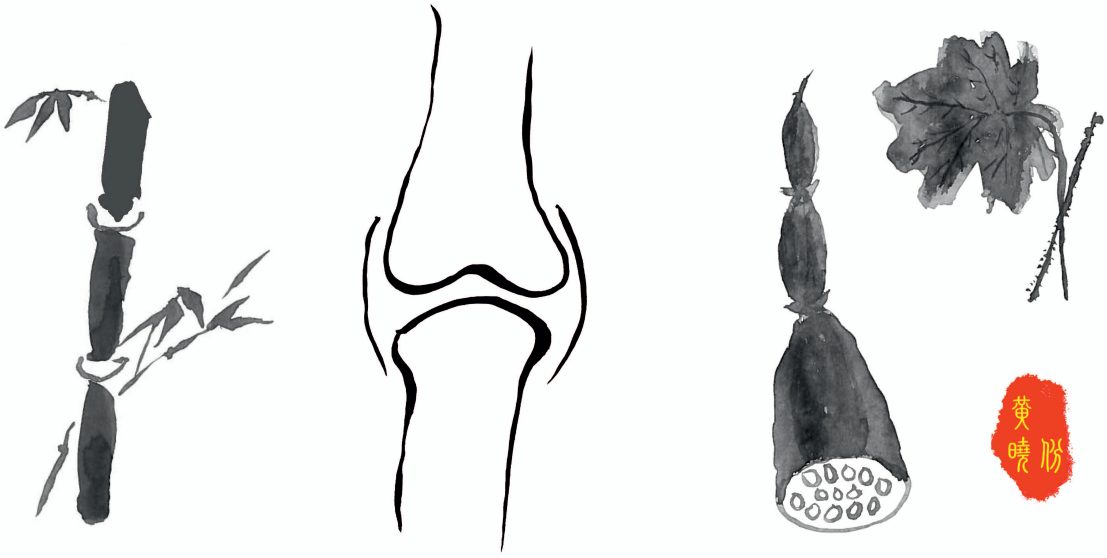
JOINT PLAYERS: ENDOGENOUS WNT ANTAGONISTS DETERMINE JOINT HEALTH



Leilei Zhong

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Leilei Zhong

2016

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PhD Thesis, University of Twente, Enschede, The Netherlands

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JOINT PLAYERS: ENDOGENOUS WNT ANTAGONISTS DETERMINE JOINT HEALTH

DISSERTATION

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Chapter 1

General Introduction and thesis outline

A journey of a thousand miles begins with a single step (千里之行, 始于足下)

Osteoarthritis (OA)

Osteoarthritis (OA) is a common joint disease and is the leading cause of pain and disability worldwide. It is a multifaceted disease characterized by progressive degradation of joint cartilage and accompanied by loss of joint function. OA symptoms including joint pain, stiffness, swelling, tenderness, and reduced mobility, associated radiographically with joint space narrowing, subchondral bone sclerosis and osteophyte formation (Figure 1) [1]. OA affects more than 70% of adults in United States [2] and cause an extremely high economic burden. OA is often diagnosed in late stages of the disease, due to the lack of specific biomarkers and very mild symptoms in early phases of the disease. There is no cure, and treatment consists of pain management and total joint replacement at the final stage of the disease process. Therefore, accumulating research efforts are devoted to understanding the molecular mechanisms underlying OA.

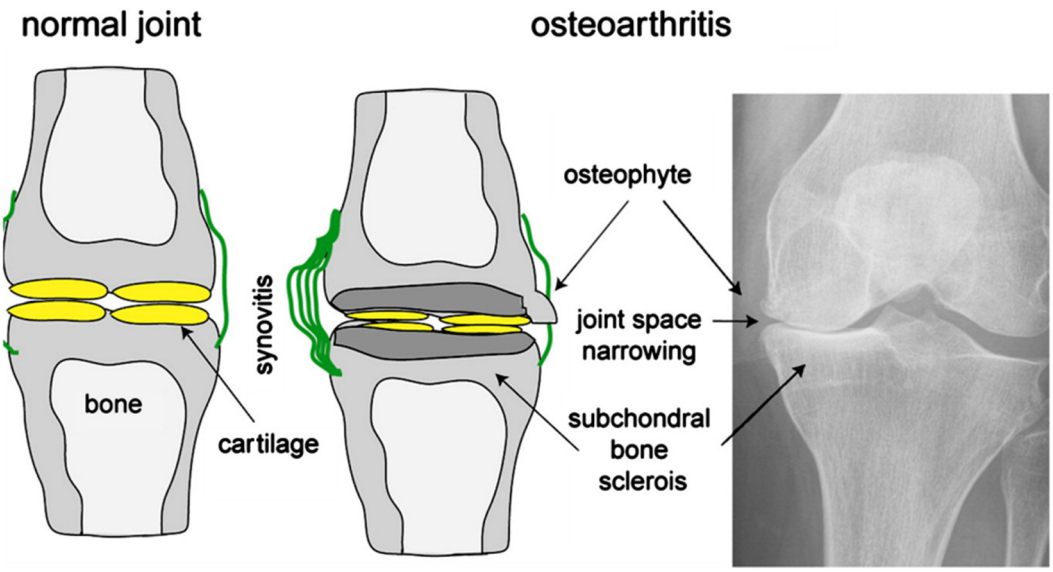

Figure 1. Anatomy of the joint and typical changes in OA. Reproduced from [1]

WNT signaling and its regulators

The conserved WNT signaling pathway is one of the fundamental pathways regulating cell fate determination, cell migration, cell polarity, neural patterning and organogenesis during embryonic development [3, 4]. The extra-cellular WNT signal stimulates several intra-cellular signal transduction cascades, including the canonical or WNT/β-catenin dependent pathway and the non-canonical or β-catenin-independent pathway [5]. The canonical WNT/β-catenin signaling is the most studied pathway. In an inactive state, WNT ligands are absent, and the

constitutively expressed cytoplasmic β-catenin protein is continually targeted for proteasomal degradation by the glycogen synthase kinase 3 (GSK3β)/the tumor suppressor *adenomatous polyposis coli* gene product (APC)/Axin complex (Figure 2A) [6]. This continual elimination of β-catenin prevents β-catenin from translocating to nucleus. In the presence of WNT ligands, WNT binds to the Frizzled receptor as well as to one of its co-receptors LRP5/6. Upon WNT ligand binding, the signal is transduced to cytoplasmic Dishevelled (Dsh/Dvl). This triggers the activation of Dvl and the disruption of the destruction complex (Figure 2B). These events lead to inhibition of GSK3β and casein kinase (CK)-mediated β-catenin phosphorylation and thereby to the stabilization of β-catenin, which accumulates and travels to the nucleus to form complexes with TCF/LEF and activates WNT target gene expression [6, 7]

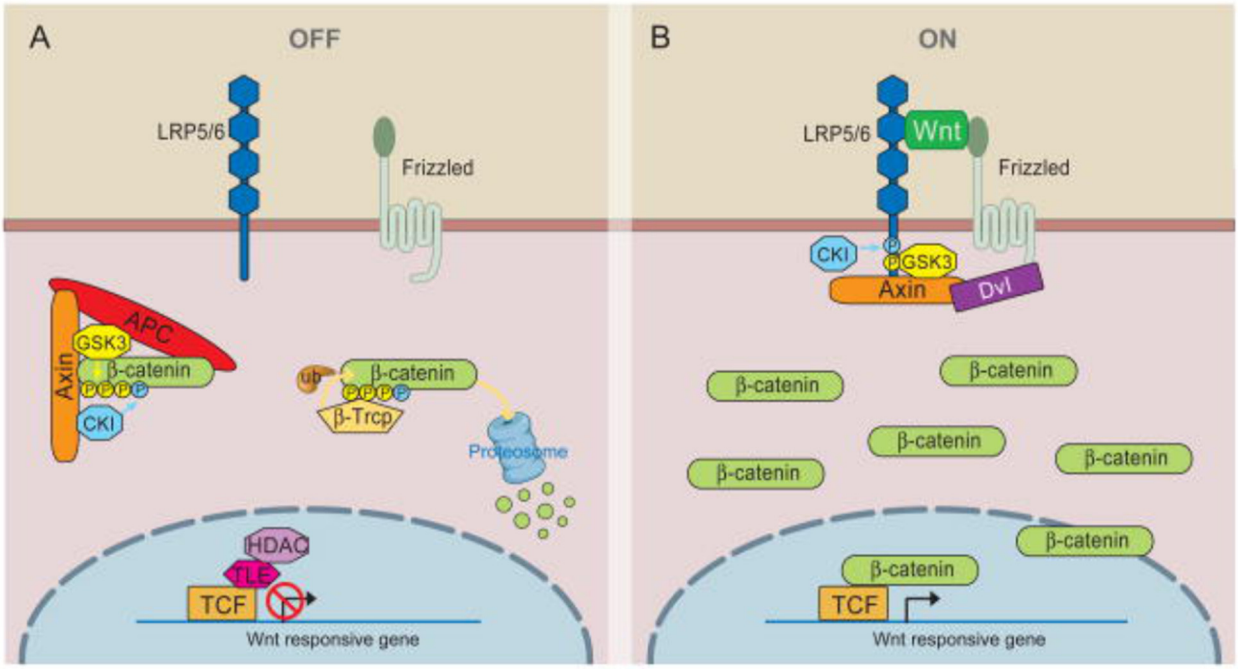

Figure 2. **WNT/β-catenin signaling.** Reproduced from [6]

WNT signaling is regulated by a number of extracellular inhibitors and activators. WNT antagonists include members of the secreted Frizzled-related protein (sFRP) class and Dickkopf (DKK) class. They antagonize WNT function through different mechanisms. The sFRP class include the sFRP family (sFRP1, 2, sFRP3 / FRZB, sFRP4 and 5), WIF-1, and Cerberus, and bind directly to WNT, thereby preventing WNT binding to its receptor complex (Fig. 1b); The Dickkopf class, which includes certain Dickkopf family proteins such as DKK1, DKK2, DKK3 and DKK4, form a ternary complex with LRP5/6 and Kremen followed by internalization of this complex and removal of LRP5/6 from the cell surface [8, 9]. For sFRP, it has been shown

to also bind to frizzled receptors to form non-functional complexes. Thus the proteins of the sFRP class will inhibit both canonical and non-canonical pathways, whereas those of the DKK class specifically inhibit the canonical pathway [10].

