) which change CaMKII may significantly influence the outcome of Wnt signaling by switching the balance between β-catenin and CaMKII. Thus IL-1-mediated pro-catabolic activity in cartilage may partially come from its enhancement of the Ca^{2+} cascade activity initiated by Wnt. The opposing induction of β-catenin pathway by IL-1 serves as a negative feedback to counteract its pro-catabolic activity (72). Due to the complicated properties that the Wnt protein activates multiple pathways and Wnt signaling components show diverse functions in human chondrocytes, more specific targeted therapy should be developed with respect to treating human joint disease by manipulating Wnt signaling.

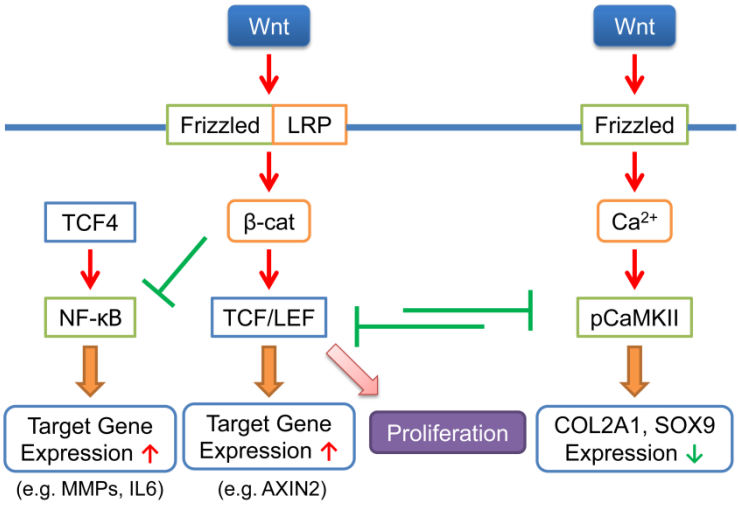

Figure 3. Function of Wnt signaling in human chondrocytes. Wnt proteins may activate both canonical and non-canonical pathways in a cellular context dependent manner, most likely involving differential expression of Fzd receptors at the cell surface. The canonical Wnt/β-catenin pathway induces proliferation and expression of target genes such as AXIN2. It represses NF-κB signaling through an inhibitory interaction of β-catenin with NF-κB, consequently inhibiting expression of target genes such as MMPs and IL6. In contrast, TCF4 is able to potentiate NF-κB signaling independent of β-catenin. The non-canonical Wnt signaling decreases COL2A1 and SOX9 expression through Ca^{2+}/CaMKII pathway.

Conclusion

A remarkable effort has unraveled the stage-dependent regulatory role of Wnt signaling in chondrogenesis and cartilage development. Consequently, cumulating evidence has suggested the involvement of Wnt signaling in cartilage disease. Although extensive animal studies have indicated that excessive Wnt signaling may lead to cartilage destruction, the exact function of Wnt signaling in human cartilage is still largely unclear. Species differences in Wnt function in chondrocytes have been observed. The future challenge for research is to properly extrapolate our knowledge of Wnt signaling in animal models to the human situation. Given the fact that Wnt signaling is a complex network, accumulation of our understanding of the involvement of specific Wnt components in cartilage degeneration will facilitate the development of more effective and specific treatments for joint disease.

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Chapter 3

Conditional Inducible Knockout Mouse Models to Study Wnt Signal Transduction in Cartilage

Conditional inducible knockout mouse models to study Wnt signal transduction in cartilage

Abstract

Wnt signaling plays a crucial role in controlling the balance between cell proliferation and differentiation during embryogenesis and postnatal life. Many of the conventional knockout mice for Wnt pathway-related genes display early embryonic lethality, highlighting the fundamental importance of Wnt signaling in early life, but precluding functional analyses at later stages. Therefore the use of conditional or inducible knockout mice enabling the study of Wnt signaling at later stages of development and adult life is warranted. By using a transgenic mouse line expressing a *Col2a1* promoter-driven tamoxifen-dependent Cre recombinase, we were able to selectively induce recombination in chondrogenic cells at defined time points. This provides us a valuable tool to study the function of Wnt pathway-related genes in cartilage development and degeneration.

Introduction

Wnt proteins constitute a large family of cysteine-rich secreted ligands which are essential for various developmental and physiological processes. Deregulated Wnt signaling is associated with a wide array of diseases such as cancer, osteoporosis and degenerative disorders like osteoarthritis (OA) (1, 2). Wnts transmit signals through different pathways by selectively activating specific receptors and co-factors (3, 4). The canonical cascade through β-catenin is the best understood pathway. In the absence of Wnt, a destruction complex consisting of Axin, adenomatosis polyposis coli (APC) and casein kinase 1 (CKI) mediates the phosphorylation of β-catenin by GSK-3β, which targets cytosolic β-catenin for degradationby the proteasome (5). Binding of Wnt to its receptors results in disruption of the destruction complex and accumulation of cytoplasmic β-catenin. Upon nuclear translocation, β-catenin will function as a co-factor of T cell factor / lymphoid enhancer-binding factor (TCF/LEF) transcription factors to switch on Wnt target gene transcription (5).

A large number of knockout mouse models have been generated to study Wnt signaling in development and disease. These models target different players spread over the pathway including ligands, receptors, intracellular intermediates and transcription factors (6). Many of these knockout mice show early embryonic lethality which precludes functional studies at later stage. With the great development of mouse engineering technology, the advent of Cre-lox technique and additional systems tools have come available allowing both spatial and temporal control over gene expression in the organism. The Cre recombinase is an integrase from bacteriophage P1 which catalyzes site-specific recombination between 34-bp repeats termed loxP sites (7). When fused to the ligand binding domain of the human estrogen receptor (ER), this chimeric Cre is only active in the presence of ER ligands such as tamoxifen (8-10). Transgenic mouse lines containing this inducible $CreER^T$ offer versatile tools to dissect functions of genes at multiple stages of development and postnatal life by having control over the time point of recombination. Moreover, tissue-specific recombination can be achieved by using tissue-specific promoters to drive the expression of $CreER^T$ (11, 12).

Conditional loss- and gain-of-function mutations of β-catenin have revealed its role as a key regulator of skeletogenesis (13-15). Conditional mutations of β-catenin were

introduced into osteo-chondrogenic progenitor cells using Prx1 and Dermo 1 promoter-driven Cre. Loss-of-function mutations of β-catenin lead to osteoblast differentiation arrest, increased chondrogenesis and ectopic cartilage formation (13-15). Conversely, β-catenin gain-of-function mutations resulted in the arrest of chondrogenesis (13). In postnatal mouse models, inducible conditional activation of β-catenin signaling in cartilage results in increased articular cartilage degeneration by stimulating endochondral ossification and other phenotypes resembling OA (16). Transient Wnt/β-catenin signaling activation in young adult mice induced growth retardation and severe deformities in knee joints (17). All these lines of evidence underscore the fundamental role of Wnt/β-catenin signaling in cartilage development and degeneration.

APC has been targeted to manipulate Wnt/β-catenin signaling. In an *APC*^{15lox} mouse model, exon 15 of *APC* is flanked by loxP sites and is deleted by Cre recombinase (18). Inactivation of APC by deletion of exon 15 results in elevated Wnt/β-catenin signaling activity. APC1638N is a truncated form of APC (19). It's a hypomorphic APC variant which preserves partial activity of wild-type APC protein therefore leading to moderately increased Wnt/β-catenin signaling activity compared to loss of the wild type allele. Observations from a Col2a1-Cre;*APC*^{15lox} mouse model indicate that a tight APC-mediated control of β-catenin levels is essential for differentiation of skeletal precursors as well as for the maintenance of a chondrocytic phenotype in a spatio-temporal regulated manner (20).

Here we report conditional inducible Col2a1-CreER^{T} and *APC* transgenic models as well as the use of in vitro Cre-induced recombination in cells from floxed mice to study the function of Wnt signaling in cartilage.

Materials and Methods

Transgenic mouse lines. The Col2a1-CreER^{T} mouse line was a kind gift from Dr. Susan Mackem (NIH, USA) (12). The Rosa26 reporter (Rosa26R) mouse line was a kind gift from Dr. Geert J. van Tetering (University Medical Center Utrecht, The Netherlands) (21, 22). Tamoxifen (Sigma) was dissolved in corn oil (Sigma) at a final concentration of 50 mg/ml for IP injection.

LacZ staining. After IP injection of tamoxifen to Col2a1-CreER^{T};Rosa26 mice, bone fragments were isolated from pups and then fixed overnight in 4% paraformaldehyde at 4°C. Whole mount LacZ staining was performed with X-Gal substrate (Sigma) as preciously described (23). Paraffin sections were made from knee joints and counterstained with fast nuclear red (Sigma-Aldrich).

Cell culture. Mouse embryonic fibroblasts (MEFs) and human embryonic kidney 293T (HEK293T) cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen). MEFs from APC$^{15lox/15lox}$ transgenic animals were isolated from E12.5 mouse embryos and were kindly provided by Dr. Razvan Miclea (Leiden University Medical Center, the Netherlands).

Lentiviral production and transduction. Lentiviral vectors pBOB-GFP (Addgene plasmid 12337) (24) or pBOB-iCRE (Addgene plasmid 12336) (25) were transfected using Fugene HD (Roche) into HEK293T cells together with packaging vectors to produce lentiviruses. Lentiviruses were harvested and used to infect MEFs in the presence of 6 µg/ml polybrene (Sigma).

DNA isolation and genomic PCR. DNA was isolated from MEFs using QIAmp DNA Mini Kit (Qiagen). PCR was performed using GoTaq Hot Start Green Master Mix (Promega). The sequences of PCR primers for genotyping Col2a1-CreER^{T} mice are: 5'-GAAAATGCTTCTGTCCGTTTGC-3' (forward primer) and 5'-ATTGCTGTCACTTGGTCGTGGC-3' (reverse primer) and the size of the PCR product is 207 bp. The sequences of PCR primers for genotyping Rosa26 reporter mice are: R1295, 5'-GCGAAGAGTTTGTCCTCAACC-3'; R523, 5'-GGAGCGGGAGAAATGGATATG-3' and R26F2, 5'-AAAGTCGCTCTGAGTTGTTAT-3'. The 600-bp PCR product was detected in wild-type mice and the 325-bp PCR product was detected in homozygous Rosa26R mice. In heterozygous Rosa26 reporter mice, both 600 and 325-bp PCR products were detected.

RNA isolation and real-time RT-PCR. Total RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel). cDNA was synthesized from total RNA with the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed using the standard curve method.

Results and Discussion

Col2-CreER^T and Rosa26R mouse lines

To specifically introduce changes in gene expression in cartilage tissue, we exploited the Col2a1-CreER^T mouse line. A chimeric CreER fusion protein is driven by a *Col2a1* promoter (Fig. 1A) (12). Since Col2a1 is a specific marker for chondrogenic cells, the recombinase is only expressed in chondrogenic progenitor cells and chondrocytes. In addition this CreER^T is only activated in the presence of tamoxifen which offers precise time control of the recombination. To examine the efficiency and specificity of recombination by Col2a1-CreER^T the Rosa26R mouse line was used. In this mouse model, a cassette consisting of neomycin and polyadenylation (polyA) sequences is flanked by two loxP sites (Fig.1B) (22). This cassette blocks the expression of the downstream LacZ gene. When the inhibitory fragment is excised out of the loxP sites, LacZ gene expression is initiated, which serves as a reporter for Cre-mediated recombination.

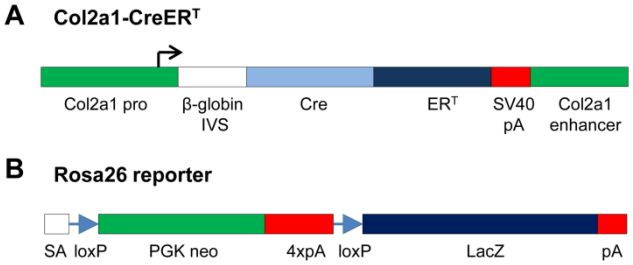

Figure 1. **Transgenic mouse lines and experimental setup. A.** Schematic diagram of transgenic Col2a1-CreER^T expression construct used to generate transgenic mice expressing the CreER^T fusion construct under the control of the cartilage specific *Col2a1* promoter. The construct contains a 1 kbp fragment of the Col2a1 promoter, the β-globin intron (IVS), the CreER^T fusion construct, the SV40 polyA signal and *Col2a1* first intron enhancer. **B.** Schematic diagram of transgenic Rosa26 reporter (R26R) construct used to generate transgenic mice. The construct includes a splice acceptor sequence (SA), a neo expression cassette flanked by loxP sites, a LacZ gene and a polyadenylation (pA) sequence.

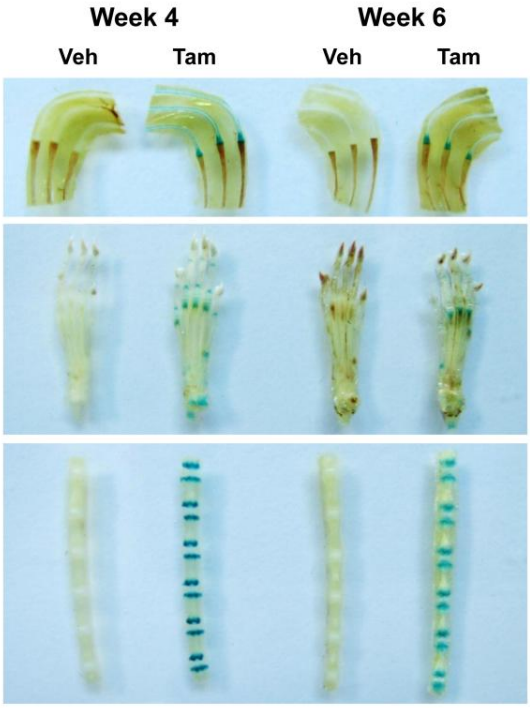

Figure 2. Whole-mount LacZ staining of bone fragments. Ribs, paws and tails were isolated from Col2a1-CreER^T; Rosa26R pups injected with a single injection of vehicle or tamoxifen 2 weeks after birth. Bones were isolated 2 and 4 weeks after the injection and then stained using X-Gal substrate.

Efficiency and specificity of tamoxifen-induced recombination

Our primary interest is the function of Wnt signaling in articular cartilage degeneration. Therefore we first evaluated the efficiency of Tamoxifen-induced recombination in chondrocytes. The Col2a1-CreER^T mouse line was crossed with Rosa26R mouse line to obtain pups carrying both Col2a1-CreER^T and LacZ reporter genes. At 2 weeks after birth, the pups were given a single injection of 2 mg tamoxifen or vehicle intraperitoneally (IP). Bone fragments were isolated from 4- and 6-week-old mice. Following LacZ staining, Col2a1-CreER^T; Rosa26R mice injected with tamoxifen exhibited apparent LacZ activity in cartilaginous tissues in ribs, paws and tails while no LacZ activity was found in mice that received vehicle (Fig. 2). In addition, we did not observe LacZ staining in other tissues than cartilage. This data suggests that CreER^T is specifically expressed in cartilage and that the recombinase activity is strictly controlled by tamoxifen.

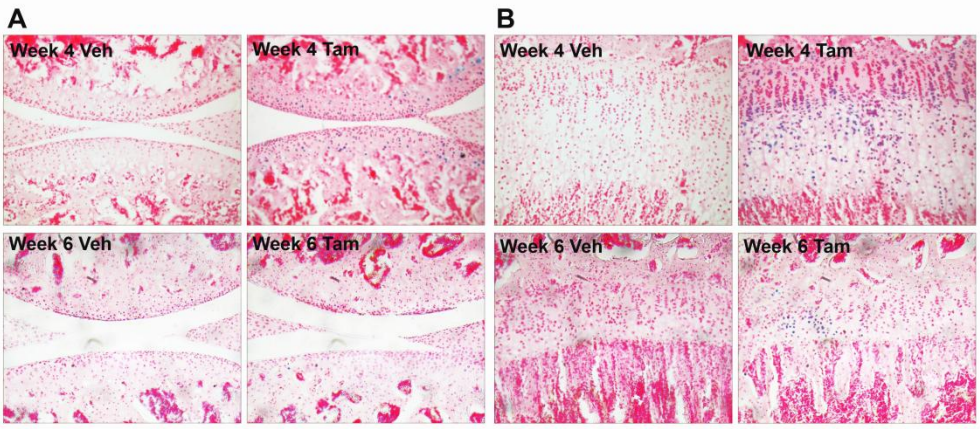

Figure 3. Histological analyses of knee joints. Knee joints isolated from Col2a1-CreER^{T}; Rosa26R pups injected with vehicle (Veh) or tamoxifen (Tam) were stained using X-Gal substrate and counterstained with nuclear fast red at week 4 and 6, respectively 2 and 4 weeks after a single tamoxifen injection. Articular cartilage (A) and growth plate (B) were examined under microscope and a representative picture is shown. Chondrocytes which underwent a recombination stained blue.

To further evaluate the Cre activity at cellular level, sections of knee joints were examined. Consistent with observations in other bone fragments, Col2a1-CreER^{T}; Rosa26R mice showed pecific recombination in knee joint and growth plate cartilage (Fig.3). At 4 weeks, two weeks after injection, tamoxifen induced recombination in approximately 30% of the articular chondrocytes in knee joint cartilage (Fig. 3A). At 6 weeks, 4 weeks after injection, recombination efficiency dropped to 5% (Fig. 3A). Growth plate chondrocytes showed relatively higher recombination efficiency than articular chondrocytes (70% at 4 weeks and 10% at 6 weeks) (Fig.3B). The fast decrease of recombination efficiency from week 4 to week 6 may be explained by the replacement of chondrocytes by new cells derived from progenitors where *Col2a1* is not expressed or expressed at low level thus having no or lower level recombination activity. During 2 to 6 weeks, mice are undergoing fast development and growth. In the joint cartilage, cells in the surface area were not affected by tamoxifen-induced recombination and most of the recombination occurred in the inner layer of cartilage, suggesting that cells in the surface layer may not express or express very low levels of *Col2a1*, and serve as the source for the renewal of articular chondrocytes. The latter is in line with current hypothesis (26). However more sophisticated time course experiments are needed to test this hypothesis. In addition, 2 weeks after a single tamoxifen injection none of the chondrocytes residing in the stem cell

zone of the growth plate did express LacZ. To substantially improve the recombination efficiency, higher doses of tamoxifen and repeated numbers of injections may be used. By varying with dosing and frequency of injection it might be possible to differentially induce recombination in more mature chondrocytes without inducing recombination in stem cells. It has been shown that recombination can be induced specifically in articular chondrocytes after growth plate closure by repeated injections of tamoxifen (16). The Rosa26R mouse line, in combination with specific promoter-driven inducible Cre mouse line, may serve as a useful and powerful tool for lineage tracing (27-29).

In vitro Cre-induced recombination

To investigate more insightful mechanisms of the phenotype observed in conditional knockout mice, cells can be isolated from floxed transgenic mice. We isolated MEFs from E12.5 *APC*$^{15lox/15lox}$ embryos and tested efficiency and effects of Cre-induced recombination in cell culture. Lentiviruses expression improved Cre (iCRE) were used to infect MEFs. A transduction efficiency of near 100% was reached with an MOI of 100. Successful recombination was demonstrated by PCR which specifically detected recombinant *APC*Δ15 whose exon 15 was deleted from the genome and the decrease in expression of the APC^{15lox} allele (Fig. 4A).

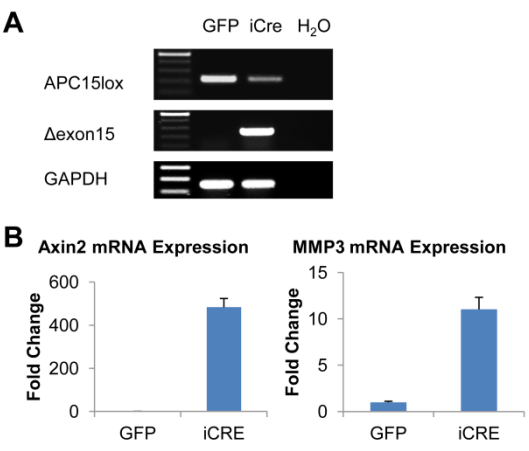

Figure 4. Efficiency and effects of lentiviral induced Cre-mediated recombination of the APC gene in MEFs. A. *APC*$^{15lox/15lox}$ MEFs were transduced with a lentivirus expressing improved Cre (iCRE). 5 days after transduction genomic PCR was isolated and PCR was used to detect the *APC*^{15lox} allele and the recombined Δexon15 allele. GFP-transduced MEFs served as control. **B.** Cre-induced recombination in *APC*$^{15lox/15lox}$ MEFs induced *Axin2* and *MMP3* mRNA expression measured using real-time RT-PCR. GFP-transduced MEFs served as control.

Since APC is a member of the degradation complex for β-catenin, inactivation of APC by deletion of exon 15 will result in accumulation of β-catenin and activation of the canonical pathway. In agreement with this, recombination induced robust increase in the mRNA expression of the canonical Wnt target gene *Axin2* (Fig. 4B), suggesting an efficient activation of canonical Wnt signaling. Furthermore mRNA expression of another β-catenin target gene, *MMP3*, was also upregulated by the recombination (Fig. 4B). This data suggests that *in vitro* Cre-induced recombination of *APC* can be used to activate canonical Wnt signaling. This represents an alternative way for activation of Wnt-signaling by other means such as direct stimulation with Wnts. Given the fact that Wnt proteins may activate multiple pathways depending on the use of distinct cell surface receptors (3), this offers a direct way to manipulate downstream pathway mediators.

Generation of APC transgenic mice

To study the function of canonical Wnt pathway in cartilage development, we crossed Col2a1-CreER^{T} with *APC*^{15lox} (18) and *APC*1638N (19) mice to generate Col2a1-CreER^{T};*APC*$^{15lox/15lox}$, Col2a1-CreER^{T};*APC*$^{15lox/1638N}$, and other control genotypes. Upon tamoxifen injection and activation of recombination, it is expected that exon 15 of the *APC* gene will be deleted. APC1638N is a truncated form of APC and retains partial activity of wild-type APC. Therefore these mice may exhibit differential APC activities, thus different β-catenin levels. These mouse models are potentially useful tools to investigate dose-dependent effects of canonical Wnt signaling in cartilage development.

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Chapter 4

A Wnt/β-catenin Negative Feedback Loop Inhibits Interleukin-1-induced Matrix Metalloproteinase Expression in Human Articular Chondrocytes

A Wnt/β-catenin negative feedback loop inhibits interleukin-1-induced matrix metalloproteinase expression in human articular chondrocytes

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Abstract

Objective. Recent animal studies suggest that activation of Wnt/β-catenin signaling in articular chondrocytes might be a driving factor in the pathogenesis of osteoarthritis (OA) by stimulating amongst others the expression of matrix metalloproteinases (MMPs). This study aimed to investigate the role of Wnt/β-catenin signaling in interleukin-1β (IL-1β)-induced MMP expression in human chondrocytes.

Methods. Primary cultures of human, murine and bovine articular chondrocytes as well as human mesenchymal stem cells and mouse embryonic fibroblasts were used. Multiple strategies for activation and inhibition of signaling pathways were used. Reporter assays and co-immunoprecipitation were used to study the interaction between β-catenin and NF-κB.

Results. In contrast to animal chondrocytes, in human chondrocytes Wnt/β-catenin is a potent inhibitor of MMP-1, MMP-3 and MMP-13 expression and generic MMP activity both in basal conditions and after IL-1β stimulation. This effect is independent of TCF/LEF transcription factors but is due to an inhibitory protein-protein interaction between β-catenin and NF-κB. Furthermore we show that IL-1β indirectly activates β-catenin signaling by inducing canonical Wnt-7B expression and by inhibiting the expression of canonical Wnt antagonists.