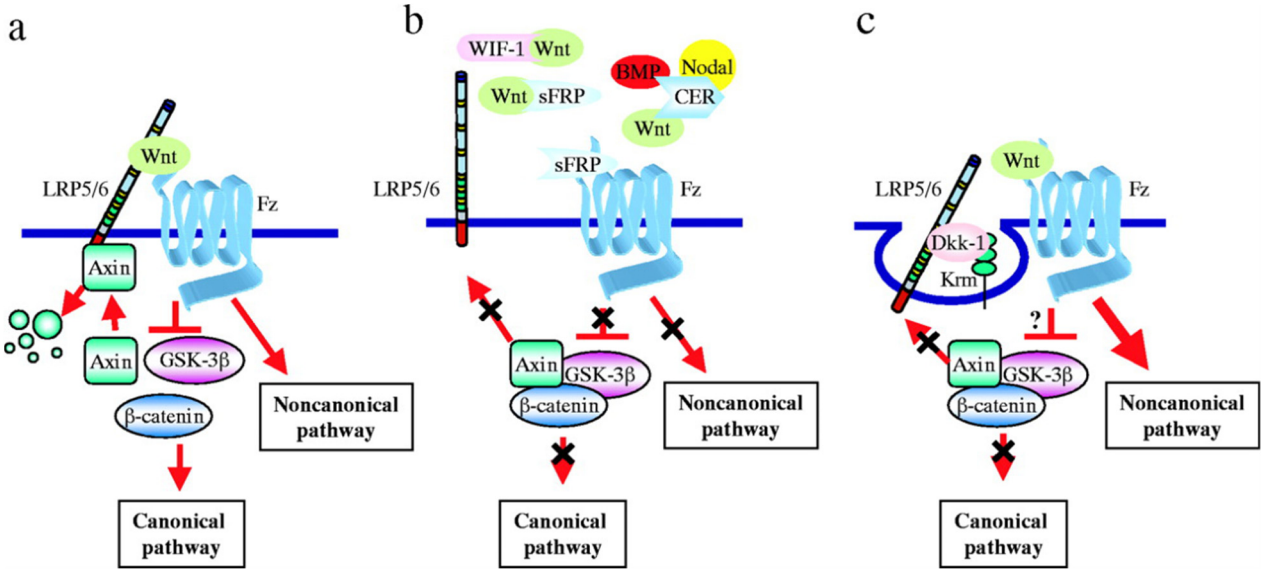

Figure 3. WNT signaling is modulated by antagonists. A. WNT signaling start from WNT binding to frizzled and LRP5/6. Then the destruction complex is disrupted. Cytoplasmic β-catenin protein is stabilized and translocates to nucleus to initiate target gene transcription. B. WNT antagonist FRZB, CER and WIF-1 prevent WNT from binding to its receptors. Therefore, blocking both canonical and non-canonical pathway. FRPs may also bind to Fz to inhibit WNT signaling. C. DKKs bind to LRP5/6 and co-receptor Kremen1/2, and triggers LRP5/6 internalization, thereby disrupting the formation of the LRP5/6-WNT-frizzled complex. β-catenin is phosphorylated by destruction complex and then canonical WNT pathway is blocked. Reproduced from [10].

The WNT family of secreted glycoproteins consists of 19 secreted WNT proteins. These proteins act as ligands to activate the distinct intracellular WNT pathways by paracrine and autocrine way [11, 3]. Two types of proteins-Norrin and R-spondin (Rspo), which are unrelated to WNTs , they are the agonists of WNT signaling. Both activate WNT signaling by binding to WNT receptors or by releasing a WNT-inhibitory protein. Norrin is a specific ligand for Fz4 and acts through Fz4 to activate LEF/TCF-medicated transcription in an LRP5/6 dependent way during retinal vascularization [12]. Rspo activates canonical WNT signaling and requires the presence of WNTs. The exact mechanism behind this activation still controversial [13].

WNT signaling in OA

Multiple signaling pathways have been shown to be involved in OA. WNT signaling appears to be a crucial player in OA pathology. Indeed, increased nuclear β-catenin staining is observed in OA cartilage. Both *in vivo* and *in vitro* data show that the activation of canonical and non-canonical WNT signaling induces expression of matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS). This indicates a switch from anabolism to catabolism followed by loss of tissue function [1, 14-16]. *In vivo*, loss of β-catenin function induces chondrocyte apoptosis and loss of superficial zone [17, 18] and *Lrp6* mutant mice display apoptosis and increase OA severity [19]. These findings suggest that although increased WNT/β-catenin signaling plays a catabolic role for OA in articular cartilage, a certain level of this signaling is required for maintenance of cartilage homeostasis.

Several WNT pathway proteins have been studied in joint disorders such as OA and rheumatoid arthritis (RA). It has been reported that the expression of WNT7b, WNT-16, β-catenin, and the WNT target gene WNT-induced signaling protein 1 (WISP-1) is upregulated in OA [20-22]. Multiple whole genome studies have shown that single nucleotide polymorphisms (SNPs) in the WNT antagonist *FRZB* [23], occur frequently with hip OA in females [24]. This association of FRZB with susceptibility for OA was further confirmed in other cohorts [25-28]. It has been reported that elevated circulating serum levels of DKK1 is associated with reduced progression of radiographic hip osteoarthritis (RHOA) in elderly women, and higher FRZB levels in serum show a tendency to be related with a modest reduction in risk of incident RHOA [29]. This is in line with our previous study showing that WNT antagonists DKK1 and FRZB have a protective role in cartilage by preventing hypertrophic differentiation [30], and that the loss of their expression is observed in OA [31]. In addition, overexpression of WIF-1 inhibits cartilage destruction in TNF-transgenic mice, and SOST is expressed in a late stage of human OA suggesting that both WNT inhibitors WIF-1 and SOST may have protective role in cartilage degeneration [32, 33]. A recent study found that WIF-1 expression is reduced in knee OA patients and is negatively correlated with Mankin score [34], further supporting the role of WIF-1 in OA progression.

In view of the complexity of the modulation of WNT signaling and the different roles of its members, including its antagonists, targeting WNT signaling in OA is a challenge, and more research should be devoted to the joint role of WNT and WNT related proteins.

Aims and Outline of this Thesis

This thesis aims to elucidate the role(s) of the endogenous WNT antagonists DKK1 and FRZB in the human joint. There are several questions which are addressed in this thesis: i) how does expression of cartilage-related genes change in cartilage and synovial fluid during OA progression (Chapter 3 and 4); ii) what is the role of WNT signaling and its antagonists (DKK1 and FRZB) in articular cartilage (Chapter 5 and 6); iii) how are WNT antagonists regulated by the inflammatory factor IL1β (Chapter 7); and iv) how can we use our knowledge to improve the understanding OA pathology and its treatment (Chapter 8).

Chapter two provides a comprehensive review on recent literature describing signaling pathways in the hypertrophy of isolated chondrocytes and MSCs-derived *in vitro* differentiated chondrocytes, with an emphasis on the crosstalk between these pathways.

OA is a disease that affects the whole joint including cartilage, synovial tissue, bone, ligaments and muscles. Cartilage and synovial tissue are the primary tissues affected in OA. In chapter three and four, we investigate the changes of cartilage-related factors in cartilage and synovial fluid respectively. **Chapter three** describes a comprehensive study on cartilage changes with varying severity of OA at the cellular and molecular levels. We find that there is a strong negative correlation between the expression of DKK1 and FRZB (WNT antagonists) and the severity of OA, and a positive correlation between the hypertrophic marker RUNX2 and IHH and OA progression.

Since OA is a local disease, the level of factors in synovial fluid may reflect what happens in the cartilage in OA. So in **Chapter four** we further investigate the expression levels of DKK1, FRZB and other factors changed in synovial fluid from patients with early and late knee injury, OA and in healthy subjects. From the two studies described above we find that the WNT antagonists are interesting factors with changed expression in joint disease. Considering their role as WNT antagonists, the alterations of their expression will influence the status of WNT signaling. It has been shown that WNT signaling is involved in OA by switching the anabolism of articular chondrocytes to catabolism and also by inducing hypertrophic differentiation of chondrocytes (chapter two). Therefore, in **Chapter five**, we first studied the role of canonical WNT signaling in human chondrocytes, focusing on the role of TCF4/LEF transcription factors as the downstream targets of β-catenin. We revealed that TCF4 was highly expressed in OA cartilage and may contribute to cartilage degeneration by potentiating NF-κB signaling. Previously our group has identified that DKK1 and FRZB are important for cartilage homeostasis by inhibiting hypertrophic differentiation and the results from Chapter 3 and Chapter 4 also point to the important role of WNT antagonists DKK1 and FRZB in cartilage.

Therefore, we further explored the fundamental role of endogenously expressed DKK1 and FRZB in chondrocytes (Chapter six) by blocking their function using variable domain of single chain heavy chain only antibodies (VHH). **Chapter six** proved that endogenous expression of the WNT antagonists DKK1 and FRZB is necessary for multiple steps during chondrogenesis: firstly DKK1 and FRZB are indispensable for the initial steps of chondrogenic differentiation of hMSCs, secondly they are necessary for chondrocyte redifferentiation, and finally they are instrumental in preventing hypertrophic differentiation of articular chondrocytes in 3D cultures.

It has been known that activation of WNT signaling has detrimental effects on cartilage and that the expression of their inhibitors WNT antagonists DKK1 and FRZB was lost in OA. This made us to postulate that the loss of WNT inhibitors might be the reason of activation of WNT signaling. In this case, studying the mechanism behind the loss of WNT inhibitor in OA appears important. Pro-inflammatory factors, such as IL1β, are shown to be upregulated in OA and activate WNT signaling via an unknown mechanism. Therefore, we hypothesized that IL1β might be involved in reducing expression levels of WNT inhibitors. In **Chapter seven**, we studied the molecular mechanism by which IL1β downregulates expression of the WNT antagonists DKK1 and FRZB. We found that IL1β downregulated DKK1 and FRZB by upregulating an inflammatory mediator iNOS, which consequently led to activation of WNT signaling. This indicates that IL1β might contribute to OA by inhibiting WNT antagonists.

In **Chapter eight**, the main findings of this thesis are discussed in a broader perspective and provides an outlook for the application of fundamental research in joint disease like OA.

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Chapter 2

The regulatory role of signaling crosstalk in hypertrophy of MSCs and human articular chondrocytes*

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Unity of knowing and doing (行是知之始, 知是行之成)

Abstract

Hypertrophic differentiation of chondrocytes is a main barrier in application of mesenchymal stem cells (MSCs) for cartilage repair. In addition, hypertrophy occurs occasionally in osteoarthritis (OA). Here we provide a comprehensive review on recent literature describing signal pathways in the hypertrophy of MSCs-derived in vitro differentiated chondrocytes and chondrocytes, with an emphasis on the crosstalk between these pathways. Insight into the exact regulation of hypertrophy by the signaling network is necessary for the efficient application of MSCs for articular cartilage repair and for developing novel strategies for curing OA. We focus on articles describing the role of the main signaling pathways in regulating chondrocyte hypertrophy-like changes. Most studies report hypertrophic differentiation in chondrogenesis of MSCs, in both human OA and experimental OA. Chondrocyte hypertrophy is not under the strict control of a single pathway but appears to be regulated by an intricately regulated network of multiple signaling pathways, such as WNT, Bone morphogenetic protein (BMP)/Transforming growth factor-β (TGFβ), Parathyroid hormone-related peptide (PTHrP), Indian hedgehog (IHH), Fibroblast growth factor (FGF), Insulin like growth factor (IGF) and Hypoxia-inducible factor (HIF). This comprehensive review describes how this intricate signaling network influences tissue-engineering applications of MSCs in articular cartilage (AC) repair, and improves understanding of the disease stages and cellular responses within an OA articular joint.

Introduction

Osteoarthritis (OA) is a multifactorial complex and chronic disease characterized by progressive degradation of joint cartilage. The underlying molecular mechanisms involved in the pathogenesis and progression of OA are still largely unknown, and currently no disease-modifying therapy is available for OA.

In cell-based cartilage regeneration therapies, the use of mesenchymal stem cells (MSCs) has shown promising results. Evidence showed that MSCs can be differentiated into chondrocytes (marked by Sex determining region Y box 9 (SOX9); Aggrecan (ACAN); Collagen type II (Col2A1)) after a condensation state (marked by Cyclic adenosine monophosphate (cAMP), Transforming growth factor-β (TGFβ), Fibronectin, Neural cell adhesion molecule (N-CAM) and N-cadherin) *in vivo* and *in vitro*) [1–3] (Figure 1a). However, in the application of human MSCs for cartilage repair *in vivo*, hypertrophic differentiation towards the osteogenic lineage is observed. Prevention of hypertrophy is becoming increasingly important for clinical application of MSCs in cartilage tissue engineering [1,4]. Interestingly, recent data indicate that the healthy chondrocyte phenotype switches toward a hypertrophic phenotype in degenerated cartilage [4–6]. Phenomena such as proliferation of chondrocytes, hypertrophic differentiation of chondrocytes, remodeling and mineralization of the extracellular matrix (ECM), invasion of blood vessels and apoptotic death of chondrocytes correspondingly also occur during OA [7]. In addition, transgenic mouse models have shown that deregulated hypertrophic differentiation of articular chondrocytes may be a driving factor in the onset and progression of OA [4]. Therefore, control of hypertrophic differentiation can be exploited as an effective strategy for cartilage repair, and used in bone regeneration, where hypertrophic cartilage could act as a template for endochondral bone formation [1]. However, the exact molecular mechanism underlying hypertrophic differentiation is not understood. Despite numerous studies about the function of single signaling pathways in hypertrophy, studies which explore comprehensive signaling pathways in hypertrophic differentiation of MSCs and chondrocytes have not been published in recent years. Here we discuss how signaling pathways are involved in hypertrophy of MSCs and chondrocytes, how these signaling pathways interplay, and how signal factors changed in OA disease.

Hypertrophy in Chondrogenesis of MSCs in Vitro

MSCs are promising candidate cells for cartilage tissue engineering, as they are present in large quantities in adipose tissue, bone marrow, synovium and cartilage [8] and can be expanded for

a number of passages without losing their ability to undergo chondrogenic differentiation. Unfortunately, the phenotype of MSCs in cartilage repair is unstable [9,10]. The expression of cartilage hypertrophy markers (e.g., collagen type X) by MSCs undergoing chondrogenesis, raises concern for a tissue engineering application of MSCs, since chondrocyte hypertrophy in neocartilage could ultimately lead to apoptosis and ossification [11].

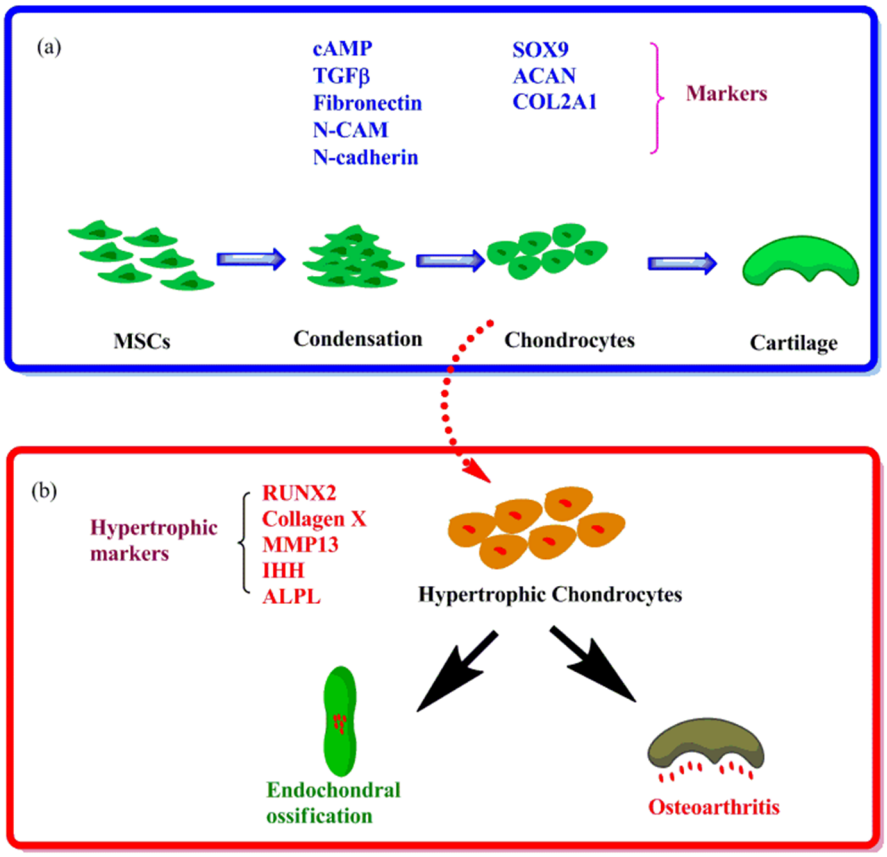

Figure 1. Chondrogenesis of MSCs and hypertrophic differentiation. (**a**) Chondrogenesis is initiated by the condensation of MSCs, and cell-cell contact. The expression of cAMP, TGFβ, Fibronectin, N-CAM and N-cadherin is involved in this process and these factors are necessary for chondrogenic induction, marked by the expression of chondrogenic genes: SOX9, ACAN and COL2A1. Mature chondrocytes begin secreting cartilage matrix primarily consisting of collagen II and GAGs, which are the main components of cartilage; (**b**) Chondrocytes from *in vitro* chondrogenesis of MSCs or *in vivo* cartilage could undergo hypertrophic differentiation, which is characterized by an increase in cell volume and the expression of hypertrophic markers (RUNX2, Collagen X, MMP13, IHH and ALPL). *In vivo*, physiological endochondral ossification and pathological osteoarthritis could be initiated after remodeling, mineralization of the extracellular matrix, and apoptotic death of chondrocytes.

Hypertrophy in Articular Chondrocytes during OA Progression

Studies have shown that the development of OA may be caused by activation of hypertrophic differentiation of articular chondrocytes [12]. As Figure 2 shows, during hypertrophic differentiation of chondrocytes in OA, chondrocytes lose the stable phenotype and the expression of Runt-related transcription factor 2 (RUNX2), Collagen type X, Matrix metalloproteinase 13 (MMP13), Indian hedgehog (IHH) and Alkaline phosphatase (ALPL) is detected [13]. Healthy articular cartilage (AC) is a stable tissue that has the potential to resist hypertrophic differentiation and maintain the normal phenotype through an unknown mechanism [14]. The interplay of multiple signaling pathways regulates the fate of chondrocytes, *i.e.*, to remain within cartilage or to undergo hypertrophic differentiation.

Signaling Pathways in Hypertrophy

Multiple signaling pathways have been involved in regulation of hypertrophy-like changes in chondrogenesis of MSCs and chondrocytes. Based on recent literature, the most important related pathways are WNT, Bone morphogenetic protein (BMP)/TGFβ, Parathyroid hormone-related peptide (PTHrP), IHH, Fibroblast growth factor (FGF), Insulin like growth factor (IGF) and Hypoxia-inducible factor (HIF) signaling pathways [15], Figure 2. In each single pathway, several distinct subtypes are involved in the regulation of chondrocyte differentiation and hypertrophy, Table 1.

WNT Signaling

WNT signaling pathways are highly evolutionarily conserved pathways with crucial roles in embryonic development, patterning, tissue homeostasis, growth, as well as in the onset and progression of a variety of diseases [16]. There are three distinct intracellular signaling cascades well known so far: the canonical WNT/β-catenin pathway, the c-Jun N-terminal kinase (JNK) pathway, and the WNT/$Ca2^{+}$ pathway [17]. The canonical WNT/β-catenin pathway is the most-elucidated pathway, mediated by β-catenin accumulation in nucleus, having strong correlation with chondrocyte hypertrophy. As shown in Figure 2b, in most cases, the presence of WNTs that bind to the Wnt receptor Frizzled, results in formation complex of Adenomatous polyposis coli protein (APC), Glycogen synthase kinase 3β (GSK3β) and Axis inhibitor (AXIN), which leads to the release of β-catenin from the complex, followed by β-catenin accumulating in the cytoplasm, and then translocation into the nucleus. There β-catenin forms a complex with T cell-specific factor (TCF)/lymphoid enhancer binding protein (LEF) transcription factors to

activate the transcription of target genes [17]. However, in the absence of a WNT ligand, β-catenin is phosphorylated by the destruction complex and subsequently ubiquitinylated and targeted for proteasomal degradation.