Conclusion. Our data reveal an unexpected anti-catabolic role of Wnt/β-catenin signaling in human chondrocytes by counteracting NF-κB-mediated MMP expression induced by IL-1β in a negative feedback loop.

Introduction

Osteoarthritis (OA) is the most common form of arthritis and a leading cause in mobility associated disability. OA affects the whole joint and is characterized by progressive degeneration of articular cartilage, mild signs of inflammation and typical bone changes (1, 2). Although all tissues in the joint are affected by the disease, it is believed that the articular chondrocyte is a major cellular mediator of OA pathogenesis through actively promoting cartilage matrix degradation by expressing matrix metalloproteinases (MMPs) and aggrecanases in response to adverse environmental signals by for example pro-inflammatory cytokines. Particularly, increased production of MMP-1, MMP-3 and MMP-13 by chondrocytes has been associated with cartilage degradation in OA (3-5). Pro-inflammatory cytokines like interleukin-1 (IL-1) are potent inducers of cartilage degradation by activating pro-catabolic NF-κB signaling in chondrocytes, which results in amongst others the expression of MMPs in cartilage (6-9).

A role for Wnt/β-catenin in OA is predominantly based on observations in animal models: i) in postnatal mouse models, conditional activation of β-catenin signaling in cartilage results in increased articular cartilage degeneration by stimulating endochondral ossification and other phenotypes resembling OA (10); ii) activation of Wnt/β-catenin signaling in rabbit and mouse chondrocytes stimulates the expression of cartilage matrix degrading MMPs (11, 12); iii) in a spontaneous guinea pig OA model, development of OA is associated with increased β-catenin expression in cartilage (11); and iv) pro-catabolic factors like IL-1 implicated in OA development induce expression of various Wnt proteins resulting in the activation of β-catenin (12, 13). These findings were subsequently corroborated by observation of increased nuclear β-catenin staining in human OA cartilage compared to control (10).

In addition, increased expression of the Wnt target gene (WISP1) was found in both mouse OA models and in human OA cartilage (14). Likewise, differential expression of various Wnt-related genes has been documented in human joint disorders (15-17). For example, canonical Wnt-1 and non-canonical Wnt-5A have been implicated in rheumatoid arthritis (RA) (15, 16) while the canonical Wnt-7B is upregulated in OA cartilage and RA synovium (17). Interestingly, inhibition of β-catenin signaling in articular chondrocytes also causes OA-like cartilage degradation in a Col2a1-ICAT transgenic mouse model (18). Taken

together, multiple lines of evidence have led to the hypothesis that low levels of Wnt/β-catenin signaling are required for maintenance of normal cartilage function and that deregulation of this pathway may contribute to the development and progression of OA. Consequently, the Wnt/β-catenin signaling pathway has been identified as a potential therapeutic target for intervention in OA.

Up to date, functional data on the role of Wnt/β-catenin signaling in human chondrocytes are however still scarce. In this study, we therefore systematically evaluated the role of canonical Wnt signaling in human chondrocytes in comparison with animal chondrocytes. Our study reveals an unexpected and remarkable species difference in regulation of MMP expression by Wnt/β-catenin signaling. In contrast to its pro-catabolic role in animal models, in human chondrocytes Wnt/β-catenin signaling potently inhibits MMP expression and can effectively counteract pro-catabolic NF-κB signaling activated by IL-1β in a negative feedback loop.

Materials and Methods

Human cartilage samples. Cartilage was obtained from 8 patients (age 62 ± 10 years) with osteoarthritis (OA) and one patient (age 67 years) with rheumatoid arthritis (RA) undergoing total knee replacement surgery. Knee cartilage was harvested from regions with no macroscopically evident degeneration.

Cell culture and cartilage explant culture. Primary human and bovine articular chondrocytes were isolated from cartilage of knee joints as described before (8). Human fetal chondrocytes were obtained as previously described (19). Passage 0 (P0) or passage 1 (P1) chondrocytes were used in all experiments. Human chondrocytes, bovine chondrocytes, HEK293T cells and mouse embryonic fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Human bone marrow-derived mesenchymal stem cells (MSCs) were isolated from aspirates as described previously (20) and cultured in α-minimum essential medium (α-MEM) with 10% FBS and penicillin-streptomycin. For cartilage explants culture, human cartilage was sliced into small cubes, and maintained in DMEM with 10% FBS and 1% penicillin-streptomycin. Mouse cartilage was isolated from femoral heads of 2-month-old C57B mice and maintained in DMEM with 10% FBS and 1%

penicillin-streptomycin. Normal human articular chondrocytes were derived from a donor (age 45 years) without joint disease (Lonza) and used as controls.

Recombinant proteins and reagents. Recombinant human Wnt-3A, IL-1β, DKK-1 and recombinant mouse Wnt-3A (R&D Systems) were used. The glycogen synthase kinase 3 (GSK-3) inhibitor BIO (6-bromoindirubin-3′-oxime) was obtained from Sigma-Aldrich.

RNA isolation and real-time quantitative polymerase chain reaction (qPCR). Total RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel). cDNA was synthesized from total RNA with the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR (qPCR) was performed with the MyiQ real-time PCR detection system (Bio-Rad) using the standard curve based method (21). GAPDH was used as internal control. The primer sequences are shown in Supplemental Table 1, available at http://www.utwente.nl/tnw/dbe/publications/2012/ma/.

Immunoprecipitation and Western blotting. Immunoprecipitation was performed using the Dynabeads Co-Immunoprecipitation kit (Invitrogen). Nuclear and cytoplasmic proteins were isolated using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific). Total cell proteins were collected in RIPA buffer (Cell Signaling) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). Antibodies used for Western blot and co-immunoprecipitation were anti-β-catenin (BD Biosciences), proMMP-1 and proMMP-13 (R&D Systems), TATA-binding protein (TBP) (Millipore), GAPDH (Sigma-Aldrich), NF-κB p50(C-19) and NF-κB p65(A) (Santa Cruz), FLAG (Origene), T cell factor 4 (TCF4; Millipore), lymphoid enhancer factor 1 (LEF1; Millipore).

MMP activity assay. Generic MMP activity in human chondrocytes and culture media was measured using the SensoLyte 520 generic MMP activity kit (AnaSpec). MMP activity was normalized for protein concentrations of total cell lysates measured using the Pierce BCA Protein Assay Kit (Thermo Scientific).

Plasmid constructs and viral transduction. Human Wnt-3 and Wnt-7B cDNAs (OriGene) were cloned in to a lentiviral vector pBOB (Plasmid 12335; Addgene) (22). shRNA sequences against human TCF4 and LEF1 were cloned into pLKO.1-TRC cloning vector (Plasmid 10878; Addgene) (23). pLKO.1 vectors containing a scrambled shRNA (Plasmid 18640; Addgene) (24) and an shRNA sequence against human β-catenin (Plasmid 18803; Addgene) (25) were used. Lentiviral vectors and packaging vectors were transfected into

HEK293T cells to produce lentiviruses. Lentiviruses were harvested and used to infect chondrocytes in the presence of 6 µg/ml polybrene (Sigma-Aldrich).

Small interfering RNA (siRNA) transfection. Mouse embryonic fibroblasts (MEFs) were transfected with ON-TARGETplus SMARTpool siRNA (Thermo Scientific) using the X-tremeGENE siRNA transfection reagent (Roche).

Reporter assay. Human chondrocytes were infected with Cignal lentiviruses containing the NF-κB responsive reporter or a TCF/LEF reporter (SA Biosciences) together with lentiviruses constitutively expressing *Renilla* luciferase (SA Biosciences) in the presence of 6 µg/ml polybrene (Sigma-Aldrich). Luciferase activity was measured using Dual-Glo luciferase assay kit (Promega). Activity of firefly luciferase was normalized for *Renilla* luciferase activity.

Statistical analysis. Data were expressed as the mean ± SD and analyzed by two-tailed student's *t*-tests or one-way ANOVA. *P* values less than 0.05 were considered statistically significant.

Results

Effect of canonical Wnt signaling on MMP messenger RNA (mRNA) expression in human chondrocytes. To investigate the effect of Wnt/β-catenin signaling on MMP expression, human articular chondrocytes isolated from knee joints of OA patients undergoing total knee replacement surgery were treated with recombinant human Wnt-3A or the GSK-3 inhibitor BIO. Both treatments activated canonical Wnt signaling in human chondrocytes as evidenced by an increase in total β-catenin protein levels, nuclear localization of β-catenin and activation of a canonical Wnt signaling responsive TCF/LEF reporter (Supplemental Fig. 1A-C). The specificity of Wnt-3A for activation of the canonical pathway was demonstrated by co-incubation with the extracellular antagonist for canonical Wnt signaling DKK-1, which blocked Wnt-3A-induced TCF/LEF reporter activity (Supplemental Fig. 1C). Furthermore, treatment with Wnt-3A stimulated chondrocyte proliferation, an established effect of canonical Wnt signaling in many cell types (Supplemental Fig. 1D) (26, 27).

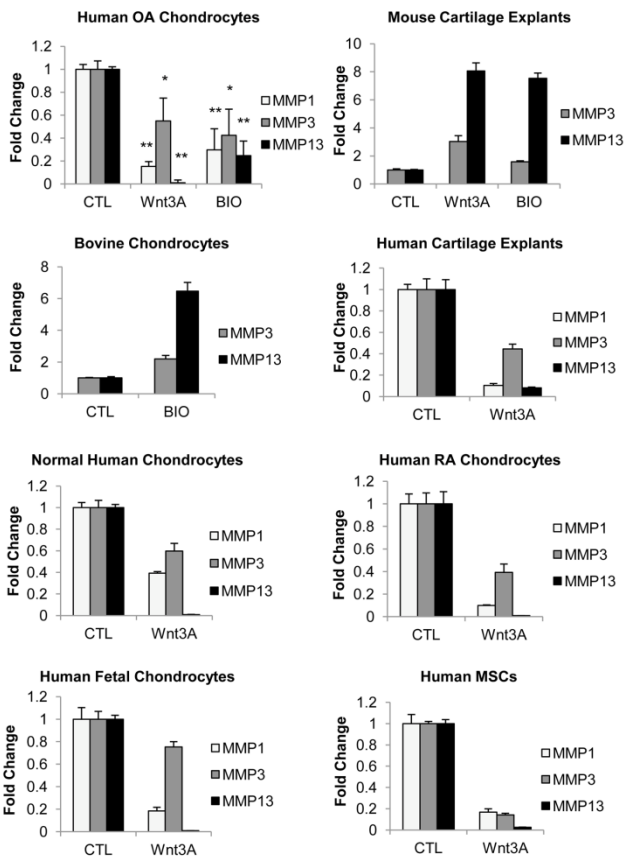


Figure 1. Effect of Wnt/β-catenin activation on MMP mRNA expression. Human articular chondrocytes from 4 paptients with OA were stimulated with 200 ng/ml recombinant human Wnt-3A or 1 µM BIO, or left untreated (control [CTL]) for 48 hours. Mouse cartilage explants (triplicate cultures) were treated with 200 ng/ml recombinant mouse Wnt-3A or 1 µM BIO for 5 days and bovine chondrocytes (triplicate cultures) were treated with 1 µM BIO for 48 hours. Human cartilage explants (triplicate cultures for 1 donor) were treated for 5 days and normal human articular chondocytes, human MSCs, human RA chondrocytes and human fetal chondrocytes (triplicate cultures for 1 donor) were treated for 48 hours with 200 ng/ml recombinant human Wnt-3A. mRNA expression was measured by qPCR. * = $P < 0.05$, ** = $P < 0.01$.

We next explored the effect of Wnt/β-catenin signaling on the expression of MMPs and chondrocyte markers. To our surprise activation of canonical Wnt signaling either by Wnt-3A or BIO significantly decreased the mRNA expression of MMP-1, MMP-3 and MMP-13 in human chondrocytes (Fig. 1). In agreement with a recent study, the expression of chondrocyte markers *COL2A1* and *SOX9* was decreased (Supplemental Fig. 1E) (27). We further explored the effect of Wnt-3A on the catabolic gene expression. The decrease in MMP mRNA expression by Wnt-3A was dose-dependent and gradual, first measurable 6 hours after the start of treatment with maximum inhibition after 72 hours (Supplemental Fig. 1F&G). The inhibition could be reversed by DKK-1, which also efficiently blocked Wnt-3A-induced expression of the established Wnt target gene *AXIN2* (Supplemental Fig. 1H&I).

Notably, blocking of endogenous canonical Wnt signaling by DKK-1 slightly, but significantly increased basal transcription levels of MMP-1 and MMP-13 mRNA (Supplemental Fig. 1H). Taken together, these data suggest that Wnt-3A represses MMP mRNA expression in human chondrocytes via a signaling cascade downstream of the Frizzled/LRP receptor complex and probably through β-catenin.

In sharp contrast, stimulation of mouse cartilage explants with recombinant mouse (rm) Wnt-3A or BIO induced MMP-3 and MMP-13 mRNA expression (Fig. 1), which is in accordance with previously reported data (12). Similarly stimulation of bovine chondrocytes with BIO induced MMP-3 and MMP-13 expression (Fig. 1). The species difference could not be explained by chondrocyte dedifferentiation, an established effect of culturing chondrocytes in monolayer, since direct treatment of human cartilage explants with Wnt3A also reduced MMP mRNA expression (Fig. 1). To exclude that the observed species difference in the regulation of MMP mRNA expression by canonical Wnt signaling in chondrocytes was due to an OA-induced change in cell response, the experiments were repeated using human chondrocytes from a donor without degenerative cartilage disease and from a donor with RA. Also in cells from these donors, Wnt3A inhibited MMP-1, MMP-3 and MMP-13 expression (Fig. 1). Similar results were obtained using human fetal chondrocytes excluding an age-related effect (Fig. 1). Finally, activation of canonical Wnt signaling in multipotent human mesenchymal stem cells (hMSCs) which were able to differentiate into chondrocytes also reduced MMP mRNA expression (Fig. 1), suggesting that a decrease in MMP-1, MMP-3 and MMP-13 mRNA expression upon activation of canonical Wnt signaling is a conserved response in various human cell types. In conclusion, these data point to a remarkable species difference in regulation of MMP mRNA expression by Wnt/β-catenin signaling in human and animal chondrocytes.

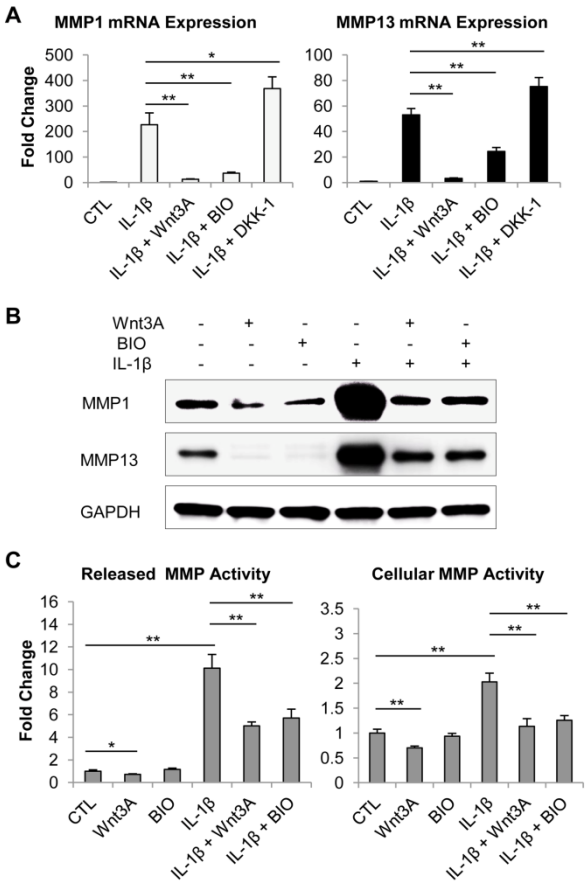


Figure 2. Wnt/β-catenin signaling reduces IL-1β-induced MMP expression and activity. A. Human chondrocytes were treated for 48 hours with 10 ng/ml IL-1β or IL-1β in combination with 200 ng/ml recombinant human Wnt-3A, 1 μM BIO or 300 ng/ml DKK-1. MMP-1 and MMP-13 mRNA expression was measured by qPCR. *p<0.05, **p<0.01, n = 3 donors each tested in triplicate. **B.** Human chondrocytes were treated for 48 hours with 200 ng/ml recombinant human Wnt-3A, 1 μM BIO and 10 ng/ml IL-1β and proteins were isolated from cell extracts. proMMP-1 and proMMP-13 were detected by immunoblotting. **C.** Culture media (left) and cell lysates (right) of human chondrocytes were collected for generic MMP activity measurements after treatment with 200 ng/ml recombinant human Wnt-3A, 1 μM BIO and 10 ng/ml IL-1β or combinations of these reagents for 72 hours. * = $P < 0.05$, ** = $P < 0.01$, n = 3 donors each tested in triplicate cultures.

Effect of Wnt/β-catenin signaling on IL-1β-induced MMP expression and activity in human chondrocytes. IL-1β is a potent activator of MMP expression in human chondrocytes and has been implicated in cartilage degradation in OA (6-9). To test the effect of canonical Wnt signaling on IL-1β-induced MMP-1 and MMP-13 mRNA expressions, human chondrocytes were co-stimulated with IL-1β and Wnt3A or BIO. As expected, IL-1β potently induced expression of MMP1 and -13 at the mRNA level and proMMP-1 and proMMP-13 at the protein level (Fig. 2A&B). Co-stimulation with Wnt3A or BIO blocked, while co-incubation with DKK-1 further increased IL-1β-induced MMP expression (Fig. 2A). Both Wnt3A and BIO inhibited proMMP-1 and proMMP-13 protein expression under basal conditions and after co-stimulation with IL-1β (Fig. 2B). The

inhibitory effect of Wnt-3A on MMP-1 and MMP-13 mRNA and protein expression was associated with a decrease in generic MMP activity secreted in the culture media and in cell extracts. This effect was observed in both basal conditions and after co-treatment with IL-1β (Fig. 2C). BIO did not decrease generic MMP activity in basal conditions but inhibited IL-1β-induced MMP activity. These data suggest that activation of Wnt/β-catenin signaling in human chondrocytes negatively regulates the expression and activity of a set of MMP family members in both basal conditions and after stimulation by IL-1β.

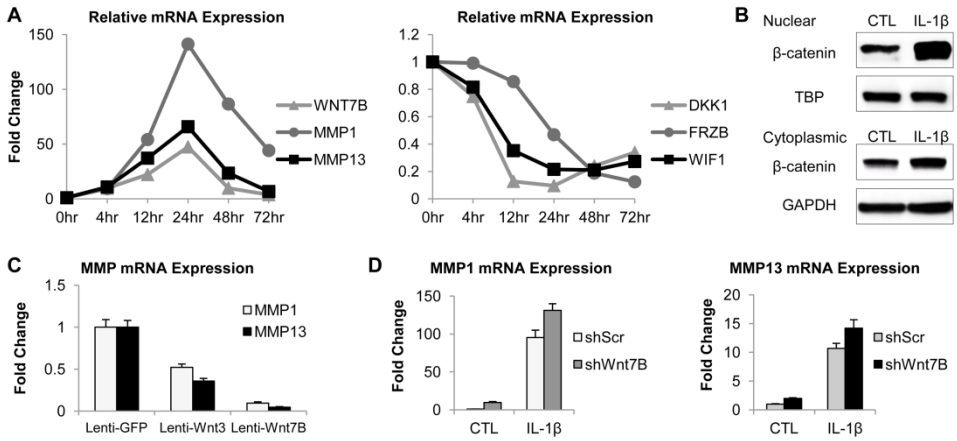


Figure 3. Crosstalk of Wnt/β-catenin and IL-1β pathways. A. Time course evaluation of mRNA expression after stimulation of human chondrocytes treated with 10 ng/ml IL-1β. mRNA expression of Wnt-7B, MMP-1 and MMP-13 and the Wnt antagonists DKK-1, FRZB and WIF-1 was evaluated at indicated time points by qPCR. Data represent the mean fold change in cells from 3 donors relative to untreated cells, which was set at 1. Each experiment was performed in triplicate. Not statistically significant (4hr: FRZB; 12hr: FRZB), At 4 hours, FRZB not significantly changed, $P < 0.05$ for MMP-1, MMP-13, DKK-1, and WIF-1 versus untreated cells,; at 72 hours, FRZB not significantly changed, $P < 0.05$ for Wnt-7B and MMP-13 versus untreated cells; for comparisons at all other time points, $P < 0.01$. **B.** Human chondrocytes were stimulated with 20 ng/ml IL-1β for 48 hours and nuclear and cytoplasmic β-catenin protein expression was detected by immunoblot. **C.** Human Wnt-3 and Wnt-7B were overexpressed using lentiviral transduction in human chondrocytes and MMP mRNA expression was measured by qPCR. Data are expressed using lentiviral GFP-transduced cells as control (Lenti-GFP). **D.** Wnt-7B was knocked down by lentivirus-mediated expression of shRNA in human chondrocytes using scrambled shRNA (shScr) as control. MMP-1 and MMP-13 mRNA expression was measured 24 hours after stimulation with 10 ng/ml IL-1β and compared to control (CTL). n = triplicate cultures for 1 donor (C&D).

Crosstalk of Wnt/β-catenin and IL-1β signaling pathways in human chondrocytes. The potentiating effect of DKK-1 on MMP-1 and MMP-13 expression in human chondrocytes in both basal and IL-1β stimulated conditions suggested the presence of endogenous canonical Wnt family members in human chondrocytes repressing MMP expression. Using a qPCR survey, we identified relatively abundant mRNA expression of the non-canonical Wnt-5A and lower expression levels of the non-canonical Wnt-4, Wnt-5B, Wnt-9A and the canonical Wnt-7B (Supplemental Table 2). Wnt-7B was the only canonical Wnt upregulated by IL-1β. The mRNA expression of Wnt-7B peaked at 24hrs after IL-1β stimulation, which coincided with the peak in MMP-1 and MMP-13 mRNA expression and after which both MMP-1 and MMP-13 mRNA and Wnt-7B mRNA started to decrease. Interestingly, IL-1β treatment simultaneously downregulated mRNA expression of several canonical Wnt signaling inhibitors including DKK-1, Frizzled-related protein (FRZB), and Wnt inhibitory factor 1 WIF-1 (Fig. 3A). DKK-1 and WIF-1 mRNA expression started to decrease from 4 hours after stimulation and reached the lowest expression levels at 24 hours (Fig. 3A). The decrease in FRZB mRNA expression was more gradual (Fig. 3A). As a consequence of the opposite effects on the expression of Wnt-7B and its antagonists, β-catenin protein accumulated in both nuclear and cytoplasmic compartments upon stimulation of human chondrocytes with IL-1β (Fig. 3B).

Lentiviral overexpressoin of Wnt-7B as well as Wnt-3, another canonical Wnt not detected in the qPCR survey as control, inhibited MMP-1 and MMP-13 mRNA expression and this inhibition was similar to that observed following stimulation with Wnt-3A in human chondrocytes (Fig. 3C and Supplemental Fig. 2A). Wnt-7B and Wnt-3 stimulated the expression of the canonical Wnt target gene *AXIN2* (Supplemental Fig. 2B). Knockdown of Wnt-7B in human chondrocytes increased MMP-1 and MMP-13 mRNA expressions in both basal condition and after stimulation with IL-1β (Fig. 3D and Supplemental Fig. 2C). Taken together, these data suggest that Wnt-7B is a likely candidate for the endogenous canonical Wnt negatively regulating MMP expression in human chondrocytes. Furthermore, these data point to a role of canonical Wnt signaling as a negative feedback loop controlling MMP expression downstream of IL-1β receptor activation.