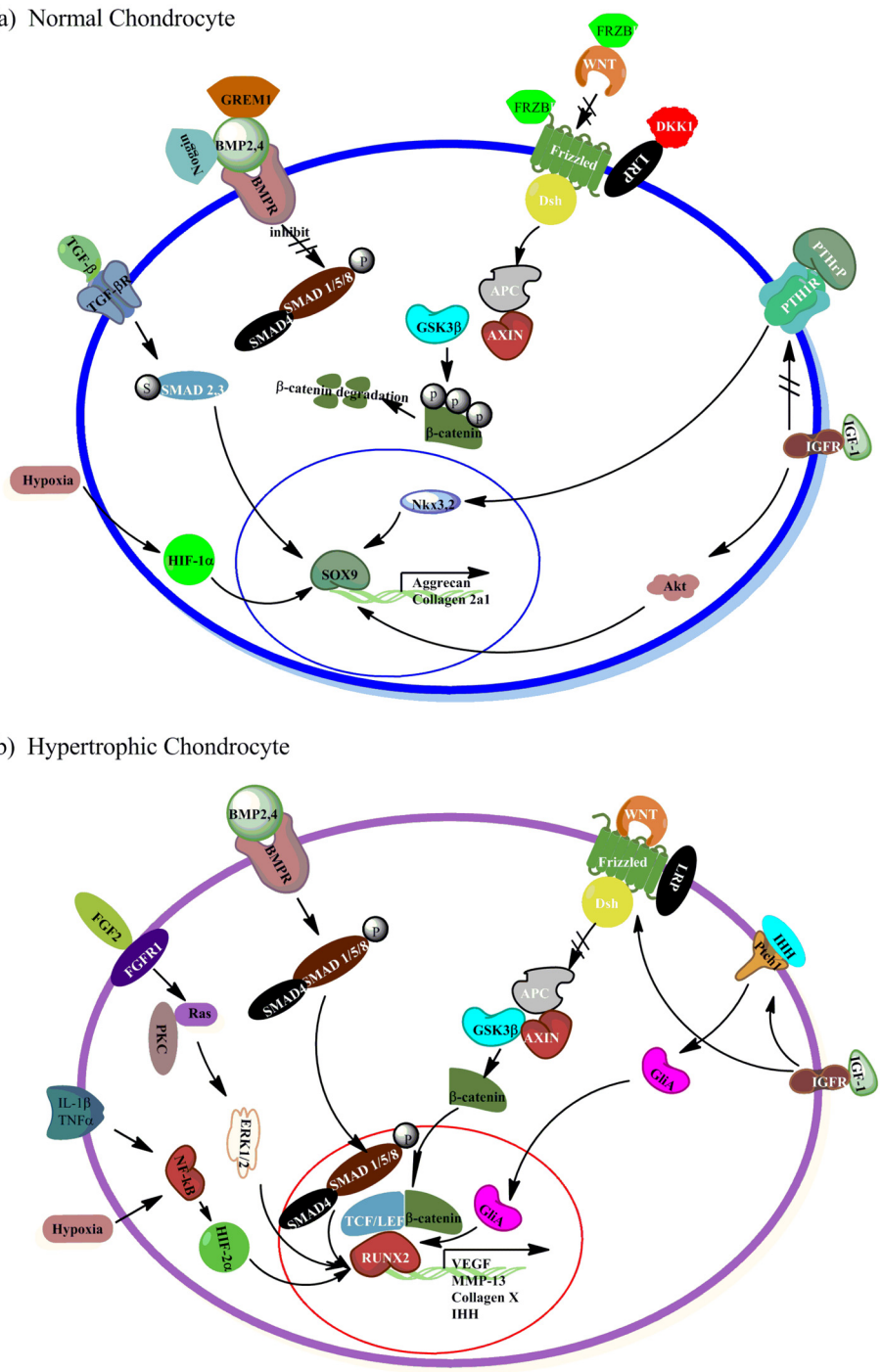

Figure 2. Signal pathways of chondrocyte hypertrophy. (**a**) In normal chondrocytes, signal pathways like WNT, BMP, IHH, *etc.* are regulated by their antagonists (DKK1 and FRZB for WNT, GREM1 for

BMP) or other signal factors to get a fine balance to maintain the chondrocyte normal phenotype. The most important transcription factor regulating chondrocytes is SOX9, which is responsible for the expression of main chondrocyte makers including collagen type II and aggrecan. Striked through arrows indicate that the signaling pathway is inhibited by its antagonists; (**b**) In hypertrophic chondrocytes, signal pathways, such as WNT, BMP, IHH, etc. are deregulated by their inhibitors or other signal factors, which consequently leads to overexpression of these pathways. Subsequently, the effects of cascade pathways result in activating the transcription factor RUNX2, which regulates the transcription of hypertrophic markers like collagen X, MMP-13, VEGF and IHH.

Signal	Subtypes	Main Functions
WNT	WNT3a	Promotes chondrogenic differentiation; delays chondrocyte hypertrophy
	WNT4	Blocks chondrogenic differentiation; promotes chondrocyte hypertrophy
	WNT5a	Promotes chondrogenic differentiation; delays chondrocyte hypertrophy
	WNT5b	Promotes chondrogenic differentiation; delays chondrocyte hypertrophy
	WNT8	Blocks chondrogenic differentiation; promotes chondrocyte hypertrophy
	WNT9a	Blocks both chondrogenic differentiation and chondrocyte hypertrophy
	WNT11	Promotes chondrogenic differentiation; stimulates RUNX2 and IHH expression
	WNT16	Upregulation is accompanied by the downregulation of FRZB
BMP/TGF-β	BMP2	Induces chondrocyte hypertrophy
	BMP4	Induces chondrocyte hypertrophy
	BMP7	Maintain chondrogenic potential and prevents chondrocyte hypertrophy;
	TGF-β	Promotes chondrogenic differentiation; inhibits chondrocyte hypertrophy
	PTHrP	Blocks hypertrophy by stimulating Nkx3.2 and prevent RUNX2 expression
	IHH	Promotes chondrocyte hypertrophy; Stimulates proliferating chondrocytes to produce PTHrP
FGF	FGF2	Promotes expression of RUNX2
	FGF8	Catabolic mediator with a pathological role in rat and rabbit articular cartilage

	FGF9	Promotes chondrocyte hypertrophy
	FGF18	Promotes chondrocyte proliferation and differentiation in the early stages of cartilage development
IGF	IGF-1	Promotes chondrocyte proliferation and maturation; augments chondrocyte hypertrophy
HIF	HIF-1α	Potentiates BMP2-induced SOX9 expression and cartilage formation, while inhibiting RUNX2 expression and endochondral ossification
	HIF-2α	Increases expression of collagen X, MMP13 and VEGF

Table 1. The subtypes involved in multiple signal pathways (WNT, BMP/TGFβ, PTHrP, IHH, FGF, IGF and HIF) and their main functions in the regulation of chondrocyte differentiation and hypertrophy.

Numerous studies have revealed a central role of WNT signaling in cartilage homeostasis. In cartilage, moderate activity of WNT is essential for chondrocyte proliferation and maintenance of their typical characteristics [18], but excessive activity increases chondrocyte hypertrophy and expression of cartilage degrading metalloproteinases [19]. For example, the conditional activation of the β-catenin gene in articular chondrocytes in adult mice leads to premature chondrocyte differentiation with collagen type X expression and the development of an OA-like phenotype [20]. However, ablation of β-catenin in the superficial zone of articular cartilage also strongly increases the expression of aggrecan and collagen type X [18]. SOX9 is the master transcription factor and thus a typical marker of chondrocytes, while RUNX2 usually is expressed highly in hypertrophic chondrocytes. This hypertrophy may be induced by the LEF/TCF/β-catenin complex promoting RUNX2 expression in the redundant WNT signal pathway [21]. Much evidence has shown that the switch between SOX9 and RUNX2 expression determines the progression of mature chondrocytes into hypertrophy in response to canonical WNT signaling [17, 22–24].

There are several types of WNT ligands, which play different roles in the chondrogenic differentiation and cartilage development. Experiments using retroviral misexpression in vivo and overexpression methods in vitro suggest distinct roles of different WNTs in the control of chondrogenic differentiation and hypertrophy. WNT4 and WNT8 block chondrogenic differentiation but promote hypertrophy [25, 26]. WNT9a blocks both chondrogenic differentiation and hypertrophy [27]. WNT3a and WNT5b promote chondrogenic

differentiation but delay hypertrophy [26]. The overexpression of Wnt11 in MSCs during chondrogenic differentiation promotes chondrogenesis and stimulates RUNX2 and IHH expression [28]. WNT16 transient expression was found associated with the activation of the canonical Wnt pathway, and was present in the early phases of osteoarthritis, its upregulation was accompanied by the downregulation of the secreted WNT inhibitor Frizzled-related protein (FRZB) [29]. WNT5a exhibit dual functions during chondrogenesis of MSCs. At early stages, WNT5a induces chondrogenesis and hypertrophy through intracellular calcium release via G-protein coupled receptor (GPCR) activation [1]. At later stages, it can act as an inhibitor of hypertrophy by activating the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB or Akt)-dependent pathway, which in-turn activates nuclear factor κ-light chain-enhancer of activated B cells (NF-κB), an inhibitor of RUNX2 [30].

Interestingly, the expression of hypertrophy-related markers in chondrogenesis of MSCs is decreased in the presence of Dickkopf (DKK1), which acts as WNT signaling inhibitor (antagonist) by binding to low density lipoprotein receptor related protein (LRP5/6) through cartilage protective mechanisms [4]. Actually, DKK1, FRZB and Gremlin 1 (GREM1) are regarded as natural brakes on hypertrophic differentiation of articular cartilage [4]. Our studies also found increased hypertrophic differentiation and mineralization and decreased expression of chondrocyte markers in the absence of the WNT inhibitors DKK1 and FRZB during chondrogenesis of hMSCs. In MSC pellet cultures, the inhibition of canonical WNT by DKK1 and FRZB increased the expression of collagen II and aggrecan, but did not affect collagen X expression [25, 31, 32]. However, the reduction of WNT antagonist secreted frizzled related protein 1 (Sfrp1) in MSCs correlated with an increased amount of cytoplasmic β-catenin and an up-regulation of RUNX2 [33].

Bone morphogenetic protein (BMP)/Transforming growth factor-β (TGFβ) Signaling

BMP Signaling

BMPs are multi-functional cytokines that belong to the transforming growth factor-β (TGF-β) superfamily. BMP signaling is mediated primarily through the canonical BMP-Smad pathway in chondrocytes [34]. The pathway will be activated when BMPs bind to receptors BMPR-I and II, which phosphorylate Sma and Mad related proteins (Smad) 1, Smad5, and Smad8 (R-Smads). The R-Smads form complexes with Smad4 and translocate into the nucleus, where they bind to regulatory regions of target genes to regulate their expression [35]. BMP has

multiple roles during embryonic skeletal development, in addition to mesenchymal condensation and chondrogenic differentiation of MSCs, BMPs induce early cartilage formation [36] and are crucial local factors for chondrocyte proliferation and maturation in endochondral ossification [37, 38].

Although BMPs have a protective effect in articular cartilage, they are also involved in chondrocyte hypertrophy and matrix degradation [39–42]. It was reported that the BMP signaling pathway was primarily activated during fracture healing via endochondral ossification and was detected in hypertrophic chondrocytes [43]. Steinert *et al.* showed that BMP2 and BMP4 induce hypertrophy during the chondrogenic differentiation of human MSC *in vitro* [37]. In another study, BMP2 was found to induce chondrocyte hypertrophy during chondrogenesis of progenitor cells ATDC5, whereas BMP-7 appeared to increase or maintain chondrogenic potential and prevent chondrocyte hypertrophy [44]. *In vivo* studies showed that overexpression of BMP4 in cartilage of transgenic mice resulted in an increased hypertrophic zone, indicating increased differentiation of hypertrophic chondrocytes [45]. As the BMPs also played role in the skeletal development, it may be that BMPs drive the chondrocytes to form bone after ossification, rather than to remain as articular chondrocytes [46]. Therefore, BMPs can be protective for articular cartilage but may have harmful effects on AC by inducing chondrocyte terminal differentiation and contributing to OA progression [31]. Our previous study has shown that addition of GREM1, the inhibitor of BMP signaling was able to slow down the hypertrophic differentiation and decrease the mineralization in the process of chondrogenesis of hMSCs [4]. In addition, another BMP inhibitor, Noggin, can block thyroid-induced hypertrophy by inhibiting BMP4 during MSC chondrogenesis [47].

TGF-β Signaling

TGFβ is a potent inducer of chondrogenesis *in vitro* [48,49]. During chondrogenesis of MSCs, TGFβ is the main initiator of MSC condensation. After aggregation, TGFβ signaling further stimulates chondrocyte proliferation while it inhibits chondrocyte hypertrophy and maturation [50–55]. Conversely, the activation of the Smad1/5/8 pathway is able to stimulate hypertrophic differentiation with the consequent expression of the hypertrophic markers collagen X, MMP13 and ALPL during chondrogenesis of MSCs [56]. Although TGFβ is clearly crucial in inhibiting chondrocyte hypertrophy during early phases of mesenchymal condensation and chondrocyte proliferation, its addition to chondrocyte differentiation medium in pellet cultures of MSCs was not sufficient to suppress the onset of hypertrophy [9–11].

Most recently, several lines of evidence have suggested that the TGFβ/Smad pathway played a critical role in the regulation of articular chondrocytes hypertrophy and maturation during OA development [57–59]. Zuscik and colleagues have shown that treatment of articular chondrocytes with 5-azacytidine (5azaC), an anti-tumor agent that functions by blocking DNA methylation, resulted in a shift of regulatory dominance from maturation suppression via TGFβ signaling to maturation acceleration by BMP-2 signaling, which confirms that a shift in signaling dominance from TGFβ to BMP is sufficient to induce AC maturation [60]. This study also raised the possibility that a similar shift in signaling dominance occurs when these cells progresses inappropriately, such as in osteoarthritis, where the balance between TGFβ and BMP signaling pathways may be broken. It has been suggested that TGFβ inhibits terminal hypertrophic differentiation of chondrocyte and maintains normal articular cartilage through Smad2/3 signals [58,61]. The Smad3 pathway can be activated by TGF-β directly to stabilize the Sox9 transcription complex and inhibits RUNX2 expression through epigenetic regulation [62,63]. Homozygous mutant mice of targeted disruption of Smad3- exon 8 developed degenerative joint disease resembling human OA, characterized by progressive loss of articular cartilage, and abnormally increased numbers of collagen type X expressing chondrocytes in synovial joints [58]. However, TGFβ1 administration has been shown to redirect expanded human articular chondrocytes towards hypertrophy [64]. Moreover, TGFβ can induce synovial lining cells to produce inflammatory factors, such as IL1β and TNFα, which further stimulates articular chondrocyte terminal hypertrophy, depositing collagen type X instead of collagen type II and aggrecan The TGF-β superfamily and its downstream phosphorylation of Smads were reported to exhibit both stimulatory and inhibitory effects on chondrocyte hypertrophy [65].

The Crosstalk between BMP/TGFβ and WNT Signaling in Regulating Hypertrophy

β-catenin crosstalk with TGFβ was reported in hypertrophy regulation in MSCs [66]. In the process of TGFβ-induced chondrogenesis of MSCs, temporal activation of β-catenin led to enhanced chondrogenic induction, further developed into hypertrophy and mineralization phenotype in vivo. However, the continuous co-activation of two signaling pathways resulted in hypertrophy inhibition, characterized by the suppressed expression of collagen type X, RUNX2, and ALPL, and did not lead to ossified tissue in vivo [66].

It was demonstrated that the crosstalk between WNT and BMP plays key roles in regulating chondrocyte activity in pathogenesis of osteoarthritis, which may be cell type-specific [67].

Papathanasiou and colleagues reported the function and crosstalk between BMP2 and canonical WNT/β-catenin signaling in regulating chondrocyte hypertrophy and matrix metalloproteinase (MMP)/aggrecanolytic ADAMTS (a disintegrin like and metalloproteinase with thrombospondin type I motif) synthesis in OA [68]. In this study, they showed human end-stage OA chondrocytes can produce BMP2 and BMP4. Interestingly, only BMP2, but not BMP4, can drive the expression of low-density lipoprotein receptor 5 (LRP5), which is one of most important co-receptors for WNT signaling that leads to β-catenin stabilization, accumulation, nuclear translocation, and activation of target genes. It can be concluded that the BMP-2-induced Wnt/β-catenin signaling pathway activation through LRP-5 induces chondrocyte catabolic action and hypertrophy [68].

This report adds to the accumulating evidence that increased or excessive activation of canonical WNT signaling in chondrocytes is detrimental and contributes to OA cartilage degradation. Recently, studies from our group also indicated that the natural WNT and BMP antagonists DKK1, FRZB and GREM1 inhibit hypertrophic differentiation of hMSCs during chondrogenesis by blocking WNT and BMP pathways [4]. Therefore therapeutic approaches to block or suppress canonical WNT and BMP2 pathways using their natural antagonists may protect cartilage damage in end-stage OA.

Parathyroid hormone-related peptide (PTHrP)/ Indian hedgehog (IHH) Signaling

PTHrP is a member of the parathyroid hormone (PTH) family that blocks hypertrophy by stimulating NK3 homeobox 2 (Nkx3.2) [69] and preventing RUNX2 expression [70]. Huang supposed SOX9 is a target of PTHrP signaling in the growth plate and that the increased activity of SOX9 might mediate the effect of PTHrP in maintaining the cells as non-hypertrophic chondrocytes [71]. IHH is an important factor involved in endochondral ossification and expressed in prehypertrophic chondrocytes [72]. In IHH knockout mice, the proliferation and hypertrophy of chondrocytes are significantly reduced [73]. Evidence has shown that IHH can positively regulate the transcription and expression of collagen type X via Runx2/Smad interactions through downstream transcription factors GLI-Kruppel family members (Gli) 1/2 [74]. Both IHH and PTHrP signaling play crucial roles in regulating the onset of chondrocyte hypertrophy. Vortkamp and colleagues [75] found that IHH stimulated proliferating chondrocytes to produce PTHrP, which in turn accelerated the proliferation of periarticular cells and prevented the onset of chondrocyte hypertrophy, thereby keeping chondrocytes in a proliferating state. This negative feedback loop regulates the balance between proliferation and maturation of chondrocytes, ensuring orderly bone formation [75]. On the other hand, resting

chondrocytes at the ends of long bones secrete PTHrP, subsequently suppressing IHH production in the proliferating zone. Chondrocytes outside of this paracrine signaling range produce IHH and undergo hypertrophy [1]. PTHrP forms a feedback loop with IHH to regulate the proliferation and onset of hypertrophic differentiation [76–78]. During endochondral bone formation, PTHrP-dependent IHH signaling inhibiting chondrocyte hypertrophy is dominant, thereby obscuring the promoting effect of PTHrP-independent IHH signaling. Other researchers reported that IHH can also function independently of PTHrP to promote chondrocyte hypertrophy [79]. In PTHrP knockout mice, the absence of PTHrP caused diminished chondrocytes and accelerated hypertrophic differentiation, and led to premature mineralization of extracellular matrix and apoptosis [75,80]. However, targeted overexpression of PTHrP under the control of the cartilage-specific collagen type II promoter resulted in the opposite effect of chondrodysplasia through delay of the terminal differentiation of chondrocytes, inhibition of apoptosis and disruption of endochondral ossification [81]. A co-culture model from Jiang and colleagues [82] demonstrated that in healthy articular cartilage PTHrP, secreted by chondrocytes from surface layers, inhibits the hypertrophic potential of chondrocytes residing in the deep layer so as to maintain the homeostasis of articular cartilage, but the effect was not confirmed in vivo. In another cell study, it was demonstrated that PTHrP from human articular chondrocytes inhibits hypertrophy of MSCs during chondrogenesis in co-culture, and intermittent supplementation of PTHrP also improves chondrogenesis of MSCs and reduces the hypertrophy [83,84]. A similar phenomenon was observed in MSCs pellet studies, it was shown that PTHrP treatment leads to suppression of hypertrophy but also down-regulates collagen II [49]. However, when cultured under hypertrophy-enhancing conditions, PTHrP could not diminish the induced enhancement of hypertrophy in the MSC pellets [85]. However, other researchers observed a selective hypertrophic inhibition upon PTHrP treatment with stable or even up-regulated expression of collagen II [86,87]. This discrepancy might be linked to the existence of both PTHrP receptor 1 (PTH1R)-dependent and PTH1R-independent pathways [1]. PTH1R knockout mice showed accelerated hypertrophy and were unaffected by treatment with PTHrP, indicating that the inhibition on hypertrophy is dependent on PTH1R receptor binding [88]. The choice of the PTHrP isoform has further been shown to affect the suppressive action on hypertrophy, with isoform 1-34 being the most effective in promotion of chondrogenesis as well as inhibition of hypertrophy [89].