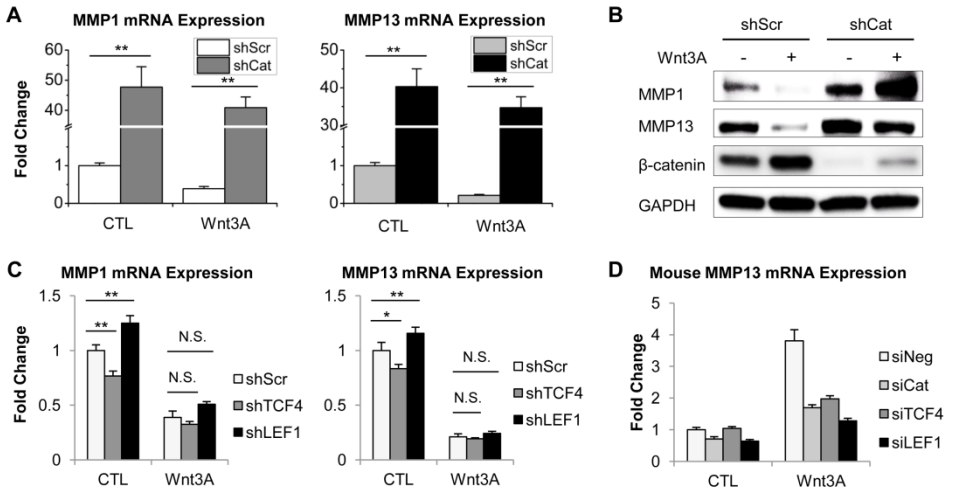


Figure 4. Effects of β-catenin, TCF4 and LEF1 knockdown. **A-C.** Human chondrocytes were transduced with lentiviruses expressing scrambled shRNA (shScr) and shRNA against human β-catenin (shCat) (A&B), TCF4 (shTCF4) and LEF1 (shLEF1) (C) and treated with 50 ng/ml recombinant human Wnt-3A for 24 hours. MMP mRNA expression was measured by qPCR and expressed as fold change relative to control (CTL) (A&C) and proteins were detected by immunoblotting (B). * = $P < 0.05$, ** = $P < 0.01$, N.S. = Not Significant, n = 3 donors each tested in triplicate cultures. **D.** MEFs were transfected with negative control siRNA (siNeg) and siRNA against β-catenin (siCat), TCF4 (siTCF4) and LEF1 (siLEF1), and then treated with or without 200 ng/ml recombinant mouse Wnt-3A for 48 hours. MMP-13 mRNA expression was measured by qPCR. n = triplicate cultures.

No involvement of TCF4 and LEF1 in Wnt-mediated repression of MMP expression in human chondrocytes. Since Wnt/β-catenin showed an inhibitory effect on MMP expression, we next tested if knockdown of β-catenin by lentiviral shRNA expression could restore MMP expression levels. The knockdown efficiently decreased β-catenin expression (Supplemental Fig. 2D) and strongly elevated MMP expression levels (Fig. 4A&B). The inhibitory effect of Wnt-3A was almost completely eliminated by β-catenin knockdown (Fig. 4A&B).

Previous studies suggested that the transcription factors TCF4 and LEF1 acting together with β-catenin might act as transcriptional repressors of target gene expression (28-30). We therefore examined the roles of TCF4 and LEF1 in regulation of MMP expression. TCF4 and LEF1 were effectively depleted in human chondrocytes by lentiviral shRNA-mediated knockdown (Supplemental Fig. 2E-G). The knockdown of both genes showed minimal effects on MMP expression (Fig. 4C), suggesting that the repression of MMP expression in

human chondrocytes is not mediated by the conventional complex of β-catenin and TCF/LEF opening the possibility that MMP repression is mediated by β-catenin independently of its DNA binding partners, for example by crosstalk with other pathways involved in MMP expression. In contrast, β-catenin, TCF4 and LEF1 were required for Wnt3A-induced MMP expression in mouse embryonic fibroblasts (MEFs) as knockdown of all three genes eliminated the pro-catabolic effect of Wnt-3A on MMP-13 expression (Fig. 4D and Supplemental Fig. 2H-K) suggesting the involvement of the β-catenin-TCF/LEF complex in regulation of MMP expression in mouse cells (31).

Suppression of MMP expression through an inhibitory interaction of β-catenin and NF-κB. It has been shown that IL-1β-induced expression of MMP-1 and MMP-13 in human chondrocytes is dependent on NF-κB activation (7-9). In addition, an interaction between β-catenin and NF-κB has been recently described in various human cell types (32-34). We therefore tested whether an interaction between NF-κB and β-catenin might explain β-catenin-mediated inhibition of MMP expression in human chondrocytes. In agreement with previous studies, knockdown of NF-κB p65/RELA abrogated the expression of basal and IL-1β-induced MMP-1 and MMP-13 mRNA expression, while knockdown of NF-κB p50, the active form of NFKB1, only diminished expression of MMP-13 while MMP-1 mRNA expression was increased (Fig. 5A and Supplemental Fig. 3A&B) (7-9). This increase in MMP1 mRNA was not reflected in an increase in MMP1 protein expression, which is most likely explained by a lag phase between an increase in mRNA expression and protein expression (Fig. 5A). MMP-3 expression was regulated by both p50 and p65 (Supplemental Fig. 3C). We next examined the effect of Wnt-3A on NF-κB activity, using lentiviral transduction of a NF-κB responsive promoter reporter construct in human chondrocytes. Treatment of Wnt-3A significantly decreased basal and IL-1β-induced NF-κB reporter activity (Fig. 5B). This effect was dependent on β-catenin, since knockdown of β-catenin abolished a Wnt-3A-induced decrease in NF-κB promoter reporter activity and enhanced IL-1β-induced reporter activation (Fig. 5B). Using a co-immunoprecipitation assay, we found that β-catenin was able to form a protein complex with NF-κB p65 in human chondrocytes under basal condition (Fig. 5C). The interaction between β-catenin and NF-

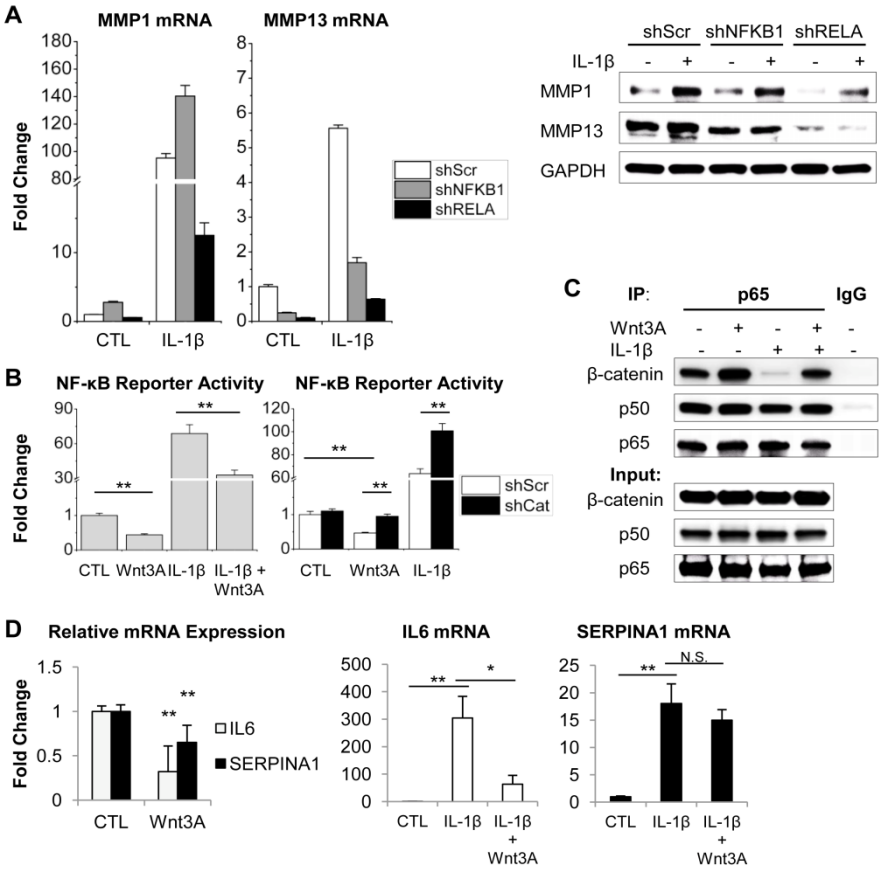


Figure 5. β-Catenin suppresses MMP expression through an inhibitory interaction with NF-κB. A. MMP mRNA was assessed by qPCR and protein expression was detected by immunoblotting in human chondrocytes transduced with lentiviruses expressing scrambled shRNA (shScr) and shRNA against *NFKB1* (shNFKB1) and *RELA* (shRELA) after treatment with 10 ng/ml IL-1β for 24 hours. qPCR data are expressed as fold change using untreated shScr-transduced cells as control. n = triplicate cultures for 1 donor. **B.** Human chondrocytes were transduced with lentiviruses expressing an NF-κB reporter and *Renilla* lucifease only or together with lentiviruses expressing scrambled shRNA and shRNA against β-catenin. Chondrocytes were treated with 100 ng/ml recombinant human Wnt-3A, 5 ng/ml IL-1β or both for 24 hours and luciferase reporter activity was measured. Data are expressed as fold change compared to control (CTL). ** = $P < 0.01$, n = 3 donors each tested in triplicate. **C.** Protein lysates of human chondrocytes stimulated with 100 ng/ml recombinant Wnt-3A, 5 ng/ml IL-1β or both for 48 hours were isolated and subjected to immunoprecitation using NF-κB p65 antibody as bait. Co-immunoprecipitated proteins were detected by immunoblotting. **D.** Human chondrocytes were treated with 200 ng/ml recombinant human Wnt-3A, 10 ng/ml IL-1β or both for 48 hours. *IL6* and *SERPINA1* mRNA expression was measured by qPCR. * = $P < 0.05$, ** = $P < 0.01$, N.S. = Not Significant, n = 4 donors each tested in triplicate cultures.

κB p65 was weakened upon activation of NF-κB by IL-1β and strengthened after Wnt3A induced stabilization of β-catenin. Our data suggest that activation of NF-κB signaling by IL-1β in human chondrocytes may require dissociation of β-catenin from the NF-κB signaling complex. Co-immunoprecipitation of β-catenin with NF-κB p65 was also found in bovine chondrocytes and mouse embryonic fibroblasts (Supplemental Fig. 4A). Wnt-3A was able to decrease basal and IL-1β-induced NF-κB activity in MEFs (Supplemental Fig. 4B), suggesting that the interaction between β-catenin and NF-κB alone may not explain the species differentiation in the regulation of MMP expression by Wnt/β-catenin signaling.

We next tested whether canonical Wnt signaling could downregulate other NF-κB target genes. We tested this hypothesis by evaluating the effect of Wnt3A treatment on expression of *IL6* and *SERPINA1*, two established target genes of the NF-κB pathway in human chondrocytes (Supplemental Fig. 3C). The mRNA expression of both genes was upregulated by IL-1β but downregulated by stimulation with Wnt3A (Fig. 5D). Wnt3A treatment was also able to decrease IL-1β-induced IL-6 mRNA expression but failed to significantly counteract the effect of IL-1β on SERPINA1 mRNA expression (Fig. 5D). These results suggest that Wnt/β-catenin signaling negatively regulates a subset of NF-κB target genes most likely via an inhibitory protein-protein interaction with NF-κB.

Discussion

Cumulating evidence, mainly based on experimental animal models for OA, has suggested an important role for Wnt/β-catenin signaling in the pathogenesis of OA by driving, amongst others, hypertrophic differentiation of chondrocytes and the expression of matrix degrading MMPs in articular cartilage. Here we propose that this hypothesis requires revisiting in human osteoarthritis. In remarkable contrast to animal models, we provide evidence that in human chondrocytes Wnt/β-catenin signaling is a potent inhibitor of MMP-1, MMP-3 and MMP-13 mRNA and protein expression and is part of a negative feedback loop counteracting pro-catabolic NF-κB signaling in human chondrocytes activated by pro-inflammatory cytokines. Thus in human articular cartilage β-catenin may have an anti-catabolic role by inhibiting MMP expression opposed to its established pro-catabolic role in animal cartilage. Similar observations were made in

human chondrocytes from OA, RA and healthy adult donors as well as in human fetal chondrocytes and adult bone marrow-derived MSCs, indicating that the inhibitory response of Wnt/β-catenin on MMP expression is conserved across various human mesenchymal cell types, irrespective of age and disease status.

It has been shown that IL-1β induces Wnt-7A expression and β-catenin accumulation in rabbit chondrocytes (13). We did not detect significant Wnt-7A expression in human chondrocytes. However, we have found that IL-1β increased Wnt-7B expression and decreased expression of several Wnt inhibitors in human chondrocytes. Together, the concerted action of increase in Wnt-7B expression and decrease in Wnt inhibitor expression may be responsible for the indirect increase in β-catenin protein levels after IL-1β stimulation. Differential expression of Wnt proteins has previously been shown in joint diseases. In particular Wnt-7B was found significantly upregulated in OA cartilage (17). We showed that overexpression of Wnt-7B repressed MMP expression while knockdown of Wnt-7B enhanced MMP expression. This implies that upregulation of Wnt7B as observed in OA cartilage may be considered as an attempt of human chondrocytes to reduce the expression of MMPs to slow down matrix degradation. Moreover, we provide evidence that Wnt-7B might be a driving factor in an anti-catabolic negative feedback loop induced by pro-catabolic IL-1 signaling, although we cannot exclude roles for other canonical Wnts. In addition, IL-1β-induced loss of Wnt inhibitor expression is another import mechanism for negative feedback on MMP expression as DKK-1 enhances both basal and IL-1β-induced MMP expression. These inhibitors may act independently of the change in Wnt-7B, or, based on time course experiments showing that the IL-1β-induced increase in Wnt-7B expression coincides with a decrease in the expression of Wnt antagonists, may potentiate the effect of increased Wnt7B expression.

It has been shown that the interaction of β-catenin with TCF/LEF transcription factors can either stimulate or repress transcription in a promoter and context dependent manner (28-30). In mouse chondrocytes β-catenin and TCF/LEF transcription factors are required for induction of MMP-13 expression (31). Indeed knockdown of either TCF4 or LEF1 abrogates Wnt3A-induced MMP expression in mouse embryonic fibroblasts. The observation that knockdown of TCF4 or LEF1 did not relieve the β-catenin-mediated repression of MMP expression in human chondrocytes, clearly demonstrates that the

canonical transcriptional function of β-catenin is not involved in inhibiting MMP expression in these cells. These data in mouse and human cells pinpoint the species specific regulation of MMP expression to a differential role of TCF/LEF transcription factors in MMP expression regulation. Why humans in evolution have lost the propensity to regulate MMP-3 and MMP-13 expression by the β-catenin-TCF/LEF transcription complex remains elusive.

Previous studies have shown that β-catenin inhibits NF-κB-mediated signaling in human cancer cells by a protein-protein interaction between β-catenin and NF-κB (32- 34). In human chondrocytes, it is well known that activation of NF-κB signaling by pro-inflammatory cytokines like IL-1β potently induces cartilage matrix degradation by stimulating the expression and activity of various MMPs. We therefore tested whether crosstalk between β-catenin and NF-κB could explain the inhibitory effects of canonical Wnt signaling on MMP expression in human chondrocytes. We extend the observations made in cancer cells to human chondrocytes and show that activated β-catenin signaling can inhibit a number of NF-κB target genes. We furthermore show that activation of NF-κB weakens the protein-protein interaction while stabilization of β-catenin strengthens the interaction between NF-κB and β-catenin. These data strongly suggest that the TCF/LEF-independent repressive effect of β-catenin on MMP expression is mediated by negative cross-regulation of the NF-κB signaling pathway. Remarkably, co-immunoprecipitation assays using mouse embryonic fibroblasts and bovine chondrocytes demonstrate the presence of a protein complex between NF-κB and β-catenin in these cells. In addition, we showed that Wnt3A was able to decrease NF-κB reporter activity in mouse cells. These data suggest that mouse β-catenin was able to inhibit NF-κB-mediated signaling and that this interaction cannot explain the species difference in regulation of MMP expression by canonical Wnt signaling. Our data suggest that in animal cells the canonical Wnt/β-catenin signaling pathway via TCF/LEF prevails over the inhibitory effects on NF-κB signaling in regulation of MMP-3 and MMP-13 expressions in remarkable contrast to human cells in which the non-canonical effect on NF-κB is dominant.

There has been longstanding debate whether animal models are suited for studying the pathogenesis of OA and it is believed that none of the current models can recapitulate all facets of human disease. Our observation of differential regulation of MMP expression by

Wnt/β-catenin signaling may explain part of this discrepancy. Although evolutionarily conserved, our data indicate that the Wnt/β-catenin pathway is able to activate distinct subsets of target genes in a species-dependent manner which is at least partly explained by differential usage of canonical and non-canonical β-catenin signaling. We propose that differential usage of crosstalk of signaling networks may be responsible for species differences in cellular responses to Wnt and other extracellular signaling pathways (26, 35). Our findings suggest that animal models may not be suited for studying the role of Wnt/β-catenin signaling in human OA and questions whether strategies aimed at inhibiting β-catenin in chondrocytes will be successful in the management of OA.

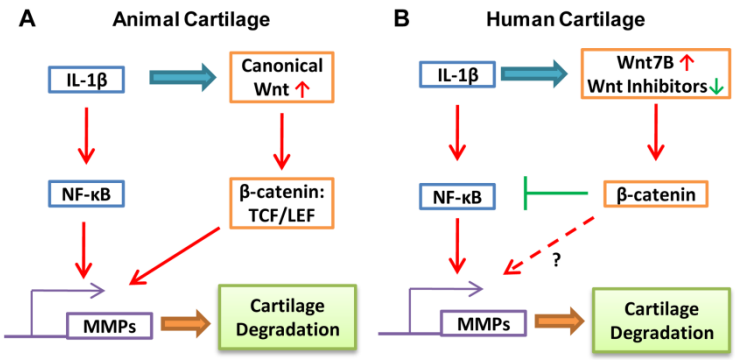


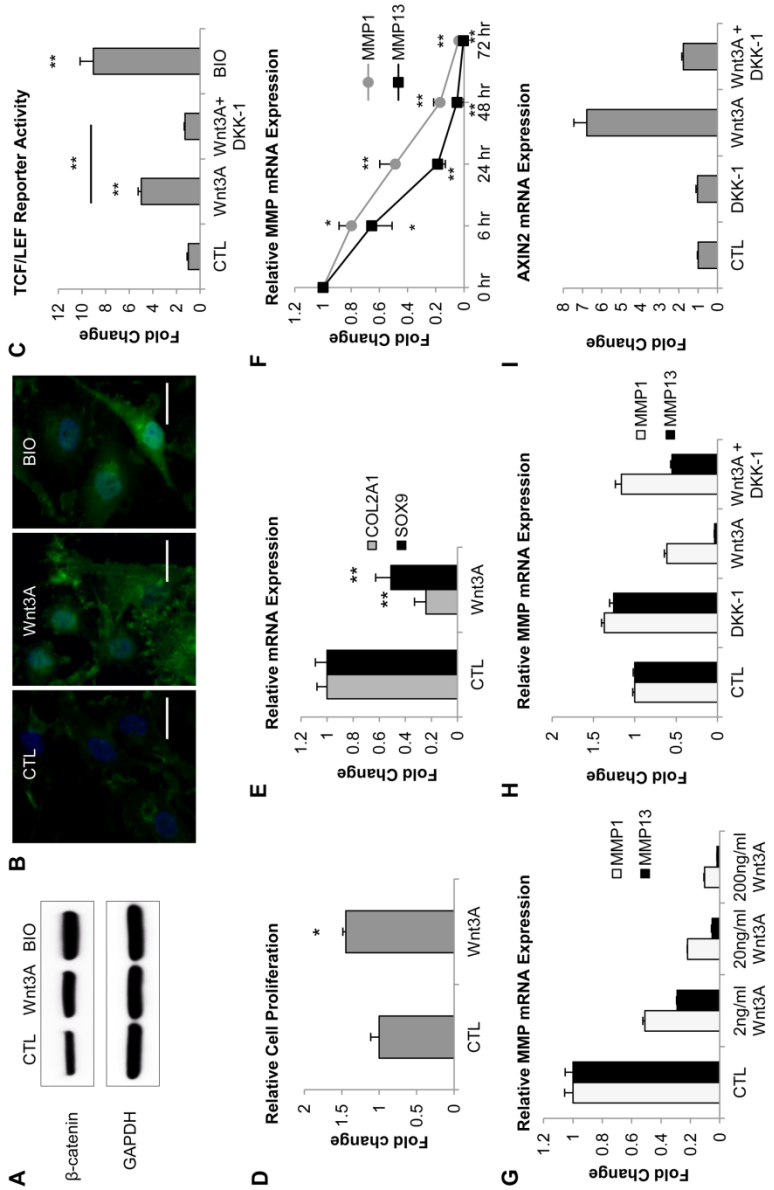
Figure 6. A Wnt/β-catenin negative feedback loop inhibits IL-1β-induced MMP expression in human chondrocytes. A. In animal cartilage, IL-1β indirectly activates canonical Wnt signaling by upregulation of Wnt ligands. Subsequently, the transcription complex of β-catenin with TCF/LEF induces the expression of MMP-3 and MMP-13 leading to cartilage destruction. **B.** In human articular cartilage, IL-1β upregulates MMP-1, MMP-3 and MMP-13 expression predominantly through activation of the NF-κB pathway resulting in cartilage degradation. Simultaneously IL-1β indirectly activates β-catenin through upregulation of Wnt-7B expression and downregulation of canonical Wnt inhibitors like DKK-1, FRZB and WIF-1. Stabilized β-catenin interacts with and inhibits NF-κB which leads to the suppression of MMP expression in a negative feedback loop. It remains unclear whether the β-catenin pathway is directly involved in human cartilage degeneration.

Recently, it has been shown that Wnt-3A can modulate the human articular chondrocyte phenotype by activating both β-catenin-dependent and -independent pathways (27). In line with our study, Nalesso et al. showed that chondrocyte proliferation and inhibition of MMP-13 expression is β-catenin-dependent. In contrast, effects of Wnt-3A on the expression of chondrocyte markers are β-catenin-independent. These data are

supported by the observation that the non-canonical Wnt-5A potently inhibits *COL2A1* expression and induces MMP expression (36, 37). Taken together, these and our data suggest prominent roles for both canonical and non-canonical Wnt signaling in articular chondrocytes though the canonical pathway might not be a key event in the pathogenesis of human OA.

In summary, our study unravels an unexpected and novel role of Wnt/β-catenin signaling in human articular chondrocytes with respect of the expression of MMPs (Fig. 6). During OA development, β-catenin signaling is upregulated in human cartilage. This is at least partly due to increased Wnt-7B expression and/or downregulation of Wnt inhibitors and can be further augmented by the activity of pro-inflammatory cytokines like IL-1β. Accumulated β-catenin in turn represses NF-κB activity and consequently expression of MMPs in human chondrocytes. Thus Wnt/β-catenin signaling is part of a negative feedback loop counteracting pro-catabolic NF-κB in osteoarthritis.