Cell studies have shown that FGF2 combined with PTHrP inhibited the TGFβ responsive COL2A1 and COL10A1 expression and ALPL induction. However, calcification of implanted

pellets was not prevented by PTHrP in vivo [49]. In another study, the combined delivery of TGF-β3 and PTHrP in nude mice reduced calcification [90]. In addition, the canonical Wnt pathway is known to promote chondrocyte hypertrophy via inhibition of the PTHrP signaling activity [91]. Therefore, PTHrP represses hypertrophic cartilage differentiation whereas WNT and IHH promote hypertrophy of chondrocytes. Hence, the fine balance of the crosstalk between signal pathways is a requirement for the normal phenotype of chondrocytes.

Fibroblast growth factor (FGF) Signaling

FGF signaling plays a critical role in controlling chondrocyte differentiation [92]. Specifically, four members of the fibroblast FGF family, FGF2, FGF8, FGF9 and FGF18, have been implicated as contributing factors in cartilage homeostasis [92–96]. FGF2 has been shown to be expressed in proliferating and prehypertrophic chondrocytes, periosteal cells and osteoblasts [97]. In human articular chondrocytes, the binding of FGF2 to FGFR1 activates Ras and Protein kinase C delta (PKCδ), which transfer the signals into the nucleus to positively regulate the expression of RUNX2 by the Raf-MEK1/2-ERK1/2 cascade[98]. Under experimental OA conditions, FGF8 has been identified as a catabolic mediator with a pathological role in rat and rabbit articular cartilage [99]. However, little is known about the precise biological function of FGF8 on human adult articular cartilage. In developing stylopod elements, FGF9 promotes chondrocyte hypertrophy at early stages and regulates vascularization of the growth plate and osteogenesis at later stages of skeletal development. $Fgf9^{-/-}$ mice have normal limb bud development and mesenchymal condensations, but show decreased chondrocyte proliferation in stylopod elements, delayed initiation of chondrocyte hypertrophy and abnormal osteogenesis in skeletal vascularization [95]. In the early stage of cartilage development, FGF18 is expressed in the perichondrium and joint spaces to promote chondrocyte proliferation and differentiation. In $Fgf18^{-/-}$ mice, the phenomenon of delayed mineralization was observed, which was found to be closely associated with delayed initiation of chondrocyte hypertrophy, decreased chondrogenesis proliferation of early stages, delayed skeletal vascularization and delayed osteoclast and osteoblast recruitment to the growth plate [100]. Further studies have shown that FGF18 is necessary to induce VEGF expression by signaling to FGFR 1 and 2 in hypertrophic chondrocytes [100]. The FGF receptor 3 (FGFR3) is a tyrosine kinase receptor, expressed in proliferating chondrocytes and early hypertrophic chondrocytes in the growth plate. Both FGF9 and FGF18 are the major ligands of FGFR3 in the growth plate [101]. Recently, Shung and coworkers found that FGFR3 expression increases the expression of SOX9 and decreases β-catenin levels in cultured mesenchymal cells [102].

The interplay of WNT and FGF signaling is important to determine the fate of MSCs and their subsequent differentiation. FGFR1 appears to act downstream of the β-catenin pathway and serves as a key determinant in the lineage decision of skeletal precursors [103]. Hypertrophic maturation of chondrocytes is highly regulated by the interplay of the FGF, IHH, BMP, and WNT signaling pathways. More specifically, FGF signaling accelerates the speed of terminal hypertrophic differentiation, and acts in an antagonistic relationship with IHH expression [104]. Another study suggests that the FGF and BMP pathways collaborate to promote aspects of hypertrophic chondrocyte maturation [105]. However, cartilage of mice carrying a targeted deletion of Fgfr3 is characterized by increased regions of proliferating and hypertrophic chondrocytes [106]. A study from Weiss and colleagues also showed that FGF2, together with PTHrP, may inhibit chondrocyte hypertrophic differentiation and is therefore necessary to obtain stable chondrocytes [49].

Insulin like growth factor (IGF) Signaling

IGF-1 has been identified as an important growth factor for skeletal development by promoting chondrocyte proliferation and maturation, while inhibiting apoptosis to form bones with appropriate size and strength. IGF-1 transmits signals via the type 1 IGF-1 receptor (IGF1R), which is expressed in the proliferating and prehypertrophic zone chondrocytes of growth plates [107]. Evidence shows that IGF-1 stimulates growth plate chondrocytes at all stages of differentiation [108]. High level of IGF-1 was detected in osteoarthritic human articular cartilage [109]. The local infusion of IGF-1 in rabbit tibial growth plate increased the numbers of both proliferative and hypertrophic chondrocytes and promoted hyperplasia of bony trabeculae within the epiphysis [110]. It has been shown that IGF-1 stimulates the chondrogenic differentiation of MSCs into chondrocytes, and into pre-hypertrophic and hypertrophic chondrocytes [111]. Recombinant adeno-associated virus (rAAV)-mediated IGF-I overexpression delayed terminal differentiation and hypertrophy in the newly formed cartilage, which may be due to contrasting effects upon the osteogenic expression of RUNX2 and β-catenin [112]. Another study demonstrates that IGF-1 enhances chondrocyte hypertrophy by insulin-like actions, and that terminal hypertrophic chondrocytes are reduced in Igf1 null mice [113]. Repudi's study showed that WNT induced secreted protein 3 (WISP3) inhibits IGF-1 induced collagen X induction, reactive oxygen species (ROS) accumulation and ALPL activity, all of which are associated with the induction of chondrocyte hypertrophy [114]. In addition, Mushtaq also found that IGF-1 stimulated chondrocyte hypertrophy and reversed the growth-

inhibitory dexamethasone effects in mouse metatarsal [115]. However, evidence shows that chick embryo chondrocytes maintained their normal phenotype and were prevented to undergo hypertrophic differentiation in the presence of IGF-1 [116]. Clearly, the IGF-I mediated improvement in growth was performed by altering the balance between proliferating and hypertrophic chondrocytes.

IGF-1 signaling also is involved in the interaction between the thyroid hormone and the Wnt/β-catenin signaling pathways in regulating growth plate chondrocyte proliferation and differentiation. Evidence showed that IGF-1 and the IGF-1 receptor (IGF1R) stimulate Wnt-4 expression and β-catenin activation in growth plate chondrocytes. Chondrocyte proliferation and terminal differentiation induced by IGF-1/IGF1R can be partially inhibited by the Wnt antagonists FRZB and DKK1 [117]. The IGF-1/IGF1R signaling and IGF-1 dependent PI3K/Akt/GSK-3β signaling can be activated by triiodothyronine (T_3) in the growth plate, and the chondrocytes undergo proliferation and differentiate to prehypertrophy. It seems that chondrocyte proliferation may be triggered by the IGF-1/IGF1R-mediated PI3K/Akt/GSK3β pathway, while cell hypertrophy is likely due to activation of Wnt/β-catenin signaling, which is at least in part initiated by IGF-1 signaling or the IGF-1-activated PI3K/Akt signaling pathway [117]. The fact that IHH expression was reduced in $Igf1^{-/-}$ mice long bones, whereas expression of PTHrP was increased, suggested that IGF-1 signaling is also required to maintain the IHH-PTHrP loop during skeletogenesis [118].

Hypoxia-inducible factor (HIF) signaling

Healthy articular cartilage is a typical avascular tissue, and chondrocytes are able to survive in low oxygen environments [119]. Hypoxia is considered to be a positive influence on the healthy chondrocyte phenotype and cartilage matrix formation. A recent study from our group has shown that the articular cartilage-enriched gene transcripts of GREM1, FRZB, and DKK1, which are established inhibitors of hypertrophic differentiation, were robustly increased in chondrogenic hMSCs pellets under hypoxic conditions, whereas under normoxia conditions these genes did not increase markedly [120]. Evidence shows that hypoxia enhances chondrogenesis and prevents terminal differentiation through a PI3K/Akt/FoxO dependent anti-apoptotic effect [121]. The hypoxic response is mainly mediated by HIF, which includes three family members, HIF-1α, -2α, -3α [122], particularly HIF-1α and HIF-2α, play an active role in chondrocyte development.

Under hypoxic conditions, the transcription factor HIF-1α accumulates and activates the transcription of genes, which are involved in energy metabolism, angiogenesis, vasomotor

control, apoptosis, proliferation, and matrix production. In subcutaneous stem cell implantation studies, HIF-1α was shown to potentiate BMP2-induced SOX9 and cartilage formation, while inhibiting RUNX2 and endochondral ossification during ectopic bone/cartilage formation. In the fetal limb culture, HIF-1α and BMP2 synergistically promoted the expansion of the proliferating chondrocyte zone and inhibited chondrocyte hypertrophy and endochondral ossification [123]. However, HIF-2α, encoded by Epas1, was identified as a regulator of endochondral bone formation, and appears to be a central positive regulator of collagen X, MMP13 and VEGF expression by enhancing promoter activities through specific binding to the hypoxia-responsive elements [124]. Inflammatory factors like IL-1β and TNF-α can increase the HIF-2α expression by NF-κB signaling in chondrocytes [124,125]. Further experiments have shown HIF-2α participates in crosstalk with the β-catenin and NF-κB pathways to promote chondrocyte apoptosis and endochondral ossification [126]. RUNX2 and IHH were identified as the possible transcriptional targets of HIF-2α related to endochondral ossification; both of them are involved in the regulation of hypertrophic differentiation of chondrocytes [124,127]. The gene corresponding to nicotinamide phosphoribosyltransferase (NAMPT) is also a direct target of HIF-2α, and plays an essential catabolic role in OA pathogenesis and acts as a crucial mediator of osteoarthritic cartilage destruction caused by HIF-2α or destabilisation of the medial meniscus (DMM) surgery [128]. There is evidence that HIF-2α causes cartilage destruction by regulating crucial catabolic genes [125] and potentiating Fas-mediated chondrocyte apoptosis [129]. However, Lafont and coworkers found that hypoxia promotes cartilage matrix synthesis specifically through HIF-2α but not HIF-1α mediated SOX9 induction of key cartilage genes [130]. The seemingly conflicting effects of HIF-2α to chondrocyte or cartilage could be induced through different pathways and the differences in experiments performed in vivo and in vitro, which need to be clarified. The balance between HIF-1α/HIF-2α activities clearly contributes to the control of cartilage homeostasis.