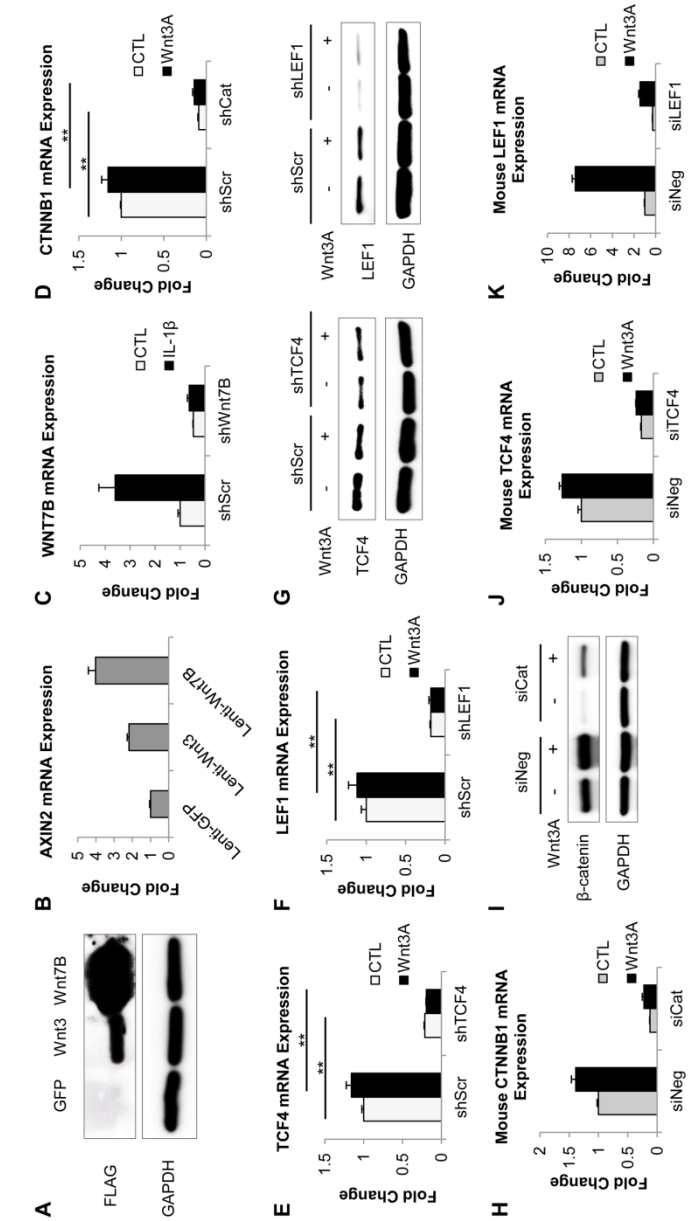
Supplemental Data



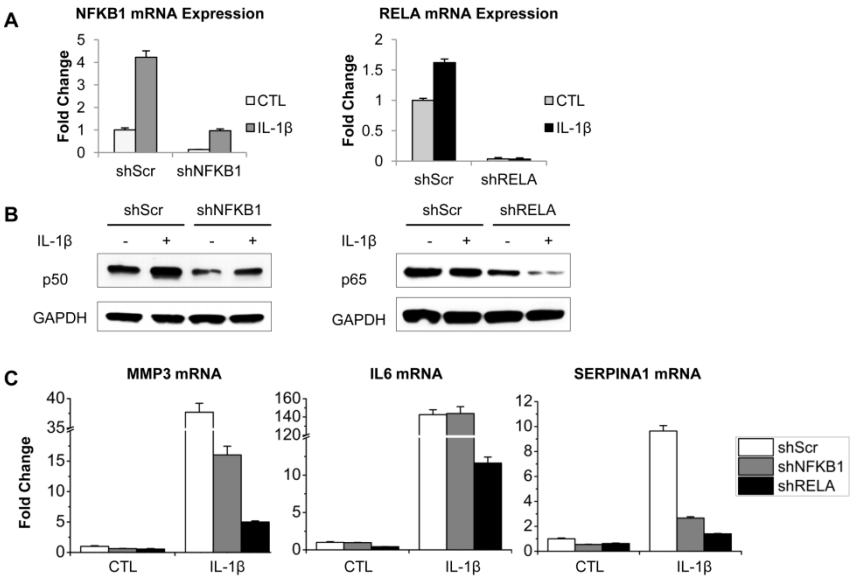
Supplemental Figure 1

Supplemental Figure 1. Effects of Wnt/β-catenin activation in human chondrocytes. A&B. Human chondrocytes were treated with 200 ng/ml recombinant human (rh) Wnt-3A or 1 μM BIO, or left untreated (control [CTL]) for 48 hours and protein expression of β-catenin was detected by immnunoblotting (A) or immunofluorescence (green: β-catenin, blue: nuclei, scale bar = 5 μm) (B). Anti-β-catenin antibody and Alexa Fluor 546 goat anti-mouse antibody were used to stain β-catenin and cell nuclei were stained by DAPI (B). **C.** To measure TCF/LEF reporter activity, human chondrocytes were infected with a lentiviral TCF/LEF reporter construct and stimulated with 100 ng/ml rhWnt-3A, 1 μM BIO or a combination of 100 ng/ml rhWnt-3A and 300 ng/ml DKK-1 for 48 hours and luciferase activity was measured. Data are expressed as fold change compared to CTL. ** = $P < 0.01$, n = 3 donors each measured in triplicate. **D.** Proliferation of human chondrocytes was measured using a BrdU proliferation assay (Roche) according to the manufacturer's instructions in the presence or absence (CTL) of 200 ng/ml rhWnt-3A for 48 hours. Data are expressed as fold change relative to control. * = $P < 0.05$, n = 3 donors each measured in triplicate. **E.** Human chondrocytes were stimulated with 200 ng/ml rhWnt-3A for 48 hours. *COL2A1* and *SOX9* mRNA expression was measured by qPCR. ** = $P < 0.01$, n = 4 donors each measured in triplicate. **F.** Human chondrocytes were stimulated with 200 ng/ml rhWnt-3A for indicated time points and MMP mRNA expression was measured by qPCR. * = $P < 0.05$, ** = $P < 0.01$, n = 3 donors each measured in triplicate. **G.** Human chondrocytes were treated with increasing doses of Wnt-3A for 48 hours and MMP mRNA expression was measured by qPCR. n = triplicate cultures for 1 donor. **H&I.** Human chondrocytes were stimulated with 300 ng/ml DKK-1, 100 ng/ml rhWnt-3A or the combination of both for 24 hours and MMP mRNA (H) or Axin2 mRNA expression (I) was measured by qPCR. n = triplicate cultures for 1 donor.

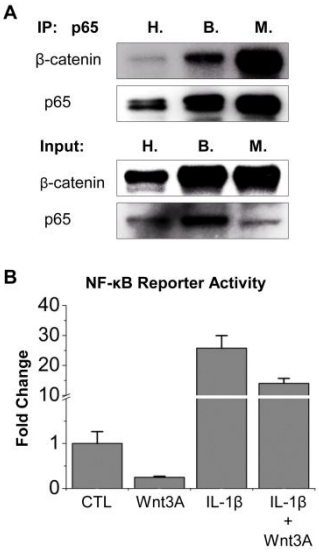
Supplemental Figure 2. Efficiency of gene overexpression and knockdown in human chondrocytes and MEFs. A&B. Lentivirus-mediated overexpression of FLAG-tagged Wnt-3 or Wnt-7B in human chondrocytes was validated by immunoblotting (A) and by upregulation of the established target gene of canonical Wnt signaling *AXIN2* meassured by qPCR, n = triplicate cultures for 1 donor (B). **C-G.** Efficiency of lentiviral shRNA-mediated knockdown was measured by qPCR after knockdown of Wnt-7B (shWnt7B) (C), β-catenin (shCat) (D), TCF4 (shTCF4) (E) and LEF1 (shLEF1) (F) in human chondrocytes treated with 10 ng/ml IL-1β or 50 ng/ml rhWnt-3A for 24 hours after lentiviral infection. Data are expressed as fold change compared to the scrambled (shScr) control. n = triplicate cultures from one donor (C). ** = $P < 0.01$, n = 3 donors each measured in triplicate (D-F). Proteins levels of TCF4 and LEF1 were measured after knockdown and treatment by immunoblotting (G). **H-K.** MEFs were transfected with siRNA and then stimulated with 200 ng/ml recombinant mouse Wnt-3A for 48 hours. β-Catenin mRNA (H) and protein expression (I) and TCF4 mRNA (J) and LEF1 mRNA (K) expression were detected by qPCR or immunoblotting. Data are expressed as fold change compared to the negative control (siNeg) (H, J, K). n = triplicate cultures.



Supplemental Figure 2



Supplemental Figure 3. Efficiency and effects of NF-κB knockdown in human chondrocytes. Human chondrocytes were infected with lentiviruses expressing shRNA against *NFKB1* (shNFKB1) and *RELA* (shRELA) and stimulated with 10 ng/ml IL-1β for 24 hours. Efficiency of lentiviral shRNA-mediated mRNA knockdown was measured at the mRNA level by qPCR for *NFKB1* and *RELA* or at the protein level by immunoblotting (B). (C) mRNA expression of MMP-3, IL-6 and SERPINA1 was measured by qPCR in human chondrocytes infected with lenviruses expressing shRNA against NFKB1 and RELA. Data are expressed as fold change compared to control. n = triplicate cultures for 1 donor.



Supplemental Figure 4. Interaction of β-catenin with NF-κB. A. Proteins from human chondrocytes (H.), bovine chondrocytes (B.) and mouse embryonic fibroblasts (M.) were immunoprecitated with NF-κB p65 antibody and subjected to immunoblotting to detect co-precipitated proteins. **B.** MEFs were treated with 200 ng/ml recombinant mouse Wnt-3A, 10 ng/ml IL-1β or both for 24 hours after transduction of lentiviral NF-κB reporter and *Renilla* control. n = triplicate cultures.

Gene	ΔCt	Expression
WNT1	23.65 ± 2.17	-
WNT3	22.05 ± 1.08	-
WNT3A	22.18 ± 2.03	-
WNT4	18.62 ± 1.90	+
WNT5A	8.31 ± 1.28	++
WNT5B	18.82 ± 1.32	+
WNT7A	28.00 ± 2.19	-
WNT7B	16.12 ± 1.34	+
WNT9A	16.65 ± 1.27	+
WNT10A	29.04 ± 2.29	-
WNT10B	24.29 ± 1.82	-
WNT11	23.03 ± 1.37	-

Supplemental Table 2. Expression analysis of Wnt members in human chondrocytes. mRNA expression levels of Wnt members were detected by real-time PCR. Data are expressed as mean ΔCt in comparison to GAPDH Ct as internal control ± SD of 4 donors. Ct, threshold of cycle. Wnt gene expression was considered present in chondrocytes if ΔCt < 20 (+). Wnt gene expression was considered strong when ΔCt was less than 10 (++). If ΔCt >20 Wnt gene expression was considered absent (-).

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Chapter 5

TCF4 is a Pro-catabolic and Apoptotic Factor in Human Articular Chondrocytes by Potentiating NF-κB Signaling

TCF4 is a pro-catabolic and apoptotic factor in human articular chondrocytes by potentiating NF-κB signaling

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Abstract

TCF/LEF transcription factors are downstream mediators of Wnt/β-catenin signaling which has been implicated in the development and progression of osteoarthritis (OA). This study aimed to investigate the role of TCF/LEF transcription factors in human articular chondrocytes. Primary human osteoarthritic cartilage predominantly expressed TCF4 and to a lesser extent, LEF1 and TCF3 mRNA. Overexpression of TCF4, but not of TCF3 or LEF1, induced matrix metalloproteinase (MMP)-1, MMP-3, and MMP-13 expression and generic MMP activity in human chondrocytes This was due to potentiating NF-κB signaling by a protein-protein interaction between TCF4 and NF-κB p65 activating established NF-κB target genes such as MMPs and IL6. LEF1 competed with TCF4 for binding to NF-κB p65. Finally we showed that TCF4 mRNA expression was elevated in OA cartilage compared to healthy cartilage and induced chondrocyte apoptosis at least partly through activating caspase 3/7. Our findings suggest that increased TCF4 expression may contribute to cartilage degeneration in OA by augmenting NF-κB signaling.

Introduction

Canonical Wnt signaling is a conserved signaling pathway implicated in many aspects of development and disease (1, 2). In the absence of Wnt, a destruction complex mediates the phosphorylation of β-catenin by GSK-3β, which induces degradation of cytosolic β-catenin through the proteasome. Binding of Wnt to its receptors results in disruption of the destruction complex and accumulation of cytoplasmic β-catenin. Upon nuclear translocation, β-catenin will function as a co-factor of TCF/LEF transcription factors to switch on Wnt target gene transcription (3). Mammals have four TCF/LEF family members: TCF1, TCF3, TCF4 and LEF1 (4). Each member is produced as a group of isoforms through alternative splicing and promoter usage. The N-terminal β-catenin-binding domain of all four TCF/LEF members is highly conserved and responsible for the binding of β-catenin. The context-dependent regulatory domain (CRD) and C-terminal tails are varied among all four members, resulting in different binding properties.

Cumulating studies mainly based on experimental animal models for OA, have suggested an important pro-catabolic role for Wnt/β-catenin signaling in the pathogenesis of OA by stimulating, amongst others, hypertrophic differentiation of chondrocytes and the expression of matrix degrading MMPs in articular cartilage (5, 6). Indeed, in animal chondrocytes it has been demonstrated that MMPs are direct β-catenin/TCF target genes and that IL-1β-induced MMP expression might indirectly involve canonical Wnt signaling (7, 8). In marked contrast, we have recently shown that in human chondrocytes IL-1β-induced Wnt/β-catenin signaling is part of a negative feedback loop inhibiting NF-κB-mediated MMP expression. In human cells β-catenin inhibits NF-κB due to a negative protein-protein interaction with p65 (9). Furthermore, also in human chondrocytes the non-canonical Wnt pathway repressed the expression of cartilage-specific extracellular matrix (ECM) molecules and might be involved in chondrocyte dedifferentiation during in vitro expansion of primary chondrocytes (9, 10). At present and in marked contrast to animal models a direct role of the canonical Wnt pathway in cartilage degeneration in human has not been identified. These findings suggest that research should be focused on human cartilage and/or human chondrocytes instead of animal models for better understanding of the role of the Wnt signaling pathway in human cartilage disease.

In this study, we have focused on the role of TCF/LEF transcription factors, the downstream targets of Wnt/β-catenin signaling in human chondrocytes. We demonstrate that TCF4 is a pro-catabolic factor by potentiating NF-κB signaling.

Materials and Methods

Human cartilage samples. The collection and use of human cartilage was approved by a local medical ethical committee. Cartilage was obtained from 8 patients (63 ± 10 years) with OA undergoing total knee replacement surgery. Knee cartilage was harvested from regions with no macroscopically evident degeneration. Healthy articular cartilage was obtained form 3 donors (66 ± 14 years) without joint diseases post mortem.

Human chondrocyte isolation and cell culture. Human articular chondrocytes were isolated from cartilage as previously described (10). Human chondrocytes and HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Plasmid constructs and viral transduction. Adenoviruses expressing null control, GFP, human TCF4 and LEF1 (Vector Biolabs) were used to infect human chondrocytes at an MOI (multiplicity of infection) of 100. Human TCF3 variant1 (E12) and variant 2 (E47) ORF sequences (Origene) were cloned in to the lentiviral vector pBOB (Addgene Plasmid 12335) (11). shRNA sequences against human TCF4 and LEF1 were cloned into the pLKO.1-TRC cloning vector (Addgene Plasmid 10878) (12). The pLKO.1 vectors containing a scrambled shRNA (Addgene Plasmid 18640) (13) was used as negative control.

The human MMP1 (-1478 to +60 relative to transcription start site) and MMP13 promoters (-1548 to +60 relative to transcription start site) were amplified by PCR using Pfu DNA polymerase (Promega) and human genomic DNA as template and cloned into pGL3-basic vector (Promega). The promoter and/or luciferase cassette was cloned into a lentiviral vector backbone (Addgene Plasmid 14715) (14). Mutations in a putative Wnt response element (WRE) in the MMP promoters were introduced using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies). All constructs were validated by sequencing.

Lentiviral vectors and packaging vectors were transfected into HEK293T cells to produce lentiviruses. Lentiviruses were harvested and used to infect chondrocytes in the presence of 6 μg/ml polybrene (Sigma-Aldrich).

Reporter assay. Human chondrocytes were infected with Cignal™ lentiviruses containing a TCF/LEF or NF-κB responsive luciferase reporter (SA Biosciences) together with lentiviruses constitutively expressing Renilla luciferase (SA Biosciences) in the presence of 6 μg/ml polybrene (Sigma). Luciferase activity was measured using a Dual-Glo luciferase assay kit (Promega). Activity of firefly luciferase was normalized to Renilla luciferase activity. MMP promoter reporter activity and the activity of a promoter-less negative control were measured using the Steady-Glo® Luciferase Assay System (Promega). Data were normalized for transduction efficiency by meassuring luciferase DNA using qPCR in chondrocytes.

RNA isolation and real-time quantitative PCR. Total RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel). cDNA was synthesized from total RNA with the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR (qPCR) was performed using the MyiQ real-time PCR detection systems (Bio-Rad). GAPDH was used as internal control. Primer sequences are available on request.

Immunoprecipitation and Western blotting. Immunoprecitaion was performed using the Dynabeads Co-Immunoprecipitation kit (Invitrogen). Total cell proteins for Western blotting were collected using RIPA buffer (Cell Signaling) supplemented with the Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). Antibodies used for immunoprecipitation and Western blotting were anti-β-catenin (BD Biosciences), proMMP-1 and proMMP-13 (R&D Systems), GAPDH (Sigma-Aldrich), NF-κB p50(C-19) and NF-κB p65(A) (Santa Cruz), IκB-α and IκB-β (Santa Cruz), FLAG (Origene), TCF4 and LEF1 (Cell Signaling).

MMP activity assay. Generic MMP activity in human chondrocytes and culture media was measured using the SensoLyte 520 generic MMP activity kit (AnaSpec). MMP activity was normalized for protein concentrations of total cell lysates measured using the Pierce BCA Protein Assay Kit (Thermo Scientific).

TUNEL assay. Apoptosis of chondrocytes was detected using The DeadEnd™ Fluorometric TUNEL assay (Promega). Nuclei were counter-stained with DAPI (Invitrogen).

Caspase activity assay. Caspase activity was measured using the caspase 3/7 Caspase-Glo 3/7 Assay Systems (Promega). Caspase activity was normalized for protein concentrations measured using the Pierce BCA Protein Assay Kit (Thermo Scientific).

Statistical analysis. Data are expressed as the mean ± SD and analyzed by two-tailed student's t-tests or one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Expression of TCF/LEF family members in human chondrocytes. We first evaluated the mRNA expression of four TCF/LEF family members in primary human cartilage samples of osteoarthritic patients. Based on mRNA expression TCF4 was the most abundant TCF member in human cartilage followed by TCF3 and LEF1 (Table 1). TCF1 mRNA was barely detectable.

Gene	ΔCt	Ratio
TCF1	17.67± 1.01	0.09%
TCF3	11.01 ± 0.50	9.15%
TCF4	7.74 ± 0.38	87.98%
LEF1	12.72 ± 0.72	2.78%

Table 1. Expression analysis of TCF/LEF members in human cartilage samples. mRNA expression levels of TCF members in human cartilage were detected by real-time PCR. Data are expressed as mean ΔCt in comparison to GAPDH Ct as internal control ± SD of 6 OA donors. Ratio of mRNA expression is expressed as percentage of total TCF/LEF mRNA expression.

Effects of TCF/LEF members on MMP mRNA expression. Viral transduction was used to overexpress TCF4, LEF1 and TCF3 in human chondrocytes. Adenoviral transduction of TCF4 and LEF1 significantly activated TCF/LEF reporter activity in human chondrocytes (Fig. 1A). MMP-1, MMP-3 and MMP-13 mRNA expression was increased by TCF4 overexpression. In contrast, LEF1 overexpression significantly decreased MMP mRNA expression (Fig. 1A). Overexpression of two TCF3 variants showed the same effect on MMP expression as LEF-1 (Fig. 1B). Knockdown of TCF4 significantly decreased MMP-1 and MMP-13 mRNA expression but failed to change MMP-3 expression (Fig. 1C). Knockdown of LEF-1 slightly but significantly upregulated MMP-1 mRNA expression only (Fig. 1C).

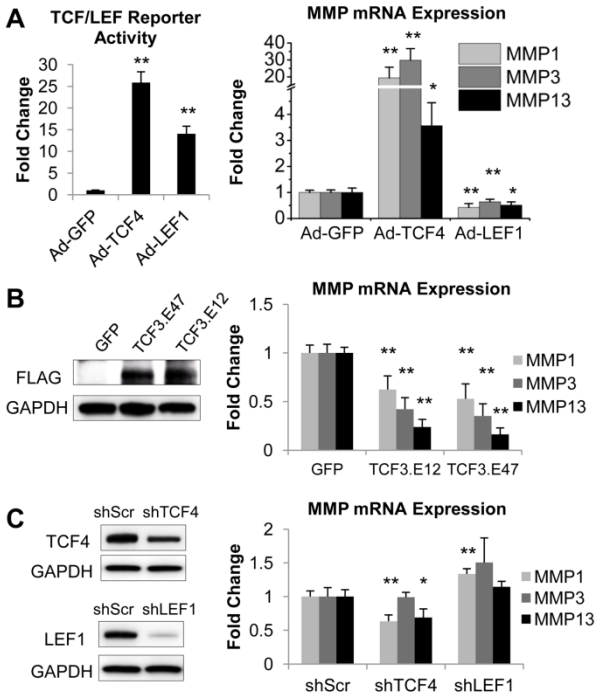

Figure 1. Effects of TCF/LEF members on MMP mRNA expression. A. Human primary chondrocytes were infected with adenoviruses expressing GFP, TCF4 and LEF1. Activation of the canonical Wnt pathway by TCF4 or LEF1 overexpression was validated by a luciferase reporter assay. * = $P < 0.05$, ** = $P < 0.01$, n = 3 donors (left panel). MMP mRNA expression was measured by qPCR. * = $P < 0.05$, ** = $P < 0.01$, n = 3 donors (right panel). **B.** Human chondrocytes were infected with lentiviruses expressing GFP, and FLAG-tagged TCF3 variant 1 (TCF3.E12) and TCF3 variant 2 (TCF3.E47). Overexpression of TCF3 was validated by Western blot using an anti-FLAG antibody (left panel). MMP mRNA expression was measured by qPCR in human chondrocytes overexpressing TCF3 variants. **p<0.01, n = 4 donors (right panel). **C.** Human chondrocytes were infected with lentiviruses expressing scrambled shRNA (shScr) and shRNA against TCF4 or LEF1. Knockdown of TCF4 and LEF1 protein levels was validated by Western blot (left panel). MMP mRNA expression was measured by qPCR. * = $P < 0.05$, ** = $P < 0.01$, n = 3 donors (right panel).

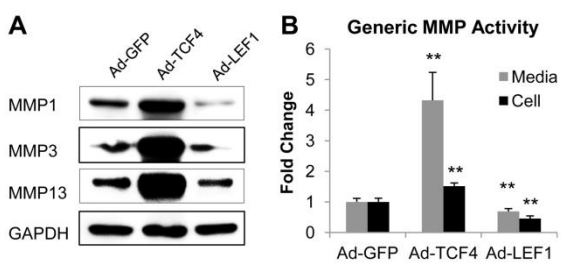

Figure 2. Effects of TCF4 and LEF1 on MMP protein expression and activity. Human chondrocytes were infected with adenoviruses expressing GFP, TCF4 and LEF1. **A.** MMP protein levels were detected by Western blot. **B.** Generic MMP activity in culture media and cell lysates was measured using a generic MMP assay kit. ** = $P < 0.01$, n = 3 donors.

Effects of TCF4 and LEF1 on MMP protein expression and activity. In agreement with their effect on MMP mRNA expression, overexpression of TCF4 upregulated the protein expression of MMP-1, MMP-3 and MMP-13 while LEF1 overexpression downregulated MMP protein expression (Fig. 2A). Increased MMP protein expression by TCF4 coincided

with an increase in generic MMP activity in human chondrocytes and in culture media. In contrast, LEF1 overexpression decreased generic MMP activity (Fig. 2B).

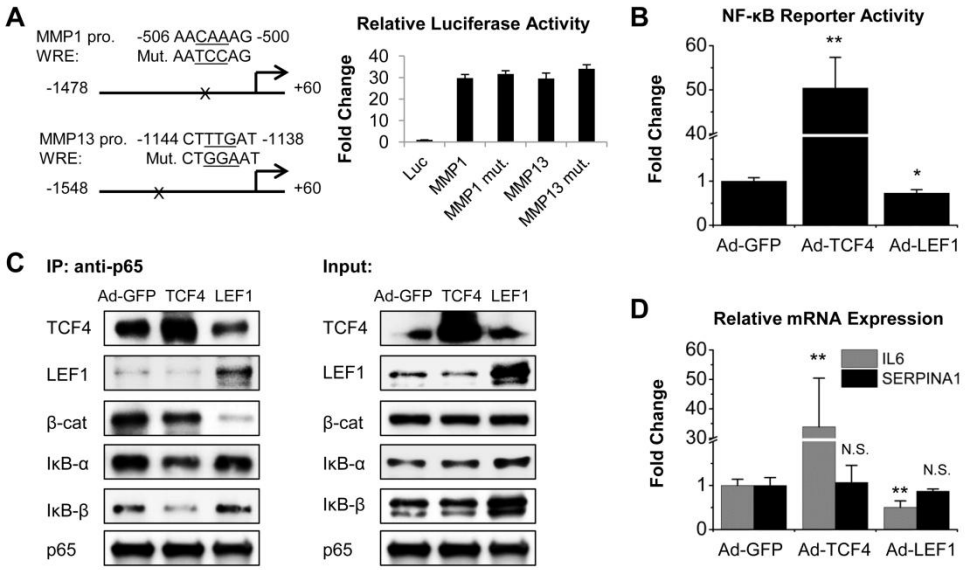

Figure 3. TCF4 potentiates NF-κB signaling in human chondrocytes. A. Schematic representation of the *MMP1* and *MMP13* promoter reporter constructs is shown. The sequence of the putative WRE and the inactivating mutants are given. Basel activities of MMP1 and MMP13 promoters and promoters containing mutant WRE were measured in human chondrocytes. n = 3 independent experiments using one donor. **B**. Human chondrocytes were co-infected with adenoviruses expressing GFP, TCF4 or LEF1, and lentiviruses containing an NF-κB reporter construct. Luciferase activity was measured. * = $P < 0.05$, ** = $P < 0.01$, n = 3 donors. **C.** Co-immunoprecipitation was performed using protein extracts of human chondrocytes and anti-p65 antibody as bait. Co-immunoprecipitated proteins were detected by Western blot. A representative experiment from 2 donors is shown. **D.** Human chondrocytes were infected with adenoviruses expressing GFP, TCF4 and LEF1. *IL6* and *SERPINA1* mRNA expression was measured by qPCR. * = $P < 0.05$, ** = $P < 0.01$, n = 4 donors.

TCF4 potentiates NF-κB signaling in human chondrocytes. Previously, we have shown that knockdown of TCF4 in mouse chondrocytes abolished Wnt/β-catenin-induced MMP expression indicating that at least in animal models MMPs are direct target genes of TCF/LEF transcription factors. To determine whether MMPs are direct target genes of TCF-4 transcription factors in human chondrocytes, we analyzed 3000 bp of promoter sequence of the *MMP1* and *MMP13* genes for the presence of consensus Wnt response elements (WRE). We identified one potential WRE in the *MMP1* promoter 506 bp

upstream of the transcription start site and one in the *MMP13* promoter 1144bp upstream of the transcription start site which matched the consensus sequence (Fig. 3A). A promoter fragment of about 1.5 kb of the human *MMP1* and *MMP13* gene was cloned in front of the luciferase reporter gene and the putative WRE sequences were mutated. Wild-type *MMP1* and *MMP13* promoters showed ~30 fold induction of promoter-activity compared to a promoter-less control. Mutation of the WREs did not influence promoter activity in human chondrocytes (Fig. 3A), suggesting that the consensus WRE is not involved in regulation of MMP expression in contrast to the knockdown of TCF-4 which significantly decreased MMP-1 and MMP-13 expression (Fig. 1C). However we cannot exclude the possible existence of functional WREs in the MMP genes outside of the analyzed promoter region.

We previously showed that Wnt-3A decreased MMP expression through an inhibitory interaction of β-catenin with NF-κB p65/RELA in human chodnrocytes (9). Therefore, we tested if TCF-4 might also influence NF-κB activity. Overexpression of TCF4 in human chondrocytes significantly increased NF-κB reporter activity 50-fold while overexpression of LEF1 slightly but significantly decreased NF-κB activity. This data suggested that TCF4 may upregulate MMP expression by potentiating NF-κB signaling rather than through its conventional function in the canonical Wnt pathway (Fig. 3B). Interestingly, as shown in Fig. 3C, TCF4 co-immunoprecipitated with NF-κB p65, a key transcription factor in the regulation of MMP expression in human chondrocytes (Fig. 3C). Overexpression of TCF4 enhanced its binding to p65. LEF1 also co-precipitated with NF-κB p65 as previously reported and this was increased by overexpression of LEF-1 (Fig. 3C) (15). In agreement with previous findings, β-catenin, IκB-α and IκB-β also co-immunoprecipitated with p65. Interestingly, overexpression of TCF4 slightly reduced co-immunoprecipitation of β-catenin, IκB-α and IκB-β, which are all inhibitors of NF-κB. Overexpression of LEF1 decreased the binding of both TCF4 and β-catenin to p65 (Fig. 3C). Since β-catenin is an inhibitor of NF-κB (9), the repression of NF-κB activity by LEF1 may be caused by competition between LEF1 and TCF4 for binding to p65. LEF1 overexpression did not change the binding of the NF-κB inhibitors IκB-α and IκB-β to NF-κB p65, though the basal expression levels seemed to be increased by LEF-1 overexpression (Fig. 3C). None of the

above-mentioned proteins were detected in Western blot when control IgG was used as bait in co-immunoprecipitation assay (data not shown).

We next evaluated whether TCF4 and LEF1 could also influence the expression of other NF-κB target gene target genes such as *IL6* and *SERPINA1*. *IL6* mRNA expression was more than 30-fold upregulated by TCF4 overexpression but repressed by LEF-1 overexpression (Fig. 3D). However, expression of *SERPINA1* was not significantly affected by either TCF4 or LEF1 overexpression (Fig. 3D). This indicated that a subset of NF-κB target genes may be selectively regulated by the interaction between NF-κB and TCF4 or LEF1 transcription factors.

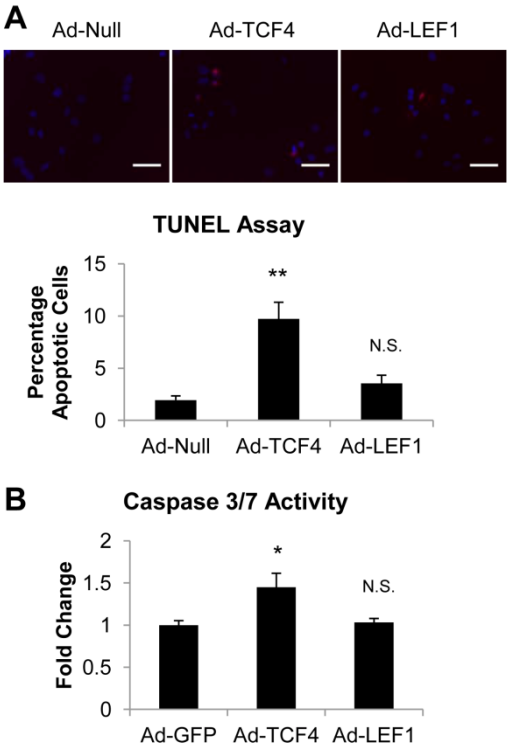

Figure 4. TCF4 induces human chondrocyte apoptosis. A. Human chondrocytes were infected with adenoviruses expressing null control, TCF4 and LEF1. Chondrocyte apoptosis was detected 4 days after adenoviral transduction using TUNEL staining (Red: TUNEL; Blue: Nuclei. Scale bar = 20 μm). Representative images are shown. Quantification of TUNEL positive cells was performed. ** = $P < 0.01$, n = 3 donors. B. Caspase 3/7 activity was measured 3 days after adenoviral transduction of human chondrocytes with Ad-GFP, Ad-TCF4 and Ad-LEF1. * = $P < 0.05$, n = 3 donors.

TCF4 induces human chondrocyte apoptosis. Overexpression of TCF4 but not of LEF-1 in primary human chondrocytes induced apoptosis as determined by a TUNEL assay (Fig. 4A). In consistence with the TUNEL assay, overexpression of TCF4 elevated caspase 3/7

activity suggesting that its effect on chondrocyte apoptosis was at least partly mediated through activating of caspase 3/7 (Fig. 4B).

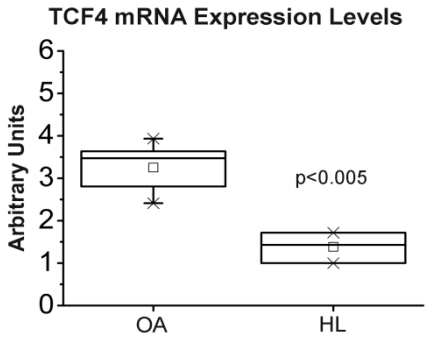

Figure 5. TCF4 mRNA expression is upregulated in OA cartilage. Human articular cartilage RNA samples were isolated from 5 OA donors and 3 healthy (HL) donors. TCF4 mRNA expression was measured by qPCR.

TCF4 mRNA expression is upregulated in OA cartilage. We finally studied the mRNA expression of TCF4 in OA and healthy articular cartilage samples using quantitative PCR. As shown in Fig. 5, mRNA expression of TCF4 was significantly elevated in OA cartilage compared to healthy human articular cartilage specimens, suggesting that increased TCF4 expression in OA cartilage may contribute to the progression of OA by potentiating the pro-catabolic NF-κB pathway and by stimulating chondrocyte apoptosis.

Discussion

Previous animal studies have suggested a catabolic and degenerative role of the Wnt/β-catenin pathway in articular cartilage. Recently we have challenged this pro-catabolic role of Wnt/β-catenin signaling in human cartilage by revealing an unprecedented species difference in the role of canonical Wnt signaling in the expression of MMP-1, MMP-3 and MMP-13 (9). In human chondrocytes Wnt/β-catenin signaling is part of a negative feedback loop counteracting IL-1-induced MMP expression by a non-canonical inhibitory protein-protein interaction of β-catenin with NF-κB. In marked contrast to animal chondrocytes, the downstream effectors of β-catenin TCF/LEF transcription factors are not involved in IL-1 induced MMP expression in human chondrocytes (9). This questions the role of TCF/LEF transcription factors, the downstream effectors of β-catenin in the canonical Wnt signaling pathway, in human chondrocytes.

Among all the catabolic factors involved in cartilage degeneration, MMPs play a crucial role in collagen and proteoglycan degradation (16-18). It has been shown that multiple pathways such as the p38, NF-κB, AP-1, MAPK and C/EBP are involved in the transcription regulation of MMPs (19-22). We demonstrated that TCF4 was a strong activator of MMP-1, MMP-3 and MMP-13 mRNA expression. Knockdown of TCF4 led to a decrease in the basal transcription of MMP-1 and MMP-13 but not of MMP-3. This might be due to the usage and/or compensation of other regulatory pathways in MMP3 transcription. Our data indicate that TCF4 may directly regulate transcription of MMPs in human chondrocytes as has been shown in mouse chondrocytes (9). Indeed, consensus WREs were identified in the promoter regions of the MMP1 and MMP13 genes. However mutations of WREs in MMP1 and -13 promoters did not change the promoter activity under basal conditions in contrast to the inhibitory effect of TCF4 knockdown. Although not conclusive, these data do suggest that the effect of TCF4 might be independent of its conventional function as canonical Wnt pathway transcription factor. We cannot, however, exclude the possibility of binding of TCF4 to other WREs in MMP genes outside of the analyzed promoter fragments. Since ectopic expression of TCF4 led to an increase in the expression at the mRNA and protein level of MMP-1, MMP-3 and MMP-13 and increased generic MMP activity, it was suggested that elevation of TCF4 levels in cartilage may result in increased cartilage degradation. In agreement with this, we found a higher mRNA expression level of TCF4 in OA cartilage compared to healthy cartilage providing support for a pro-catabolic role of TCF4 in OA.

Since it has been shown that in human chondrocytes MMPs are direct target genes of NF-κB signaling (9), we further explored if the effect of TCF/LEF on MMP expression was due to a crosstalk with NF-κB. TCF4 was found to augment NF-κB reporter activity in human chondrocytes. By co-immunoprecipitation assay, we observed an unexpected interaction between TCF4 and NF-κB p65, suggesting that this interaction might be responsible for the increase in MMP expression. LEF1 also forms a complex with p65 in consistence with previous findings (15). How the protein complex of TCF4 and p65 increases NF-κB activity is not clear. It might be due to modification and/or recruitment of other co-factors to the p65-TCF4 complex, such as for example CBP/p300. It is known that transcriptional activity of NF-κB can be enhanced by many co-factors such as CBP/p300

and ribosomal protein S3 (23-25). Interestingly, TCF4 contains a unique domain in the C-tail which binds to CBP/p300, while its family members TCF3 and LEF1 lack this domain (26). In addition, the C-terminal binding protein (CtBP) binds to TCF4 but not to LEF1 (27). The potentiating effect of TCF4 and inhibitory effect of LEF1 and TCF3 on NF-κB might be explained by the different interactions with co-factors. For example, it's possible that TCF4 stabilizes the interaction between NF-κB and its positive co-factors such as CBP/p300 while TCF3 and LEF1 fail to do so because of the lack of the binding domain for CBP/p300. Alternatively, TCF4 overexpression may reduce the binding of β-catenin, IκB-α and IκB-β to p65 which are known negative regulators of NF-κB (9, 28). In marked contrast to TCF4, LEF1 is a negative regulator of NF-κB. It's likely that LEF1 negatively regulates NF-κB activity by competing with TCF4 for binding to NF-κB p65 thereby counteracting TCF4's potentiating effect on NF-κB. This is based on our observation that overexpression of LEF1 decreased the binding of TCF4 to NF-κB p65, although a direct negative effect from LEF1 cannot be excluded. Vice versa TCF4 overexpression decreased the binding of LEF1 to NF-κB p65. The potentiating effect of TCF4 on NF-κB-mediated gene transcription was not limited to MMPs but was also found for other established target genes such as *IL6*. Remarkably, TCF4 could not potentiate the expression of the NF-κB target gene *SERPINA1*. This might be explained by the recruitment of different co-factors which might be less sensitive to TCF4 (28).

We previously showed that β-catenin interacts with and inhibits NF-κB in human chondrocytes (9). It's not clear whether or how TCF/LEF members interact with NF-κB in cooperation with β-catenin. Decrease in NF-κB-associated β-catenin levels by overexpression of TCF4 may contribute to its effect on NF-κB activity. In contrast, although LEF1 overexpression weakened the interaction of NF-κB p65 with its inhibitor β-catenin, NF-κB activity was not increased by LEF1, possibly due to competition between LEF1 and TCF4 to NF-κB p65 binding. It remains to be elucidated if the effect of β-catenin on NF-κB is dependent on its interaction with TCF/LEF.

Besides its pro-catabolic effects, it has been suggested that NF-κB may also play a role in chondrocyte apoptosis (29-31). A number of studies have described NF-κB's involvement in apoptotic events in articular chondrocytes. For example, it has been shown that NF-κB activation mediates the apoptotic effect of Nitric Oxide in articular

chondrocytes, by activating caspase 3-induced apoptosis through activation of p53 (30, 31). In our study, we found that overexpression of TCF4 induced chondrocyte apoptosis. Since TCF4 is an enhancer of NF-κB activity, the effect of TCF4 on apoptosis might be at least partly due to its potentiating effect on NF-κB signaling. This is also supported by the fact that TCF4 overexpression activates caspase 3/7 which are prime mediators of NF-κB-induced apoptosis.

Our results indicate that ectopic expression of TCF4 in human chondrocytes induces cartilage catabolism by increasing MMP expression and activity and by inducing apoptosis. We provide evidence that this action of TCF4 is independent of its function as canonical Wnt pathway transcription factor, but instead is due to a potentiating interaction with NF-κB. This is in marked contrast to the inhibitory effect of β-catenin on NF-κB activity as described previously (9). Our findings suggest that TCF4 might be a pathogenic factor in human cartilage degeneration, which is further supported by an upregulation of TCF4 mRNA expression in OA cartilage. Therefore, targeting TCF4 activity and/or expression might be a promising avenue for the treatment of degenerative cartilage disease.

Acknowledgements. The authors would like to thank Dr. Simon Mastbergen (Utrecht University, The Netherlands) for providing healthy human articular cartilage samples.

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Chapter 6

Involvement of ERK, Wnt and BMP2 Signaling in Human Articular Chondrocyte Dedifferentiation in Monolayer Culture

Involvement of ERK, Wnt and BMP2 signaling in human articular chondrocyte dedifferentiation in monolayer culture

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Abstract

Objective. When primary chondrocytes are cultured in monolayer, they undergo dedifferentiation during which they lose their phenotype and their capacity to form cartilage. Dedifferentiation is an obstacle for cell therapy for cartilage degeneration. In this study, we aimed to systemically evaluate the changes in gene expression during dedifferentiation of human articular chondrocytes to identify underlying mechanisms.

Methods. RNA was isolated from monolayer-cultured primary human articular chondrocytes at serial passages. Gene expression was analyzed by microarray. Based on the microarray analysis, relevant genes and pathways were identified. Their functions in chondrocyte dedifferentiation were further investigated in detail.

Results. In vitro expanded human chondrocytes showed progressive changes in gene expression during dedifferentiation. Strikingly, an overall decrease in total gene expression was detected. Genes in the Wnt and BMP pathways exhibited significant changes in expression. The non-canonical rather than the canonical Wnt pathway was found to be involved in the loss of collagen II synthesis. BMP2 was able to decelerate the dedifferentiation and reinforce the maintenance of chondrocyte phenotype in monolayer culture. DNA methylation was in part responsible for the expression downregulation of a set of genes.

Conclusion. Our study revealed the roles of ERK, Wnt and BMP pathways as well as DNA methylation in chondrocyte dedifferentiation in monolayer culture.

Introduction

Articular chondrocytes are the sole cell type in articular cartilage and maintain the homeostasis of cartilage by synthesis of extracellular matrix (ECM) molecules including type II collagen and proteoglycans (1). This homeostasis is impaired in cartilage diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA), ultimately leading to cartilage destruction. The phenotype of differentiated chondrocytes is unstable and rapidly lost with exposure to inflammatory factors such as interleukin-1 (IL-1) and retinoic acid, as well as during monolayer culture (2, 3). This process is designated dedifferentiation and is characterized by the loss of synthesis of hyaline cartilage associated ECM molecules such as type II collagen (COL2) (4). When isolated chondrocytes are cultured in monolayer, they lose their typical round shape and transform into flattened fibroblast-like cells. Interestingly, dedifferentiated chondrocytes chondrocytes better preserve their normal phenotype and start to re-express type II collagen when they are cultured three-dimensionally (5-7).

Autologous chondrocyte implantation (ACI) is the golden therapy for patients with focal lesions of articular cartilage (8, 9). The ACI procedure consists of removal of a small piece of intact articular cartilage, isolation and expansion of chondrocytes to obtain sufficient number of cells for implantation. The dedifferentiation of chondrocytes during *in vitro* expansion is a major obstacle in ACI therapy. Although many factors and signaling pathways have been implicated in dedifferentiation, the exact mechanism remains elusive. Upregulation of extracellular regulated protein kinase (ERK) and downregulation of protein kinase C (PKC) contribute to the dedifferentiated phenotype of rabbit chondrocytes (2). Increased β-catenin is associated with rabbit chondrocyte dedifferentiation (3) and canonical Wnt-3A and Wnt-7B stimulation also induce loss of differentiated phenotype of rabbit chondrocytes. Both studies suggest that Wnt/β-catenin signaling may be a driving factor in chondrocyte dedifferentiation (10, 11). IL-1β as well as Wnt-3A has been shown to induce rabbit chondrocyte dedifferentiation through the c-Jun/activator protein-1 (AP-1) pathway (10, 12). IL-6 in the presence of soluble IL-6 receptor (sIL-6R) downregulates the expression of cartilage-specific ECM molecules through the JAK/STAT pathway (13). It has been reported that growth and differentiation factor 5 (GDF-5) is affected by dedifferentiation in human chondrocytes (14). Recently, it

has been shown that αvβ5 integrin is involved in dedifferentiation of human articular chondrocytes in monolayer culture (15). Although many pathways are involved in chondrocyte dedifferentiation, most studies have been performed in animal chondrocytes and the number of studies using human chondrocytes is limited.

A systemic analysis of mechanisms for human articular chondrocyte dedifferentiation is still lacking. In this study, we obtained gene expression profiles of dedifferentiating human articular chondrocytes during monolayer culture. We identified many genes of the Wnt, BMP, and ERK signaling pathways to be differentially expressed in early vs. late passages. We further explored the roles of these altered pathways in dedifferentiation. Our findings not only improve our knowledge of the intricate signaling network regulating maintenance of chondrocyte phenotype, but also contributes to improved chondrocyte expansion and chondrogenic performance for ACI procedures.

Materials and Methods

Human chondrocyte isolation and cell culture. The use of patient material was approved by the medical ethical committee of the Medisch Spectrum Twente in Enschede, The Netherlands. Cartilage was obtained from human OA patients (62 ± 11 years) undergoing total knee replacement surgery. Cartilage was harvested from regions without macroscopically evident degeneration. Human cartilage was incubated with 1 mg/ml collagenase II (Sigma-Aldrich) prepared in DMEM (Invitrogen) at 37 °C overnight under agitation. Chondrocytes recovered from the digestion were seeded at a density of 25,000 cells/cm^2. Chondrocytes, and HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen).

To redifferentiate dedifferentiated chondrocytes, 200,000 cells per well were seeded in a round-bottom 96-wells plate and centrifuged for 3 min at 2000 rpm to form high-density micromass cell pellets. Cell pellets were cultured in the chondrogenic differentiation medium (DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, 0.2mM ascorbic acid 2-phosphate, 0.4mM proline, 10 ng/ml TGF-β1) for 3 weeks. All supplements were from Invitrogen.

Recombinant proteins and reagents. Recombinant human BMP-2 and Wnt-5A were from R&D Systems. ERK inhibitor PD98059 and 5-Aza-2′-deoxycytidine (5-AzaC) were from Sigma-Aldrich. Wnt inhibitor PKF115-584 was a kind gift from Novartis (16).

Histochemistry. Cell pellets were fixed with 10% formalin for 15 min and embedded in paraffin using routine procedures. Sections of 5 µm were cut and stained for sulfated glycosaminoglycans (GAGs) with alcian blue combined with counterstaining of nuclear fast red to observe nuclei, or stained with safranin O or toluidine blue alone.

RNA isolation and real-time quantitative PCR. Total RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel). cDNA was synthesized from total RNA with the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR (qPCR) was performed with the MyiQ real-time PCR detection system (Bio-Rad) using the standard curve based method (17). GAPDH was used as internal control.