Conclusions

Chondrocyte differentiation is regulated by multiple signal transduction pathways. Maintaining a normal chondrocyte phenotype and avoiding hypertrophy is important for cartilage repair. SOX9 and RUNX2 are two typical markers in chondrocyte development. SOX9 is expressed in chondrocytes, while RUNX2 is highly expressed in hypertrophic chondrocytes. In most cases, a hypertrophic phenotype was accompanied by high expression of RUNX2 through activation of either of the WNT, BMP, IHH, FGF and HIF signaling pathways. However, TGFβ,

IGF-I and PTHrP promote the proliferation of chondrocytes. Here we propose a model in which the balance of these signal pathways adjusts the state of chondrocyte proliferation or hypertrophy through the shifting between SOX9 and RUNX2 transcriptional activities. In the WNT pathway, the LEF/TCF/β-catenin complex can promote RUNX2 expression. BMP/TGF-β signaling has a dual role in the chondrocyte development. TGF-β induces collagen II and SOX9 deposition through Smad2/3 phosphorylation pathway, while BMP2/4 promotes chondrocyte hypertrophy and cartilage mineralization via Smad1/5/8 phosphorylation. PTHrP represses hypertrophic cartilage differentiation whereas IHH signaling positively regulates the hypertrophic phenotype by high transcription and expression of collagen type X and RUNX2. IGF-1 signaling stimulates chondrocyte proliferation by the IGF-1/IGF1R-mediated PI3K/Akt/GSK3β pathway, while cell hypertrophy is likely due to activation of Wnt/β-catenin and IHH signaling by IGF-1. HIF-1α and HIF-2α have a distinct role in the chondrocyte development. The former inhibits the RUNX2 expression, while the latter enhances the expression of collagen X, MMP13 and RUNX2 and promotes the hypertrophic differentiation of chondrocytes. The fine balance of the crosstalk between these signaling pathways is a requirement for normal chondrocyte differentiation and cartilage development.

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Chapter 3

Correlation between gene expression and osteoarthritis progression in human*

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knowledge is infinite (学无止境)

Abstract

Osteoarthritis (OA) is a multifactorial disease characterized by gradual degradation of joint cartilage. This study aimed to quantify major pathogenetic factors during OA progression in human cartilage. Cartilage specimens were isolated from OA patients and scored 0–5 according to the Osteoarthritis Research Society International (OARSI) guidelines. Protein and gene expressions were measured by immunohistochemistry and qPCR, respectively. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were used to detect apoptotic cells. Cartilage degeneration in OA is a gradual progress accompanied with gradual loss of collagen type II and a gradual decrease in mRNA expression of *SOX9*, *ACAN* and *COL2A1*. Expression of WNT antagonists *DKK1* and *FRZB* was lost, while hypertrophic markers (*RUNX2*, *COL10A1* and *IHH*) increased during OA progression. Moreover, *DKK1* and *FRZB* negatively correlated with OA grading, while *RUNX2* and *IHH* showed a significantly positive correlation with OA grading. The number of apoptotic cells was increased with the severity of OA. Taken together, our results suggested that genetic profiling of the gene expression could be used as markers for staging OA at the molecular level. This helps to understand the molecular pathology of OA and may lead to the development of therapies based on OA stage.

Introduction

Osteoarthritis (OA) is a multifactorial disease of the joints, affecting many parts of the joint, including bone, synovium, ligaments, and articular cartilage (AC). It is characterized by the progressive destruction of the articular cartilage matrix [1]. Cartilage damage in OA is likely to result from the aggregate effect of multiple genetic, environmental, mechanical and cell biological factors driving changes in gene expression [2].

The pathology of OA is complex, the underlying mechanism behind OA development and progression is still unknown. Many studies of OA progression are based on animal models [3-5] that may not be translatable into human disease and therapy. Although gene expression has been studied in normal and advanced OA [6, 7], little is known about what happen in transition stages during OA progression. Studies about gene expression in different steps of OA based on OA scores have not been reported and may provide clues for comprehensive understanding of the progression of OA. This would mean that if we measure the expression of these genes in any given patient, we may tailor therapy based on the stage of OA of the individual patient.

It has been shown that the cartilage-specific transcription factor sex determining region Y box 9 (SOX9) expression is decreased at the mRNA and protein levels in OA cartilage [8]. Collagen type II, alpha 1 (COL2A1) is reduced while collagen type I, alpha 1 (COL1A1) is increased during the progression of human OA [9, 8]. The loss of cartilage markers is not the only known characteristic of OA, as derailed hypertrophic differentiation in articular cartilage has been implemented in the pathogenesis of OA, at least in a subset of patients [10]. We have previously shown that dickkopf 1 homolog (DKK1), frizzled-related protein (FRZB) and bone morphogenetic protein (BMP) antagonist gremlin1 (GREM1*)* as antagonists of the wingless-type MMTV integration site (WNT) or Bone Morphogenetic Protein (BMP)-signaling pathways respectively, are key factors in controlling the articular chondrocyte phenotype. In addition, creating permanent cartilage under hypoxia condition correlates with the expression of these three natural antagonists [11].

It has been indicated that runt-related transcription factor 2 (RUNX2), is a positive regulator for chondrocyte maturation [12] and that it is highly expressed in OA cartilage as compared to normal cartilage [13]. Collagen type X, alpha1 (COL10A1) is a typical hypertrophic marker and a direct transcriptional target of RUNX2 [14]. Upregulation of COL10A1 expression is observed in human OA cartilage [15]. Indian hedgehog (IHH) is mainly produced by prehypertrophic chondrocytes and regulates chondrocyte hypertrophic differentiation [16]. *IHH* and matrix metallopeptidase 13 (*MMP13*) are upregulated in human OA and are correlated with

OA progression [17]. *BMP2* and the canonical WNT target gene *AXIN2* are upregulated in injured human articular cartilage explants [18].

Although expression of various genes has been reported in OA, as above mentioned, very little is known about the expression of these genes at different stages of OA and the correlation with OA severity. In order to investigate the cellular changes and the changes in gene expression that are directly involved in cartilage degeneration, we performed IHC to detect the expression of important proteins. Quantitative Polymerase chain reaction (qPCR) assays were used to quantify the expression of OA related genes: the cartilage markers: *SOX9*, *ACAN*, and *COL2A1*; WNT antagonists: *DKK1*, and *FRZB* and *GREM1*; hypertrophic markers: *RUNX2*, *COL10A1* and *IHH*; *AXIN2*, *BMP2,* and a dedifferentiation marker *COL1A1*. Finally, we investigated the correlation between gene expression, apoptosis and severity of OA.

This is, to our knowledge, the first comprehensive study determining the correlation between the expression of different major signal transduction factors and apoptosis and different stages of OA in human. Based on our data, the specific gene or protein expression can be coupled to the stage of OA disease, which may ultimately improve OA diagnosis and treatment.

Materials and Methods

Cartilage samples collection

The collection and use of human cartilage was approved by the local hospital ethical committees and for all samples informed written consent was obtained. Cartilage specimens were isolated from 12 patients (mean age ±SD: 68±6 years) with OA undergoing total knee replacement surgery (Supplemental table 1). In order to get comparable cartilage samples for histology and RNA extraction, several cartilage pieces were removed from the same joint (Supplemental figure 1) and for each of the specimens a cartilage score was determined. Each cartilage specimen was cut into 2 equal parts. One half was used for histology for grading of OA; the other half was used for RNA isolation and qPCR analysis. For RNA isolation, subchondral bone was removed from the cartilage, and samples were cut into small pieces (1-2 mm) and quickly snap frozen into liquid nitrogen (LN2). The frozen samples were stored at -80° C until use.

Histological analysis

Cartilage samples were collected into 10 mL tubes, washed twice with PBS and fixed using 10% phosphate buffered formalin (pH=7, Sigma Aldrich, USA) overnight at 4 °C. In the next step, samples were decalcified for 4 weeks in 12,5% (w/v) EDTA solution containing 0.5%

phosphate buffered formalin (pH=8.0). After decalcification, samples were dehydrated using graded ethanol and embedded in paraffin.

Safranin O and Alcian blue staining

A microtome (Shandon, Thermo Scientific, France) was used to cut 5µm thick sections. Sections were then deparaffinized in xylene and hydrated using a graded ethanol series. Slides were either stained for sulfated glycosaminoglycans (GAG) with a 0.1% solution of Safranin O (Sigma Aldrich, USA) dissolved in distilled water for 5 min, and counterstained with haematoxylin (Sigma Aldrich, USA) dissolved in water, to visualize nuclei, or stained for sulfated GAG with a 0.5% solution of Alcian blue (pH=1, adjusted with HCL) for 30 min, and counterstained with nuclear fast red to visualize nuclei as described previously [19].

Immunohistochemistry (IHC)

Immunohistochemical staining of COL2A1, COL10A1 and GREM1 was performed on 5 µm tissue sections, which were pre-incubated with 5 µg/mL proteinase K (Sigma Aldrich, USA) for 10 min in Tris-EDTA (TE) buffer at room temperature (RT) followed by incubation with 1 mg/mL hyaluronidase dissolved in PBS (Sigma Aldrich, USA) for 40 min at 37 °C. Rabbit polyclonal collagen type II (Abcam, UK) and rabbit polyclonal GREM1 (Santa Cruz biotechnology, USA) were diluted 1:200, mouse monoclonal antibody against collagen type X (Quartett chemicals, Germany) was diluted 1:100 in PBS containing 1.5% normal goat blocking serum (Santa Cruz Biotechnology, USA) and incubated overnight at 4°C. Non-immune controls underwent the same procedure without primary antibody incubation. The biotinylated secondary antibody was diluted 1:200 in normal goat blocking serum and incubated for 30 minutes at RT. Staining was developed using a rabbit or mouse ABC staining system (Santa Cruz biotechnology, USA) according to the manufacturer's protocol and imaged using a Nanozoomer (Hamamatsu Photonics, Japan).