Whole genome gene expression and pathway analysis. The Ambion Illumina total prep 96 kit was used to generate biotinylated cRNA from the RNA samples. 750 ng of the obtained cRNA of each sample was hybridized onto the Illumina HumanHT-12 v3 Expression BeadChips. The BeadChips were scanned using the Illumina iScan array scanner. Gene expression profiling was performed using llumina's Genomestudio v. 2010.1 software with the default settings advised by Illumina. The raw fluorescence intensity values were normalized applying quantile normalization. Differential gene expression was analysed using the commercial software package Genespring, version 11.5.1. (Agilent Technologies). Genes with at least a two-fold difference being significantly differentially expressed according to a one-way ANOVA with a Benjamini-Hochberg FDR correction and TukeyHSD post-hoc test using a cut-off rate of $P = 0.05$ were selected. A Venn diagram was generated based on the selected genes of the comparisons passage 0 (P0) with P2, P0 with P4 and P0 with P8 using the Genespring software. Changes in gene expression of annotated canonical pathways and bio-functions were visualized using ingenuity pathway analysis (IPA) software (Ingenuity Systems) to identify biological processes and pathways that may be associated with the modulated gene expression.

Western blotting. Total cell proteins were collected using RIPA buffer (Cell Signaling) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). Approximately 10 - 20 µg of protein was subjected to SDS-PAGE and transferred to PVDF

membrane (Millipore). After primary antibody and HRP-conjugated secondary antibody (Dako) incubation, proteins were visualized using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific). Antibodies used for western blot were anti-β-catenin (BD Biosciences), active β-catenin (Millipore), p-ERK1/2 (Santa Cruz), ERK2 (Santa Cruz), COL2A1 (Abnova), GAPDH (Sigma-Aldrich).

Reporter assay. Human chondrocytes were infected with Cignal™ lentiviruses containing ERK or TCF/LEF responsive reporter (SA Biosciences) together with lentiviruses constitutively expressing Renilla luciferase (SA Biosciences) in the presence of 6 μg/ml polybrene (Sigma). Luciferase activity was measured using Dual-Glo luciferase assay (Promega). Activity of Firefly luciferase was normalized to Renilla luciferase activity.

Plasmid constructs and viral transduction. shRNA sequence against human Wnt5A was cloned into pLKO.1-TRC cloning vector (Addgene plasmid 10878). pLKO.1 vectors containing a scrambled shRNA as control (Addgene plasmid 18640) and an shRNA sequence against human β-catenin (Addgene plasmid 18803) were used. Lentiviral vectors and packaging vectors were transfected into HEK293T cells using FuGENE HD (Roche) to produce lentiviruses. Lentiviruses were harvested and used to infect chondrocytes in the presence of 6 μg/ml polybrene (Sigma-Aldrich). A transduction efficiency of at least 90% was achieved in all experiments.

Quantitative GAG and DNA assay. Cell pellets were digested and measured for GAG quantification as previously reported (18). Relative cell number was determined by quantification of total DNA using a CyQuant DNA Kit (Invitrogen) according to the manufacturer's instructions. GAG content was normalized for DNA content.

Statistical analysis. Microarray data were analyzed as described above. Other data were expressed as the mean ± SD and analyzed by two-tailed student's *t*-tests or one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Loss of differentiated phenotype and re-differentiation ability in monolayer culture. Human articular chondrocytes were isolated from knee joints at end stage osteoarthritis. To study chondrocyte dedifferentiation, chondrocytes were cultured in monolayer up to passage 8 and assessed at passage 0, 2, 4, 6 and 8. Human articular chondrocytes showed

typical phenotypic changes during monolayer culture. Cells lost their round shape and became flattened (Supplemental Fig. 1A). To test if dedifferentiated chondrocytes maintained their ability to produce cartilage-specific ECM, passage 2 and 8 chondrocytes were cultured in pellets and chondrogenic differentiation media and then subjected to histochemical analysis. As shown by histological staining, passage 2 cells were still able to produce a cartilage specific ECM while passage 8 cells had lost their ability to regain chondrocyte characteristics (Supplemental Fig. 1B). These data suggest that after extensive expansion for 8 passages human chondrocytes have lost their capacity to produce a cartilaginous matrix at least *in vitro*.

Whole genome gene expression analysis of dedifferentiating chondrocytes . RNA samples from passage 0, 2, 4, 6, and 8 human articular chondrocytes from four donors were analyzed by microarray. Gene expression levels at later passages were compared to that of passage 0 (P0). All genes with at least a two-fold change in expression in at least one of the four later passages are detailed in Supplemental Table 1 (available upon request). 137 genes showed over two-fold significant change at passage 2. Over time, an increasing number of genes showed significant differences (Fig. 1A). 93 genes showed consistent changes through all passages compared to P0 (Fig. 1A). Interestingly, the 10 most changed genes were all down-regulated during all passages as compared to P0 (Supplemental Table 2). In agreement with this, the global gene expression level of all significantly changed genes decreased from P2 to P8 (Fig. 1B). The average changes in expression level between two adjacent passages (e.g. P0 - P2, P2 - P4, P4 - P6, P6 - P8) showed constant reduction (Fig. 1C). Thus the dedifferentiation is likely to be a gradual and cumulative process.

Functional annotation using Ingenuity Pathway Analysis (IPA) showed that significantly modulated genes were involved in multiple biological processes (Fig. 1D). Notably, cellular growth and proliferation, cell death and cell cycle were significantly changed, most obvious in P4 and P6. This is consistent with a decrease in proliferation in monolayer culture (19). Other pathways directly related to cartilage biology, such as skeletal and muscular system development and function and connective tissue development also changed. The cell morphology pathway also showed a significant change, which is in agreement with the phenotypic changes observed (Supplemental Fig. 1A). Genes that

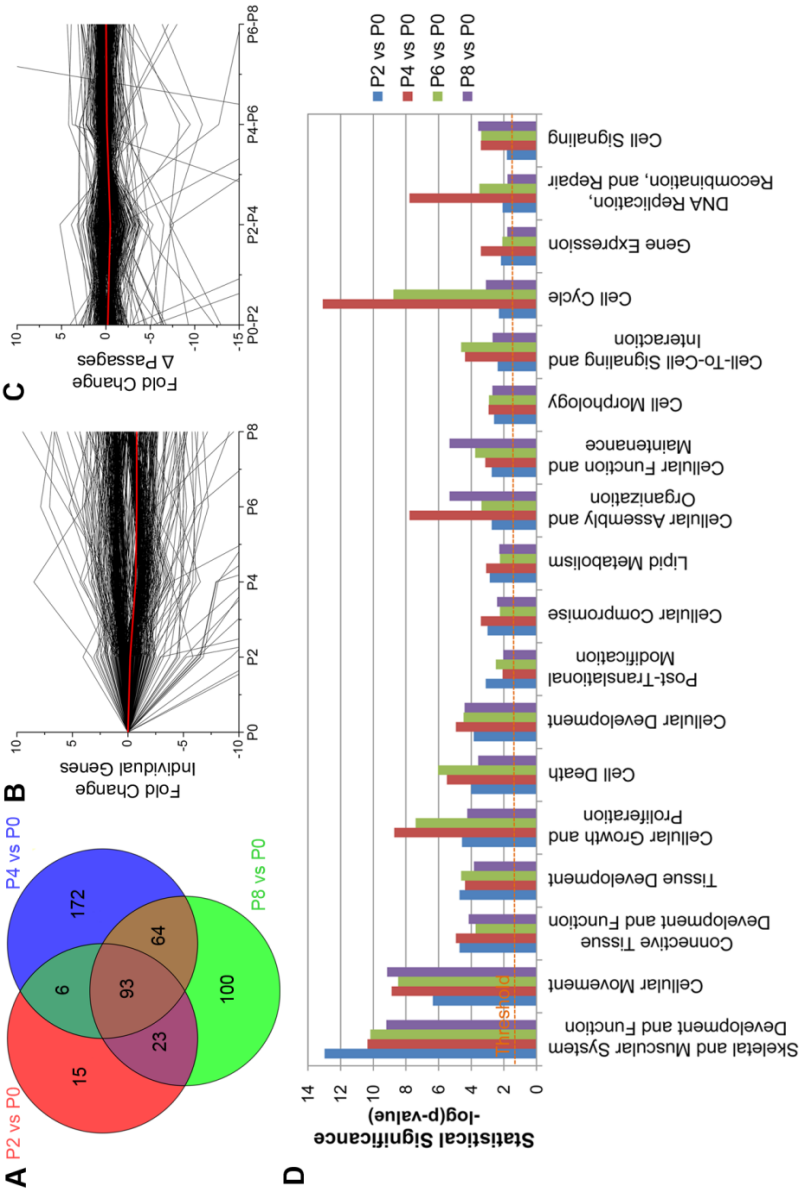


Figure 1. Whole genome gene expression analysis of dedifferentiating chondrocytes. **A.** Venn diagram depicting the number of significantly changed genes with >=2.0 mean fold change at P2, P4 and P8 compared to P0 respectively of 4 donors. **B.** Mean fold changes of all individual genes showing significant changes at each passage of 4 donors. The average fold change is indicated by a red line. **C.** Mean fold changes of each gene between adjacent passages (P0-P2, P2-P4, P4-P6, P6-P8). Average of difference between passages is indicated by a red line. **D.** Significantly changed biological processes during dedifferentiation according to ingenuity pathway analysis (IPA). The orange line denotes the threshold for significance (P = 0.05). Data are based on input of 4 donors.

exhibited significant changes in expression and are associated with these biological processes are shown in Supplemental Table 3 (available upon request).

Network analysis based on gene expression changes between P8 and P0 chondrocytes revealed the involvement of multiple signaling pathways (Supplemental Fig. 2). Key signaling mediators such as Akt, ERK1/2, PI3K, p38 MAK and NF-κB formed the cores of these networks. This indicated that these signaling pathways may play a role in the dedifferentiation process.

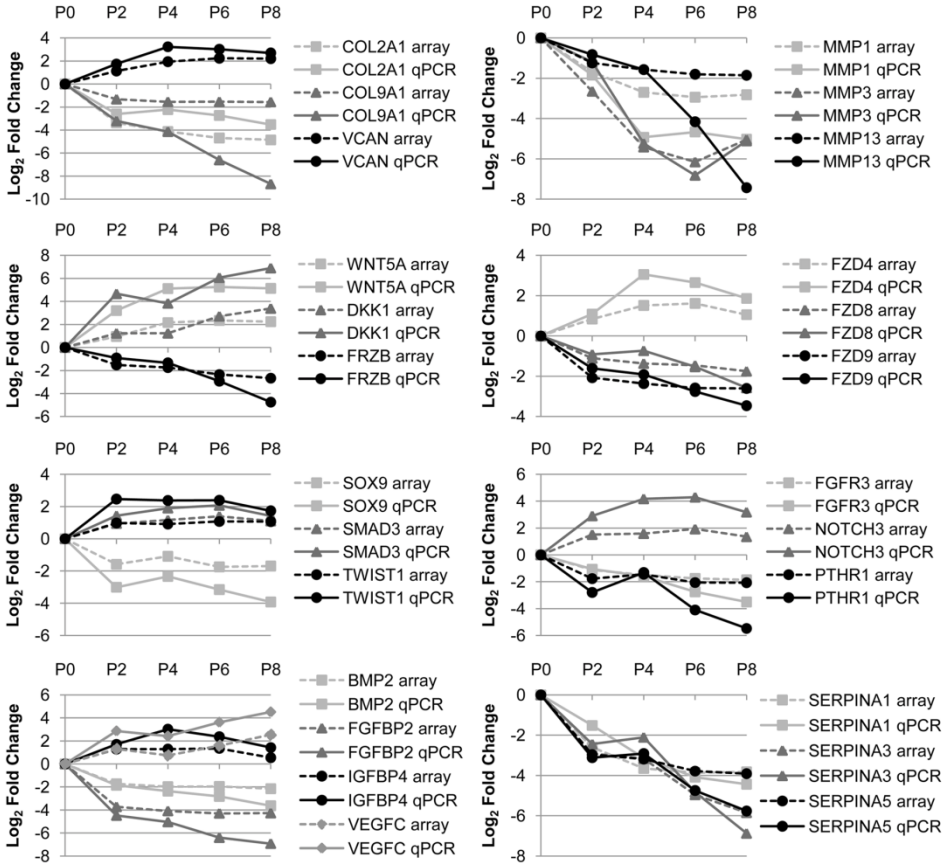


Figure 2. Validation of microarray results by quantitative PCR. Data from microarray and real-time PCR are indicated as "array" (dashed lines) and "qPCR" (straight lines) respectively and are expressed as mean Log_2 fold change compared to expression levels at P0 of the same 4 donors. Same RNA samples were used for microarray and qPCR.

Validation of microarray results by quantitative PCR. To validate the microarray data, expression of a number of significantly changed genes was measured by qPCR. All qPCR results and microarray data showed strong correlation indicating valid and successful microarray analysis (Fig. 2). Gene expression of ECM molecules *COL2A1* and *COL9A1* decreased while VCAN expression increased, characterizing the chondrocyte dedifferentiation process. Matrix degrading enzymes *MMP1*, *MMP3* and *MMP13* were down-regulated. Many Wnt pathway genes were changed including *WNT5A*, *DKK1* and *FRZB*. Interestingly, the expression pattern of the Wnt signaling receptor Frizzled (FZD) changed with an upregulation of *FZD4* and a downregulation of *FZD8* and *FZD9*. Chondrogenic transcription factor *SOX9* expression decreased during dedifferentiation. Other transcription factors, receptors and extracellular ligands involved in several signaling pathways such as BMP, FGF, IGF and Notch also showed changes in expression, suggesting that chondrocyte dedifferentiation may be controlled by a complex network of several pathways. In addition, expression of several members of serine protease inhibitors (SERPIN) was down-regulated.

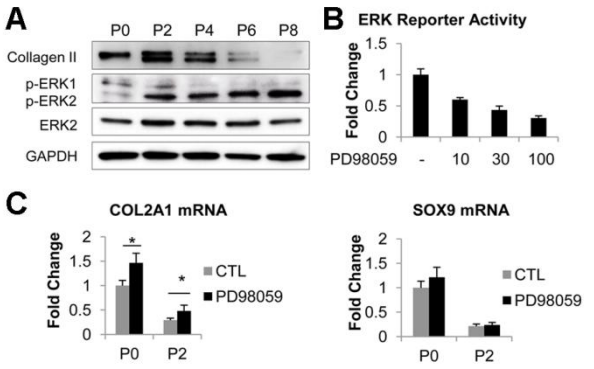


Figure 3. Role of ERK signaling in chondrocyte dedifferentiation. A. Protein levels of Collagen II, phospho(p)-ERK, total ERK, and GAPDH were detected by immunoblotting. Blots from one representative donor out of 3 donors are shown. **B.** ERK reporter activity was measured after treatment with different concentrations of PD98059 for 24 hours as indicated (in µM) in chondrocytes lentivirally transduced with luciferase promoter reporter construct. Data are expressed as mean fold change of triplicate cultures from one donor. **C.** Human chondrocytes were cultured with or without 10 µM PD98059 from P0 until P2. mRNA expression of *COL2A1* and *SOX9* was measured by qPCR. * = $P < 0.05$, n = 3 donors.

Role of ERK signaling in chondrocyte dedifferentiation. As shown in the pathway analysis, ERK is connected with many changed genes (Supplemental Fig. 2). We further explored the role of ERK in human chondrocyte dedifferentiation. Western blot was

performed to examine the chondrocyte marker COL2A1 and ERK protein expression. Type II collagen protein showed decreased expression with increasing passaging, in agreement with decreased *COL2A1* mRNA expression. Remarkably, in culture chondrocytes started to express a smaller isoform of type II collogen which might be a transcription variant or cleaved protein from P2 onwards which also decreased over time (Fig. 3A). Over time, there was an increase in phospho-ERK2 and a decrease in phospho-ERK1, with ERK2 as the dominant form of ERK in these human chondrocytes (Fig. 3A). Total ERK2 did not show a substantial change during dedifferentiation. To test the function of increased ERK activity during dedifferentiation, we used the MEK1 inhibitor PD98059 which showed nearly 50% inhibition of ERK activity at 10 μM (Fig. 3B). We cultured human chondrocytes in the presence of 10 μM PD98059 from P0 to P2. *COL2A1* expression was slightly but significantly increased at both P0 and P2 (Fig. 3C), which is consistent with previous findings (2, 16). However, the key chondrogenic transcription factor *SOX9*, which is also important for the maintenance of the chondrocyte phenotype (20, 21), was not affected by ERK inhibition (Fig. 3C). The weak effect of ERK inhibition suggests that it may not be the main driving factor of chondrocyte dedifferentiation.

The Wnt signaling pathways in dedifferentiation. Many genes encoding proteins of the canonical and non-canonical Wnt signaling pathways showed changes in expression during dedifferentiation, indicating that Wnt signaling may play a role in this process. The key mediator of the canonical Wnt pathway, β-catenin, showed low level expression at P0 and the active/unphosphorylated form of β-catenin was hardly detectable (Fig. 4A). Active and total β-catenin protein levels were substantially increased at later passages (Fig. 4A). By using the β-catenin/TCF blocker PKF115-584 (16), canonical Wnt pathway activity can be inhibited by 50% at a concentration of 0.5 μM (Fig. 4B). Human chondrocytes were cultured for 2 passages in the presence or absence of 0.5 μM PKF115-584. *COL2A1* expression was decreased by PKF115-584 and *Sox9* was slightly upregulated at P0 but not affected at P2 (Fig. 4C). Knockdown of β-catenin in P0 chondrocytes showed the same effect as chemical inhibition of the canonical Wnt pathway (Fig. 4D). In contrast to its role in animal chondrocytes where canonical Wnt pathway causes the loss of the chondrocyte phenotype (3, 10, 11), canonical Wnt signaling alone does not seem to be responsible for dedifferentiation of human chondrocytes. The non-canonical *WNT5A* exhibited increased

expression during chondrocyte expansion (Fig. 2). Stimulation of human chondrocytes with Wnt-5A significantly decreased *COL2A1* expression, but not *SOX9* (Fig. 4E). Knockdown of *WNT5A* reversed *COL2A1* expression (Fig. 4E). These data suggest that the increase in *WNT5A* expression during dedifferentiation contributes to the loss of COL2 production and possibly changes in expression of other genes.

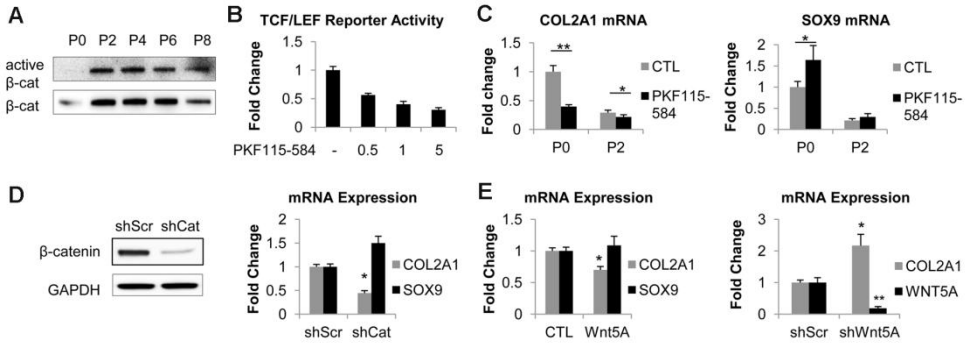


Figure 4. The Wnt signaling pathways in dedifferentiation. A. Protein levels of active and total β-catenin (β-cat) were detected by western blot. Blots from one representative donor out of 3 donors are shown. **B.** Human chondrocytes were transduced with a TCF/LEF reporter construct and activity was measured after treatment with different concentrations (in µM) of PKF115-584 for 24 hours as indicated. Data represent mean fold change of triplicate cultures from one donor. **C.** Human chondrocytes were cultured with or without 0.5 µM PD98059 from P0 until P2. mRNA expression of *COL2A1* and *SOX9* was measured by qPCR. * = $P < 0.05$, ** = $P < 0.01$, n = 3 donors. **D.** Human chondrocytes were infected with a lentivirus encoding scrambled shRNA (shScr) which serves as control or an shRNA against β-catenin (shCat). Protein levels of β-catenin and GAPDH were detected by immunoblotting. Blots from one representative donor out of 3 donors are shown. mRNA expression of *COL2A1* and *SOX9* was measured by qPCR. * = $P < 0.05$, n=3 donors. **E.** Human chondrocytes were treated with 300 ng/ml WNT-5A or infected with a lentivirus encoding scrambled shRNA (shScr) or a shRNA against *WNT5A* (shWnt5A). mRNA expression levels were measured by qPCR. * = $P < 0.05$, ** = $P < 0.01$, n = 3 donors.

Maintenance of differentiated chondrocyte phenotype by BMP-2. It has been shown that BMP-2 induces the expression of chondrocyte-specific genes such as *COL2A1* and *SOX9* (22-24). Our microarray and qPCR data showed that *BMP2* expression decreased during dedifferentiation, implying that it may be involved in loss of chondrogenic markers and the chondrocyte phenotype. Direct stimulation of P0 human chondrocytes with BMP-2 increased the expression of *COL2A1*, *SOX9*, *FGFR3* and *PTH1R*, all of which showed decreased expression during dedifferentiation (Supplemental Fig. 3A). When 100 ng/µl

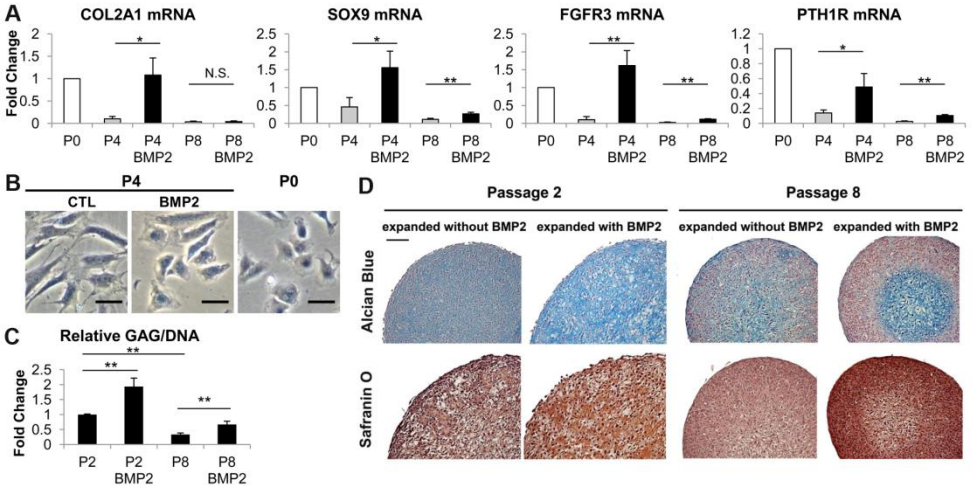


Figure 5. Maintenance of differentiated chondrocyte phenotype by BMP-2. A. Human chondrocytes were cultured with or without 100 ng/ml BMP-2 from P0 until P8. mRNA expression levels were measured by qPCR. * = $P < 0.05$, ** = $P < 0.01$, N.S. = Not Significant, n = 3 donors. **B.** Light microscopic images of chondrocytes cultured with or without BMP-2 were taken at P4. Scale bar = 5 µm. Images from one representative donor out of 3 donors are shown. **C&D.** P2 and P8 chondrocytes expanded with or without 100ng/ml BMP-2 were re-differentiated in pellet culture in chondrogenic differentiation medium. (C) GAG production of chondrocyte pellets from chondrocytes expanded with or without BMP-2 was measured and normalized for DNA content. ** = $P < 0.01$, n = 3 donors. (D) Histological staining of GAG / sulphated GAG in pellet sections was performed. Scale bar = 100 µm. Images from one representative donor out of 3 donors are shown.

BMP-2 was added during extensive expansion until P8, downregulation of these genes was counteracted at P4 and decelerated at P8 except for *COL2A1* (Fig. 5A). Chondrocytes cultured in the presence of BMP-2, in comparison to cells cultured in the absence of BMP-2, showed a reduction in proliferation at P2, but demonstrated no significant difference in proliferation rate between control and BMP-2-expanded chondrocytes at P8 (Supplemental Fig. 3B). In addition, BMP-2 was able to reduce phenotypic changes of the chondrocytes during dedifferentiation (Fig. 5B). Chondrocytes expanded without BMP-2 became flattened and elongated while BMP-2-treated chondrocytes stayed rounded through culturing from P0 to P4 (Fig. 5B). In agreement with previous findings, the growth of chondrocytes decreased during dedifferentiation (Supplemental Fig. 3B) (19). To test whether the presence of BMP-2 during culture expansion enhances the capacity of expanded chondrocytes (P2 and P8) to produce cartilage matrix, chondrocytes cultured

with or without BMP-2 were re-differentiated in pellet cultures in chondrogenic differentiation media. GAG production was significantly higher in chondrocyte pellets formed by cells expanded in the presence of BMP-2 at both P2 and P8 (Fig. 5C). Moreover, histological staining of chondrocyte pellet sections revealed that BMP-2 stimulated the production of cartilage-specific ECM (Fig. 5D). Taken together, these data suggest that loss of *BMP2* expression may be an important trigger of chondrocyte dedifferentiation. Moreover, it suggests that BMP-2 signaling seems to be upstream of many other events in the dedifferentiation, especially at early stage of dedifferentiation.

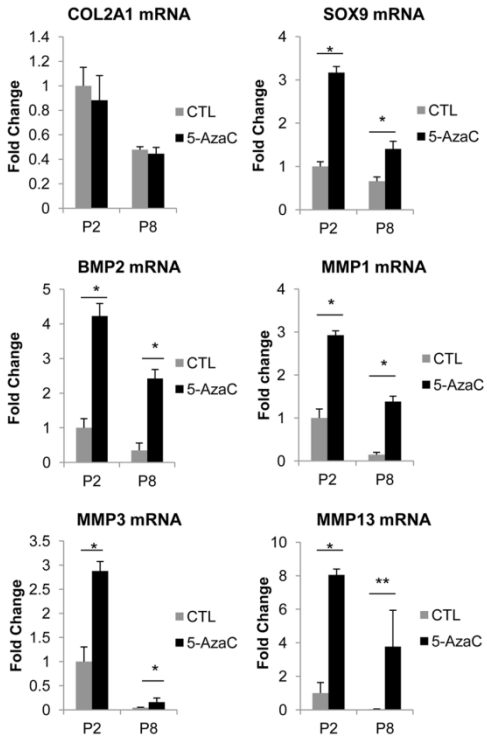


Figure 6. Involvement of DNA methylation in downregulation of gene expression. P2 or P8 chondrocytes were treated with 2 μM 5-AzaC for 48 hours. mRNA levels were measured by qPCR. * = $P < 0.05$, ** = $P < 0.01$, n = 3 donors.

Involvement of DNA methylation in downregulation of gene expression. Since more genes showed down-regulation of expression during chondrocyte dedifferentiation, we explored the role of gene expression silencing by DNA methylation. P2 and P8 chondrocytes were transiently treated with the chemical analog of cytidine 5-AzaC, which inhibits DNA methylation. *SOX9* and *BMP2* expression was increased by DNA methylation inhibition, though *COL2A1* was not affected (Fig. 6). *MMP1*, *MMP3* and *MMP13* also showed increased expression in response to 5-AzaC treatment (Fig. 6). Therefore DNA methylation may be an important mechanism for the down-regulation of a subset of genes during dedifferentiation of human articular chondrocytes.

Discussion

In this study, we analyzed gene expression profiles of human articular chondrocytes during dedifferentiation in monolayer culture. Global gene expression changes occurred gradually and cumulatively during extensive expansion. The ability of chondrocytes to produce ECM is inversely correlated with the culture period. While P2 chondrocytes can still be induced to produce substantial amounts of ECM, the ability of P8 chondrocytes to produce ECM is severely compromised. This is confirmed by our observation that expression of *COL2A1* and *COL9A1* decreases in time. Other markers such as *BMP2* and *FGFR3*, which predict the capacity of expanded human chondrocytes to form stable cartilage in vivo (25), also showed continuous decrease during dedifferentiation. The chondrogenic transcription factor *SOX9* exhibited a steep reduction in expression from P0 to P2. SOX9 is the key transcription activator driving *COL2A1* expression (26-28) and overexpression of SOX9 enhances the ability of chondrocytes to maintain their differentiated phenotype during expansion (20, 21). Loss of these important cartilaginous markers may eventually lead to the loss of ability of chondrocytes to produce cartilage-specific ECM and form stable cartilage in vivo.

In order to shed light on possible factors that play an important role in regulating or preventing dedifferentiation, we investigated the role of ERK, Wnt and BMP signaling in preventing dedifferentiation of primary human chondrocytes in culture. These signaling pathways were at the crossroads of many genes shown to be gradually but significantly altered during prolonged culturing. Perturbation of these signaling pathways during extended culturing provides opportunities for the prevention of chondrocyte dedifferentiation and preservation of the chondrocyte phenotype. The outcome of ACI procedures would be greatly improved with preservation of the chondrocyte phenotype.

Pathway analysis of microarray data revealed that ERK is connected to many of the genes that are affected by the extensive passaging. MEK inhibitor increased *COL2A1* expression in human chondrocytes, which is consistent with previous findings that ERK is involved in chondrocyte dedifferentiation (2). In addition, ERK is linked to the role of αvβ5 integrin in chondrocyte dedifferentiation (15). In our microarray data we did not observe significant changes in αvβ5 integrin expression. Despite this, inhibition of the ERK pathway showed only weak effects on marker gene expression in dedifferentiated chondrocytes. Therefore

the change of ERK activity may be a secondary event in dedifferentiation and blocking of ERK activity is not sufficient to maintain the chondrocyte phenotype. Other signaling mediators such as JNK, PI3K and p38 MAPK also appeared in the pathway network, indicating that many of these regulatory pathways are affected by dedifferentiation. These signaling networks may cooperate in prevention of dedifferentiation.

Many genes encoding proteins in the Wnt signaling pathway showed changes in expression during dedifferentiation. Consistent with the observation in animal chondrocytes (3), β-catenin protein gradually accumulated in dedifferentiating human chondrocytes. However, in contrast to the animal study (3), inhibition of canonical Wnt pathway by using a β-catenin/TCF inhibitor or gene knockdown failed to preserve chondrocyte marker gene expression in human chondrocytes. We recently found that β-catenin is a negative regulation of MMP expression in human chondrocytes (29). This suggests that the increase in β-catenin protein may at least partly contribute to the decrease in MMP expression during dedifferentiation. The expression of the non-canonical *WNT5A* gradually increased in expanded chondrocytes. Stimulation of chondrocytes with Wnt-5A decreased *COL2A1* expression while knockdown of WNT5A increased COL2A1, suggesting that increase of *WNT5A* may regulate expression of *COL2A1* and possibly other genes during dedifferentiation. There was a change in the expression pattern of Wnt receptor Frizzled (FZD). *FZD4* expression was upregulated while *FZD8* and *FZD9* expressions were downregulated. Given the fact that different receptors selectively activate distinct pathways (30), there may be a change in the response and sensitivity of chondrocytes at later passages to extracellular Wnts.

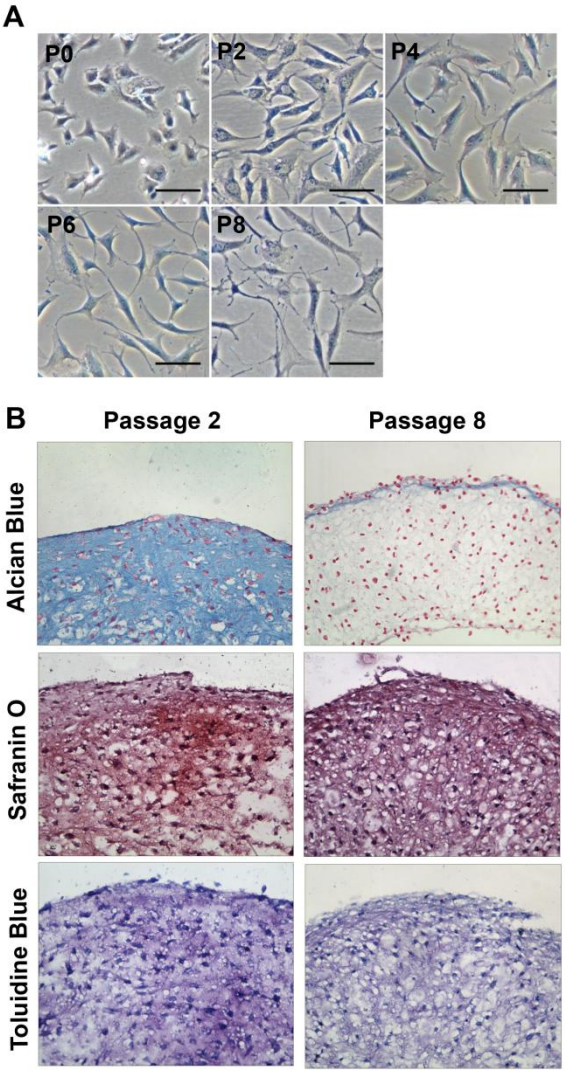
BMP-2 is a stimulator of chondrocyte marker genes including *COL2A1* and *SOX9* (22-24). Loss of *BMP2* may be involved in human chondrocyte dedifferentiation. Stimulation of P0 chondrocytes with BMP-2 increased the expression of *COL2A1*, *SOX9* as well *FGFR3* and *PTH1R*. BMP-2 enhances the ability of human chondrocytes to maintain their differentiated phenotype and exhibits more pronounced effects at early stages of dedifferentiation. However, treatment with BMP-2 alone was not sufficient to rescue *COL2A1* expression at P8. Chondrocytes expanded with BMP-2 produced significantly more ECM than chondrocytes expanded without BMP-2. FGFR3 has been involved in cartilage development and homeostasis (33-35). Parathyroid hormone-related protein

(PTHrP)/PTH1R signaling may also be important for homeostasis of chondrocytes as it promotes survival of in vitro cultured chondrocytes (36) and PTH1R expression is decreased in OA chondrocytes (37). Loss of these important factors for chondrocytes may contribute to the loss of differentiated chondrocyte phenotype. Therefore the effect of BMP-2 on chondrocyte dedifferentiation might at least partly depend on its effect on the expression of these factors. This also implies that chondrocytes expanded with BMP-2 may form more stable cartilage in vivo and may therefore improve the therapeutic outcome.

Dedifferentiating chondrocytes may become increasingly inactive as demonstrated by the increase in population doubling time at later passages and the microarray data, which show that the expression of most genes is progressively down-regulated. DNA methylation is an epigenetic mechanism for gene expression silencing and is implicated in chondrogenesis and cartilage degeneration (38-40). Treatment of human chondrocytes with the DNA methylation inhibitor 5-AzaC increased the expression of a set of genes down-regulated during dedifferentiation. Most importantly, expression of chondrogenic markers *BMP2* and *SOX9* was upregulated upon inhibition of DNA methylation. It suggests that expression of a set of genes might be cumulatively repressed by DNA methylation or other epigenetic effects during chondrocyte dedifferentiation, which contributes to the gradual loss of differentiated phenotype.

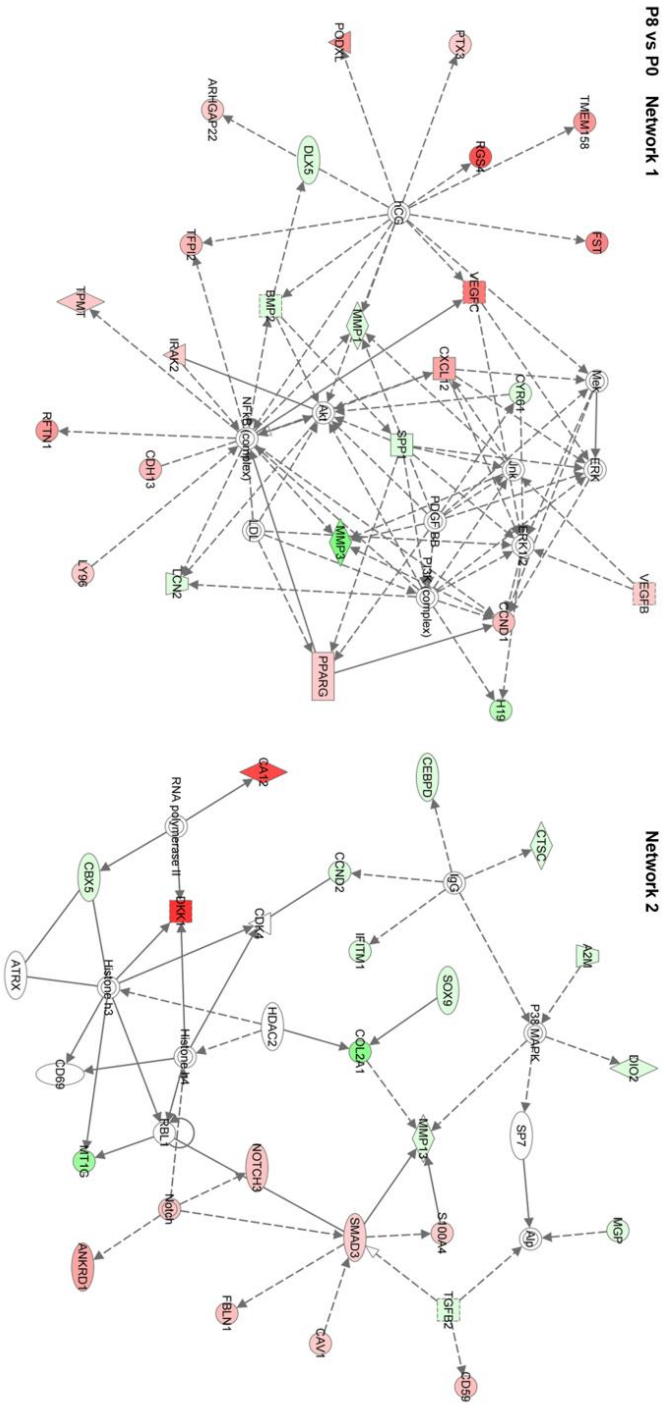
Whole genome gene expression profiling enabled us to identify relevant pathways in human chondrocyte dedifferentiation, which allows the evaluation of potential molecular mechanisms for human chondrocyte dedifferentiation. Such studies provide useful knowledge for modifying culture conditions of human articular chondrocyte and eventually improve the outcome of cell therapy to repair cartilage lesions.

Supplemental Data

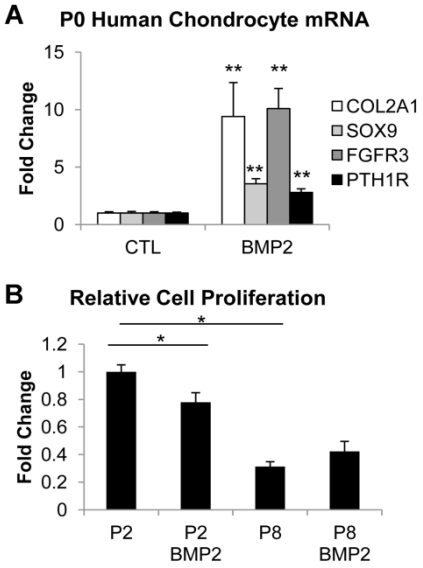


Supplemental Figure 1. Loss of differentiated phenotype and re-differentiation ability in monolayer culture. A. Microscopic images of human chondrocytes were taken at passage 0, 2, 4, 6, and 8. Images of a representative donor (n = 4) are shown. Scale bar = 10 µm. **B.** Histology of redifferentiated human chondrocyte pellets formed by chondrocytes from P2 and P8. Images of a representative donor (n = 3) are shown.

Supplemental Figure 2. Pathway analysis of chondrocyte dedifferentiation. Pathway networks were reconstructed using significantly changed genes comparing P8 with P0 using microarray data of 4 donors. Two major hubs were identified and are shown. Red color indicates upregulation of gene expression and green indicates downregulation. Pathway intermediates such as ERK, PI3K, Akt and NF-κB in the left panel and p38 in the right panel are connected with genes showing significant changes in dedifferentiation.



Supplemental Figure 2



Supplemental Figure 3. Effects of BMP-2 on human chondrocytes. A. P0 human chondrocytes were cultured in the presence or absence of 100 ng/ml BMP-2. mRNA expression levels were measured by qPCR. ** = $P < 0.01$, n = 3 donors. **B.** Proliferation of human chondrocytes cultured in the presence or absence of 100 ng/ml BMP-2 was measured using a BrdU proliferation assay (Roche) according to the manufacturer's instructions. * = $P < 0.05$, n = 3 donors.

Supplemental Table 2

	P2 vs P0		P4 vs P0		P6 vs P0		P8 vs P0	
	Gene	Fold Change	Gene	Fold Change	Gene	Fold Change	Gene	Fold Change
1	UNQ830	-13.94	MMP3	-42.60	MMP3	-71.02	C2orf40	-59.87
2	FGFBP2	-13.19	MT1G	-33.10	C2orf40	-38.54	SERPINA3	-57.50
3	COL2A1	-10.28	UNQ830	-21.18	UNQ830	-32.02	UNQ830	-38.36
4	SERPINA5	-7.79	COL2A1	-17.46	SERPINA3	-31.05	MMP3	-32.90
5	SERPINA3	-7.53	FGFBP2	-16.97	MT1G	-29.29	CHI3L2	-32.73
6	PLA2G2A	-7.47	C2orf40	-14.55	COL2A1	-25.74	COL2A1	-28.89
7	C2orf40	-7.24	SERPINA1	-12.57	FGFBP2	-19.61	MT1G	-24.94
8	H19	-7.16	H19	-11.94	PLA2G2A	-18.43	PLA2G2A	-20.36
9	CHI3L2	-6.87	MT1F	-11.55	CHI3L2	-17.60	FGFBP2	-19.33
10	MMP3	-6.26	PODN	9.48	H19	-15.18	H19	-17.30
11	SERPINA1	-6.06	SERPINA5	-9.12	SERPINA1	-14.50	GLDN	-15.18
12	GLDN	-5.66	SERPINA3	-8.97	SERPINA5	-13.79	SERPINA5	-15.07
13	SPP1	-5.45	PLA2G2A	-8.93	MT1F	-13.06	SERPINA1	-14.12
14	C13orf15	-5.35	ARHGDIB	-7.53	CA12	8.87	MT1F	-12.12
15	FOSB	5.05	MT1H	-7.11	MT1X	-8.25	SMOC2	-10.54

16	LCN2	-4.75	C13orf15	-6.85	MFAP5	8.06	DKK1	10.48
17	PODN	4.29	CDC20	-6.63	MMP1	-7.66	COL11A1	-10.15
18	FZD9	-4.21	MMP1	-6.48	COL11A1	-7.58	FOXQ1	8.82
19	ACTG2	4.07	MT1X	-6.33	GLDN	-7.17	C20orf82	-8.18
20	MT1G	-3.96	SPP1	-5.96	TCEAL2	-7.01	CA12	7.87
21	MT1F	-3.93	CA12	5.87	C13orf15	-7.01	MT1X	-7.77
22	RGS4	3.84	LCN2	-5.81	MT1H	-6.92	FLNC	7.63
23	ACTA2	3.74	MT1M	-5.75	DKK1	6.57	MFAP5	7.62
24	MT1H	-3.72	GLDN	-5.70	DRAM	6.31	RGS4	7.45
25	C20orf82	-3.54	SVEP1	5.33	MT1M	-6.30	TCEAL2	-7.29
26	TAGLN	3.48	TK1	-5.21	FZD9	-6.00	MMP1	-7.05
27	PTHR1	-3.40	FZD9	-5.16	SPP1	-5.98	MT1H	-6.78
28	TNFRSF11B	-3.40	MFAP5	5.14	FLNC	5.84	ALCAM	6.45
29	GPNMB	3.38	PTTG1	-5.06	LCN2	-5.70	FRZB	-6.35
30	SOX8	-3.36	AGT	4.99	FOXQ1	5.68	ITM2A	-6.32
31	SERPINE1	3.36	CHI3L2	-4.90	CRTAC1	-5.56	CCND2	-6.24
32	BMP2	-3.31	TYMS	-4.85	SOX8	-5.52	MGP	-6.13
33	PC-3	-3.31	SOX8	-4.83	ITM2A	-5.46	FZD9	-6.07
34	MMP1	-3.23	CCNB2	-4.81	RGS4	5.45	C13orf15	-6.07
35	DUSP5	3.21	UBE2C	-4.65	ARHGDIB	-5.37	SPP1	-6.06
36	MATN3	-3.13	KIF20A	-4.58	FRZB	-5.10	OGN	-6.05
37	RPESP	-3.09	WNT5A	4.48	WNT5A	5.10	VEGFC	5.79
38	CDO1	-3.03	PTTG3	-4.45	PODN	5.03	SOX8	-5.69
39	CRTAC1	-3.01	TCEAL2	-4.39	C20orf82	-5.02	MT1M	-5.68
40	SOX9	-3.00	DBC1	4.33	MGP	-4.97	LCN2	-5.38
41	TMEM119	3.00	COL11A1	-4.30	PDE5A	4.92	PC-3	-5.38
42	CD248	2.99	RFTN1	4.25	GPNMB	4.89	COLEC12	-5.37
43	ALCAM	2.96	LOC399942	-4.22	PC-3	-4.84	TUBB2B	-5.36
44	CCND2	-2.96	GPNMB	4.07	VCAN	4.74	ACTA2	5.34
45	SLC7A2	-2.94	DRAM	4.03	KIAA1199	4.64	FST	5.32
46	NOTCH3	2.86	GINS2	-4.01	FST	4.49	CRTAC1	-5.22
47	MYL9	2.86	TOP2A	-3.93	ALCAM	4.33	SLC7A2	-5.02
48	FRZB	-2.84	BMP2	-3.92	CCND2	-4.26	PODXL	4.72
49	SCIN	-2.84	MGP	-3.91	RFTN1	4.26	WNT5A	4.71
50	COL11A2	-2.82	LDB2	3.86	MST4	-4.23	OMD	-4.71

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Chapter 7

General Discussion

Role of chondrocytes in cartilage degeneration

Cartilage consists primarily of matrix such as collagens and proteoglycans and is sparsely populated by chondrocytes. These chondrocytes perform matrix-generation and maintenance functions. Homeostasis of cartilage tissue is maintained by the balance of synthesis and degradation of extracellular matrix (ECM) by chondrocytes. Articular cartilage is the major target tissue for destruction in both osteoarthritis (OA) and rheumatoid arthritis (RA), which both disturb ECM homeostasis. Although all tissues in the diseased joint are affected, it appears that articular chondrocytes are the key mediators in joint cartilage destruction by responding to adverse environmental stimuli. For example, chondrocytes can respond to direct biomechanical perturbation by up-regulating tissue formation or by increasing the production of inflammatory cytokines which drive tissue degradation. These inflammatory cytokines are also produced by other joint tissues (1). Inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor α (TNFα) trigger up-regulation of catabolic gene expression and activity in chondrocytes resulting in subsequent cartilage degradation (2, 3).

Cartilage has limited regenerative capacity once damaged. The golden standard for repair of focal cartilage defects is autologous chondrocyte implantation (ACI). This procedure relies on the isolation of chondrocytes out of a biopsy taken from a non-weight bearing site of the affected joint and their subsequent expansion by in vitro cell culture. Dedifferentiation of chondrocytes during *in vitro* culture is a major obstacle in ACI procedures. The molecular mechanisms underlying this process are largely unknown. Therefore, investigation of chondrocyte biology in cartilage degeneration and dedifferentiation is crucial for development and improvement of therapeutic strategies for cartilage disease. In addition to animal models, human articular chondrocytes are the primary target of study in this thesis.

Wnt function in human chondrocytes

In contrast to the considerable knowledge regarding the role of Wnt signaling in chondrogenesis and cartilage development, relatively little is known regarding the role of Wnt signaling in the maintenance and destruction of cartilage tissue. Evidence predominantly from animal studies suggests that ectopic Wnt signaling may lead to

cartilage degradation by inducing catabolic gene expression, endochondral ossification and chondrocyte apoptosis (4-6). Indeed, canonical Wnt signaling potently induces the expression of matrix metalloproteinases (MMPs) driving cartilage degradation in mouse chondrocytes and mouse embryonic fibroblasts. In contrast to the findings in animal chondrocytes, in human chondrocytes Wnt/β-catenin activation represses the expression of catabolic MMPs including MMP-1, -3 and -13 (Chapter 4). Wnt signaling was also shown to regulate gene expression in human and mouse mesenchymal stem cells in opposite ways (7). Such species difference leads to the question whether Wnt/β-catenin is a pathogenic factor in human cartilage diseases like osteoarthritis. Indeed, species differences in the function and activity of other pathways have been described (8, 9). Therefore, knowledge obtained from animal studies particularly with respect of the role of Wnt/β-catenin signaling in osteoarthritis should be carefully extrapolated to human disease.

The ability of β-catenin to decrease MMP expression in human chondrocytes indicates that Wnt/β-catenin pathway may have anti-catabolic activity in human cartilage. Wnt/β-catenin activation forms a negative feedback loop downstream of the stimulation of IL-1β, a pro-inflammatory cytokine involved in both OA and RA. One of the key Wnt members potentially involved in the process is Wnt-7B. We showed that knock down of Wnt-7B effectively pertubated this feedback loop and potentiated IL1 induced MMP expression (Chapter 4). Interestingly, Wnt-7B expression is also upregulated in OA cartilage and RA synovium (10). Such feedback mechanism might be an attempt of the cells to counteract the excessive catabolic activity induced by proinflammatory cytokines during cartilage destruction. The use of Wnt-7B for the treatment of cartilage degeneration requires more investigation.

The effect of Wnt activation on MMP expression is independent of the transcriptional activity of the canonical pathway, but instead is due to an inhibitory interaction of β-catenin with NF-κB. A direct function of the canonical Wnt pathway is to promote chondrocyte proliferation (11). The function of transcription factors of Wnt/β-catenin pathway was further explored (Chapter 5). TCF4 was found to increase MMP expression and chondrocyte apoptosis by potentiating NF-κB signaling while LEF1 and TCF3 showed opposite effects. This action of TCF4 is also independent of its conventional transcriptional

function. We provided evidence that this effect is likely independent of β-catenin. Instead we demonstrated that TCF4 directly interacted with NF-κB augmenting transcription from NF-κB target genes. Elevated TCF4 mRNA expression was observed in human osteoarthritic cartilage indicating that increased TCF4 might be a pathogenic factor contributing to the development and progression of OA. Disturbing the interaction between TCF4 and NF-κB opens a new window for developing therapeutic strategy for cartilage degeneration.

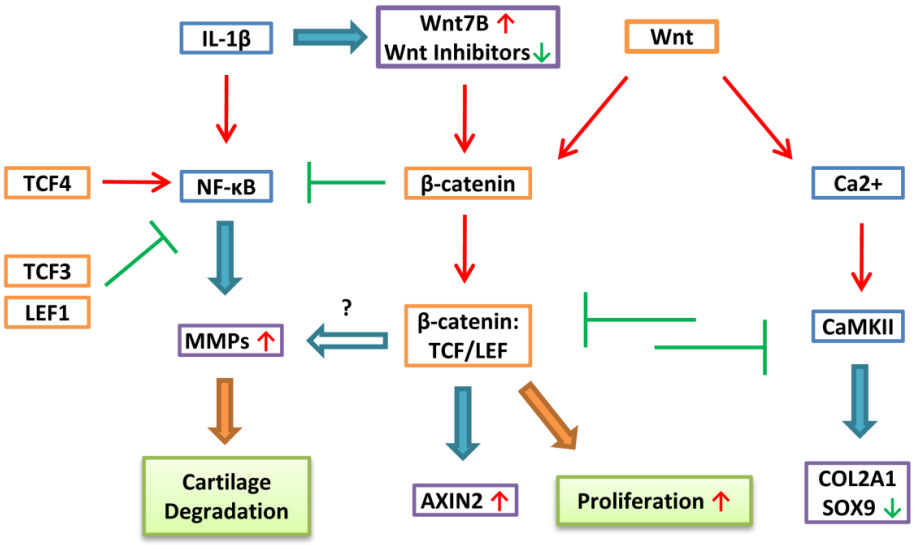

Figure 1. Function of Wnt signaling in human cartilage. Wnt proteins are able to activate both canonical and non-canonical pathways. The β-catenin-dependent pathway induces cell proliferation and the expression of established β-catenin target genes such as AXIN2. The non-canonical pathway decreases the expression of typical chondrocyte markers like COL2A1 and SOX9 via the Ca^{2+}/CaMKII cascade. The canonical and non-canonical pathways reciprocally inhibit each other. IL-1β up-regulates MMP-1, -3 and -13 expression predominantly through activation of the NF-κB pathway resulting in cartilage degradation. Simultaneously IL-1β indirectly activates β-catenin through up-regulation of Wnt-7B expression and down-regulation of canonical Wnt inhibitors like DKK-1, FRZB and WIF-1. Stabilized β-catenin interacts with and inhibits NF-κB which leads to the suppression of MMP expression in a negative feedback loop. TCF4 potentiates while TCF3 and LEF1 repress NF-κB activity. It remains unclear whether the transcriptional activity of β-catenin-TCF/LEF is directly involved in human cartilage degeneration.

Previous studies using animal chondrocytes suggest that increased Wnt/β-catenin signaling is a trigger for chondrocyte dedifferentiation which is associated with monoculture expansion of chondrocytes. However, in human chondrocytes, β-catenin appears not to be involved in loss of expression chondrocyte markers such as COL2A1 or SOX9 during serial passaging of monolayer cultures of primary chondrocytes (Chapter 6). This is in agreement with a recent study showing that Wnt-3A decreases chondrocyte marker gene expression through a β-catenin-independent non-canonical Wnt pathway (11). In contrast, non-canonical Wnt-5A represses COL2A1 expression in both human and animal chondrocytes (12). In line with a role in dedifferentiation are our observations that the expression of Wnt5A gradually increases and that knock down of Wnt-5A during serial passaging of primary human chondrocytes restores Col2A1 expression and preserves the rounded chondrocyte phenotype. Besides down regulation of COL2A1 and other chondrocyte markers, Wnt-5A also up-regulates MMP expression (13). Collectively, current evidence suggests that the non-canonical Wnt signaling contributes to chondrocyte dysfunction and cartilage degeneration while activation of the canonical pathway might not be a key event in the pathogenesis of human OA.

Taken together, a complicated role of Wnt signaling has been unraveled in human chondrocytes (Fig.1). The same Wnt protein may transduce both canonical and non-canonical pathways at the same time. For example, Wnt-3A, which was considered as a canonical Wnt protein, activates both β-catenin and Ca^{2+}/Calmodulin-dependent Protein Kinase II (CaMKII) pathways (11). Similarly, Wnt5A, which was considered as a non-canonical Wnt protein, is able to activate β-catenin-TCF cascade under certain conditions (14). Therefore it has been proposed that Wnts themselves are not intrinsically canonical or non-canonical but that the multiple pathways that these ligands initiate are determined by distinct sets of receptors expressed at the cell surface. The expression of several Wnt members (Chapter 4) and Frizzled receptors (11) in human chondrocytes has been validated. Expression of other (co-)receptors for Wnts such as Ror2 and Ryk remains to be evaluated. It's a big challenge for future research to study the function of these Wnt proteins in the context of distinct receptors and dissect multiple pathways initiated by the same Wnt. It has been suggested that canonical and non-canonical pathways reciprocally inhibit each other (11). According to this model, blockade of the canonical Wnt/β- catenin

pathway may indirectly contribute to articular chondrocyte dedifferentiation through de-repression of the Ca^{2+}/CaMKII pathways. This is supported by our observations that knockdown of β-catenin or use of a β-catenin/TCF inhibitor represses COL2A1 expression similarly as Wnt-5A does in the non-canonical pathway (Chapter 6). These data substantiate the role of the non-canonical cascade in the deregulation of chondrocyte function. Stimuli such as IL-1 (15) and biomechanical loading of chondrocytes (16) which change the expression of CaMKII may significantly influence the outcome of Wnt signaling by switching the balance between β-catenin and CaMKII. CaMKII plays a critical role in the non-canonical Ca^{2+} pathway. Thus IL-1-mediated pro-catabolic activity in cartilage may partially come from its enhancement of the non-canonical Ca^{2+} cascade initiated by Wnt. The opposing induction of β-catenin pathway by IL-1 serves as a negative feedback to counteract its pro-catabolic activity.

The downstream effectors of the Wnt/β-catenin pathway exhibit different actions in human chondrocytes. TCF4 potentiates NF-κB signaling whereas β-catenin, TCF3 and LEF1 inhibit NF-κB activity (Chapter 4 & 5). Such diverse actions of Wnt components may result from their different binding properties (17). Since NF-κB is a potential therapeutic target for osteoarthritis (OA) and rheumatoid arthritis (RA) (18), the interaction of Wnt components with it will provide new opportunities for treatment for joint diseases. Similarly, it has also been shown that β-catenin activity negatively regulates bacteria-induced inflammation via inhibition of NF-κB (19). Therefore the interaction between Wnt and NF-κB pathways may apply to many other diseases where they are involved. Despite the indirect effect of Wnt/β-catenin pathway, a recent study in human chondrocytes also suggests the direct binding of LEF1 to the promoter of *MMP13* on a site different from the site we identified (20, chapter 5). However, knockdown of LEF1 failed to decrease MMP13 expression and LEF1 overexpression down regulated MMP-13 expression under basal condition, suggesting that the dominant effect of LEF1 is its negative effect on MMP expression via inhibition of NF-κB. Activation of specific Wnts and sets of receptors might also selectively recruit a TCF/LEF member, which adds to the complexity of signaling networks downstream Wnt activation. Therefore it's important to dissect the involvement and function of each TCF member.

Crosstalk of Wnt signaling with other pathways

It has been shown that the Wnt signal transduction pathway may crosstalk with many other signaling pathways such as BMP- (21), hypoxia- (22), oxidative stress- (23), and androgen signaling pathways (24). The crosstalk occurs at various levels. For example the Wnt pathway may regulate expression of members in other pathways, or components from two pathways may directly interact with each other. This may explain why the Wnt pathway has widespread involvement in developmental and physiological processes as well as in disease, and attracts intensive research focus.

The interaction between β-catenin and NF-κB was first reported in cancer cells (25). β-Catenin was also found to interact with and inhibit NF-κB in human chondrocytes (Chapter 4). In addition, TCF4 is able to interact with and potentiate NF-κB while TCF3 and LEF1 show the opposing effect (Chapter 5). It's unclear how exactly β-catenin and TCF/LEF interact with NF-κB. One possibility is that TCF4 and NF-κB form a new transcription complex and synergistically mediate induction of NF-κB target gene expression. Other downstream Wnt effectors like LEF1 and TCF3 which fail to do so may compete for the binding of TCF4 to NF-κB, thus reducing NF-κB activity. Previous studies have shown that another transcription factor C/EBP directly interacts with NF-κB bound to gene promoters (26) and enhances NF-κB activity by displacing histone deacetylases from NF-κB (27). Similar mechanisms may also apply to the interaction of NF-κB with TCF4 which may recruit a transcription co-factor or displace a transcription repressor. The underlying mechanism requires more investigation. However, these findings have provided important implications for the development of new therapeutic strategies for cartilage disease.

Wnt signaling as therapeutic target for osteoarthritis

It has been proposed that inhibition of Wnt/β-catenin signaling could be a potential therapeutic strategy for OA. Downregulation of DKK-1 expression with an antisense oligonucleotide ameliorates chondrocyte apoptosis, cartilage destruction, and subchondral bone deterioration in osteoarthritic knees of rats (28). A recent study also shows that DKK-1 expression in chondrocytes inhibits experimental osteoarthritic cartilage destruction in mice (29). However, according to our model (Fig. 1), a decrease in intracellular β-catenin levels in human chondrocytes, for example, by the use of an

extracellular inhibitor such as DKK-1, may lead to increased MMP levels which eventually accelerate cartilage destruction. Moreover, inhibition of β-catenin causes chondrocyte apoptosis (30). This indicates that methods targeting at β-catenin are likely to fail for the treatment of human OA.

From the pharmacological point of view, the fact that Wnt proteins conduct multiple processes has important implications. Understanding how downstream biological events are individually regulated by Wnts in different cells represents an opportunity to achieve a higher degree of target specificity. Molecules and drugs that selectively interfere with the non-canonical Ca^{2+} pathway may offer the possibility of targeting pathological mechanisms in OA and avoid adverse effects. Other methods such as targeting TCF4 expression and/or activity could be a potentially effective strategy for OA.

Future perspectives

A remarkable effort has contributed to our understanding of Wnt signaling in cartilage development and degeneration. However, the role of Wnt signaling in human cartilage disease is still poorly understood. In our studies we have shown remarkable species difference in Wnt function. Therefore, in the future more research should be focused on human material to elucidate the complex role of canonical and non-canonical Wnt signaling in the pathophysiology of OA. Given the complexity of Wnt pathway, it is important to dissect the functions of individual components and understand more insightfully the crosstalk of Wnt signaling with other pathways in order to develop more specific and effective therapeutic interventions.

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Summary

Articular cartilage provides mechanical support for joints and is responsible for smooth and pain free joint movement. It is the primary tissue affected in joint diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA) which are the leading causes of mobility-associated disability. Wnt signaling is widely involved in development and disease and has been proposed as a therapeutic target for the treatment of OA. A remarkable effort has contributed to the elucidation of the role of Wnt signal transduction in cartilage development and degeneration. Given the complexity of Wnt signaling and joint diseases, it's still far from transferring our current knowledge of Wnt signaling in cartilage to applications in the treatment for human joint diseases. This thesis aims to provide more insightful understanding of Wnt signaling function in cartilage and consists of studies using both animal models and human materials with the emphasis on human materials.

Chapter 1 introduces the background and problems of research on Wnt singling in cartilage development and degeneration, and the strategies used in this thesis to answer the research questions. Chapter 2 gives an overview of current understanding of Wnt signaling in cartilage and raises the challenges for future research.

Since cartilage development is a complicated process, chapter 3 describes an *in vivo* method to study the role of canonical Wnt signaling in cartilage using a conditional inducible knockout mouse model. This model can be crossed with transgenic mouse models carrying genetically engineered floxed *APC* alleles. APC is a member of the degradation complex of the central mediator in the canonical Wnt signaling pathway β-catenin. The results indicate that recombination can be specifically induced in cartilage tissue after birth upon injection with tamoxifen. By generation of series of transgenic mice with completely inactivated or partially active *APC* alleles, it may be possible to obtain mice with different degrees of APC activity in cartilage. This enables the investigation of the dose-dependent effects of Wnt signaling on cartilage development. Chapter 3 also describes lentiviral transduction of cells obtained from conditional APC^{15lox} knockout mice with Cre enzyme expressing lentiviruses which can induce the deletion of the APC gene to activate Wnt signaling and study its function *in vitro*. Using these validated methods, these studies are now underway.

Chapter 4 and 5 provide detailed investigation of Wnt/β-catenin signaling in human articular chondrocytes. Evidence predominantly based on animal models suggests that ectopic Wnt signaling is a pathogenic factor for cartilage degeneration. However, the function of Wnt signaling in human cartilage is largely unknown. Therefore, the effects of Wnt activation was explored in human articular chondrocytes isolated from both OA and healthy cartilage. Wnt-3A was found to stimulate cell proliferation and repressed matrix metalloproteinase (MMP) expression in human articular chondrocytes (Chapter 4). In contrast, canonical Wnt activation increased MMP expression, via the β-catenin-TCF/LEF transcription complex, in bovine and mouse cells. The repressive effect of Wnt activation on MMP expression in human chondrocytes was independent of its interaction with TCF/LEF transcription factors, but due to an inhibitory interaction of β-catenin with NF-κB. NF-κB is the key transcription factor involved in induction of MMPs in human chondrocytes. In addition, interleukin-1β (IL-1β), a pro-inflammatory factor with a possible role in arthritis, indirectly up-regulated Wnt/β-catenin signaling by increasing Wnt-7B expression and decreasing Wnt inhibitor expression. The IL-1β-induced Wnt activation formed a negative feedback loop to counteract the pro-catabolic effect of IL-1β mediated by NF-κB. The species difference in the Wnt action in this chapter suggests that more future research should focus on human models. The expression profile of Wnt members in human chondrocytes was also evaluated (Chapter 4). To further disclose the role of Wnt signaling in human cartilage, the function of TCF/LEF members was investigated (Chapter 5). Overexpression of TCF4 was found to potentiate NF-κB signaling and induce apoptosis, while overexpression of TCF3 or LEF1 repressed NF-κB activity. These effects appear to occur independently of TCF4's interaction with β-catenin. Taken together, the distinct actions of Wnt signaling components have important implications for the development of specific and effective treatments for cartilage degeneration targeting the Wnt signaling pathway.

Cartilage has limited regenerative capacity once damaged. Autologous chondrocyte implantation (ACI) is the golden standard for repair of focal cartilage defects and relies on chondrocyte expansion in culture to obtain sufficient cells for implantation. This process is associated with chondrocyte dedifferentiation. Dedifferentiation of chondrocytes during in vitro expansion is a major obstacle for ACI procedures. In chapter 6, changes in global

gene expression during human chondrocyte dedifferentiation in monolayer culture was analyzed using microarray analysis. Based on these studies several relevant pathways were identified that might play a role in governing dedifferentiation. Some of these pathways were studied in more detail in relation to this process. The non-canonical Wnt pathway, rather than the canonical Wnt pathway, is involved in human chondrocyte dedifferentiation. Loss of BMP-2 signaling may be a contributor to the dedifferentiation. These results provide useful knowledge for modifying culture conditions of human articular chondrocyte and will eventually improve the outcome of cell therapy to repair cartilage lesions.

In the general discussion (Chapter 7), a novel model for the role of canonical and non-canonical Wnt signaling in cartilage degeneration is presented and perspectives for future research are provided.

In conclusion, this thesis described an *in vivo* method to study Wnt signaling in cartilage development and unraveled the complicated functions of Wnt signaling in human articular chondrocytes.

Acknowledgements

With the completion of my PhD thesis, first I would like to thank Prof. Marcel Karperien and Prof. Clemens van Blitterswijk for giving me the opportunity to perform my PhD research in your group. Marcel, thank you for all the guidance during my PhD! You introduced me the idea of animal experiments and explained the complicated processes so well. Your insightful thoughts helped me to solve all the problems and develop many new ideas in my research. Clemens, thank you for all the inspiration you brought to me! You have shown me a unique and successful way of doing science.

It's my great pleasure to meet so many nice collegues and friends in this international group in Enschede. Jan, thank you for the scientific advice and you also let me understand the importance of always being "young". Thank you, Janine, for all the discussions and help! Many thanks to Anouk and Jacqueline for the technical support and coordination in the labs! I also want to thank Audrey for all the assistance. Liliana and Jeroen, I had very good time with you in the same office and thank you for all the helpful discussions and interesting conversations. Ellie, it's glad to work on a similar topic with you. It had been a tough project for us, but we still survived. Nicole and Chris, it's nice to meet you and it's also a pity that I didn't manage to talk with you in German. Emily, you are probably the most mysterious girl in the lab, in addition to being so cute. Ana and Hugo, you don't only make a perfect couple, but also an energetic one. Tim, hope you are doing well with your fancy bioreactor. Aart, I'm grateful for your useful suggestions during work discussions. Lorenzo, you have shown me an impressive Italian style and Italy will be my next stop right after my defense. Gustavo, you have been such a funny guy, it's happy to spend my last few months in Enschede with you in the same office. Eelco, it's really good to meet friend from Groningen in the same office in Enschede. Karolina, you have successfully demonstrated the feminine power in the lab. Roman, you have been such a kind friend and thanks for all the interesting talks. Hey, Nicolas, thank you for being such a fantastic friend! We definitely need to have more dinner sessions and long discussions in the future. All the people doing their PhD – Janneke, Anne, Nathalie, Charlene, Bach, Eric, Wim, Björn, Parthiban, Mijke, Febriyani, Andrea, Niloofar, Honglin and Leilei (and people I might have missed here) – I wish you all a joyful and fruitful PhD.

I would like to thank all my Chinese friends in Enschede. The sweet couples: Jun and Nan, Xiaoqiong and Ling, Jingwei and Zhonghua, thank you for all the help and happy moments together: the fabulous dinners, San Guo Sha and Wii games … Liang, you were my teacher for fitness, thank you for showing me that a tough guy also needs to act in a slightly different strong way! I also want to thank Li Jiaping, Yuan Huipin and Bao Chongyun for all the nice talks, which absolutely made me grow up. Zhang Qiwei, thank you for sharing with me the apartment and your taste on red wine when I first arrived in Enschede.

Six years in the Netherlands is one of the most important and memorable time in my life. My journey started from Groningen and here I would like to give my special thanks to friends in Groningen. Teng Yinglai, as my comrade-in-arm, we went through all the hard time in Groningen which is actually the most valuable gift for us. Let the courage lead our way. I will never forget my first meal in Holland you cooked for me, without salt or any other spices, but it's probably the most heart-warming dinner I have ever had. I would like to thank Eveline and Arja for your guidance in my research project! Frank, thank you for all the help in the lab and funny conversations! Robert, as my first research supervisor, thanks for being such an inspiring and kind scientist! Duan Li, thank you for all the though-provoking discussions! Li Xiangyi, as my hometown fellow, it's really great to meet you in Groningen.

I would like to thank all my friends back in China. Names are not mentioned here but you know whom I'm talking about. Thank you for the friendship, the support, and the wonderful time we spent together all these years!

Finally, I would like to express deepest love to my family. Thank you for all the care and support! All my achievements belong to you.

Sincere acknowledgments to all the people who have helped me, cared about me, inspired me, criticized me or depressed me!

Fortune favors the bold!

Curriculum Vitae

Bin Ma was born on November 6th, 1983, in Zhengzhou City, Henan Province, China. He obtained his Bachelor's degree in Biological Sciences at Lanzhou University, Lanzhou, China, in 2005, after which he continued his Master program in Medical & Pharmaceutical Sciences at University of Groningen, Groningen, The Netherlands. His study in Groningen was supported by scholarships. In 2007, he received his Master's degree with distinction *cum laude* and moved on to his PhD program in the Department of Tissue Regeneration at University of Twente, Enschede, The Netherlands. His PhD research focused on the role of Wnt signaling in cartilage development and degeneration and was performed under the supervision of Prof. Marcel Karperien and Prof. Clemens van Blitterswijk. In 2012, he completed his PhD thesis entitled "Wnt Signaling in Cartilage Development and Degeneration" and won the 2011 Chinese Government Award for Outstanding Self-financed Students Abroad.

List of Publications:

Ter Elst A*, **Ma B***, Scherpen FJG, de Jonge HJM, Douwes J, Wierenga ATJ, Schuringa JJ, Kamps WA, de Bont ESJM. Repression of vascular endothelial growth factor expression by the runt-related transcription factor 1 in acute myeloid leukemia. Cancer Res 2011; 71:2761-71. *contributed equally

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Ma B, Leijten J, Wu L, Kip M, Post JN, van Blitterswijk CA, Karperien M. Involvement of ERK, Wnt and BMP2 signaling in human articular chondrocyte dedifferentiation in monolayer culture. Submitted.

Ma B*, Landman E*, Miclea RL*, Wit JM, Robanus-Maandag EC, Post JN, Karperien M. Wnt signaling and cartilage: of mice and men. Submitted. *contributed equally