RNA isolation and qPCR

Cartilage pieces of samples with the same OA grade from different patients were pooled in a pre-cooled Cryo-Cup Grinder for crushing. The obtained cartilage powder was collected into 50 mL tubes and samples were weighed. One ml TRIzol reagent per 50-100 mg sample was added. Total RNA was isolated from the lysate according the manufacturer's protocol (ThermoFisher scientific, USA). The precipitated RNA was dissolved in RNase-free water and subsequently treated with RNase-free DNase I (Invitrogen life technologies, USA). The

concentration of RNA was measured using the Nanodrop 2000. cDNA was obtained from 1µg of RNA with a cDNA synthesis kit (Bio-Rad, USA). QPCR was performed using the SYBR Green sensimix (Bioline, UK). PCR Reactions were carried out using the Bio-Rad CFX96 (Bio-Rad) under the following conditions: cDNA was denatured for 5 min at 95°C, followed by 39 cycles consisting of 15s at 95°C, 15s at 60°C and 30s at 72°C. For each reaction a melting curve was generated to test primer dimer formation and non-specific priming. Gene expression was normalized using GAPDH as housekeeping gene. Primers sequences are listed in Supplemental table 2.

Apoptosis assay

Apoptosis of cells was detected in paraffin-embedded tissues using The DeadEnd™ colorimetric TUNEL assay (Promega, USA) following the manufacturer's procedure. Apoptotic nuclei were stained dark brown.

Statistical analysis

Data are expressed as the mean. Correlations between the gene expression levels with severity of OA were assessed using Pearson's correlation analysis. $P<0.05$ was considered statistically significant.

Results

During OA progression, the expression of *COL2A1* decreases, while expression of *GREM1* and *COL10A1* increases

Cartilage samples showed microscopic changes, mostly related to a gradual reduction of Alcian blue and Safranin O staining and loss of cartilage integrity with the severity of OA (Figure 1), Based on the histological features, the severity of OA was represented by scores from 0-5 by assessing structural damages and cellular abnormalities according to the OARSI guidelines [20]. From OARSI grades 0-5, cartilage showed histological changes, from intact and smooth surfaces to discontinued surfaces, from fissures in the superficial layer to fissures extending into the deep layers and from slight loss to extensive loss of Alcian blue and Safranin O staining (Figure 1). The IHC results (Figure 2A and B) confirmed the loss of Collagen type II with increasing severity of OA, the expression of Collagen type II was measured in each patient (Supplemental figure 2). The expression of GREM1 and collagen type X were increased in high grade OA, their expression was measured in each patient (Supplemental figure 3 and 4). Statistical analyses of IHC quantification indicated that the expression of collagen type II ($r = -0.972$, $p = 0.001$) was negatively correlated with OA grading, while collagen type X ($r = 0.972$, $p = 0.001$) and GREM1 ($r = 0.987$, $p = 0.0002$) were positively correlated with OA severity.

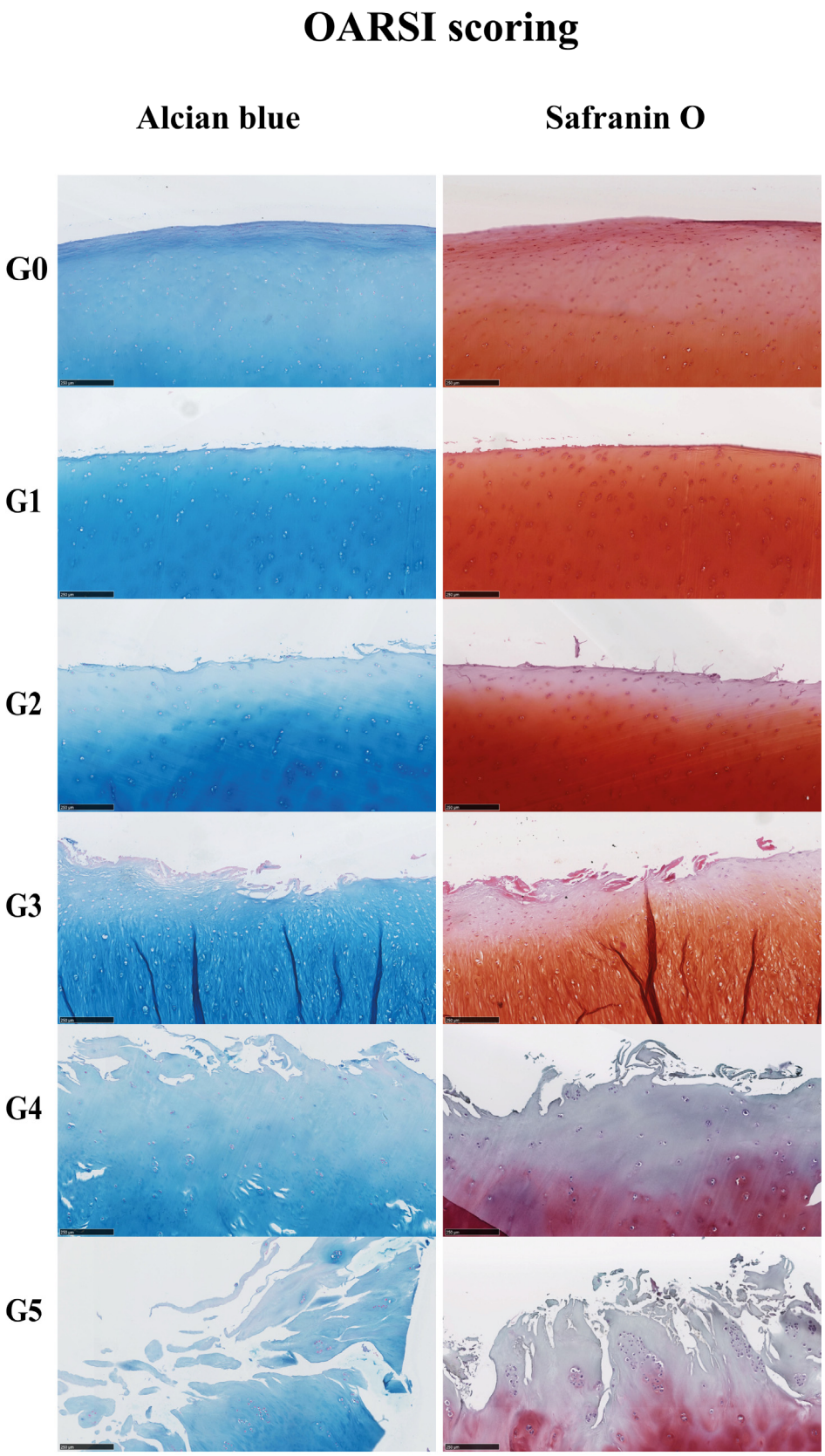
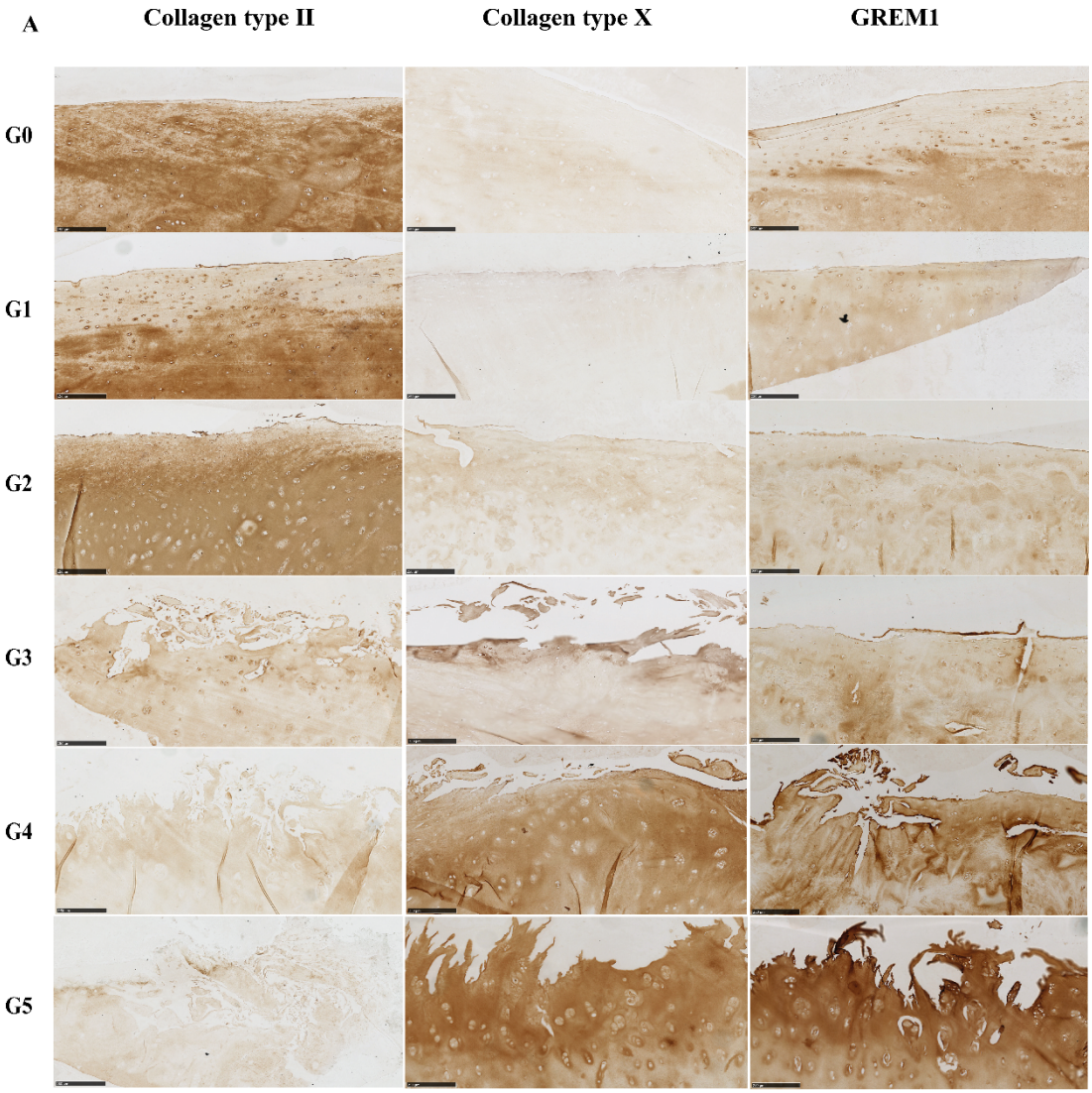


Figure 1. Histological changes of OA cartilage. 5µm Paraffin sections of cartilage stained by Alcian blue and Safranin O (scale bar 250µm). The severity of the OA lesion was graded on a scale 0-5.


A
Collagen type II
Collagen type X
GREM1
G0
G1
G2
G3
G4
G5

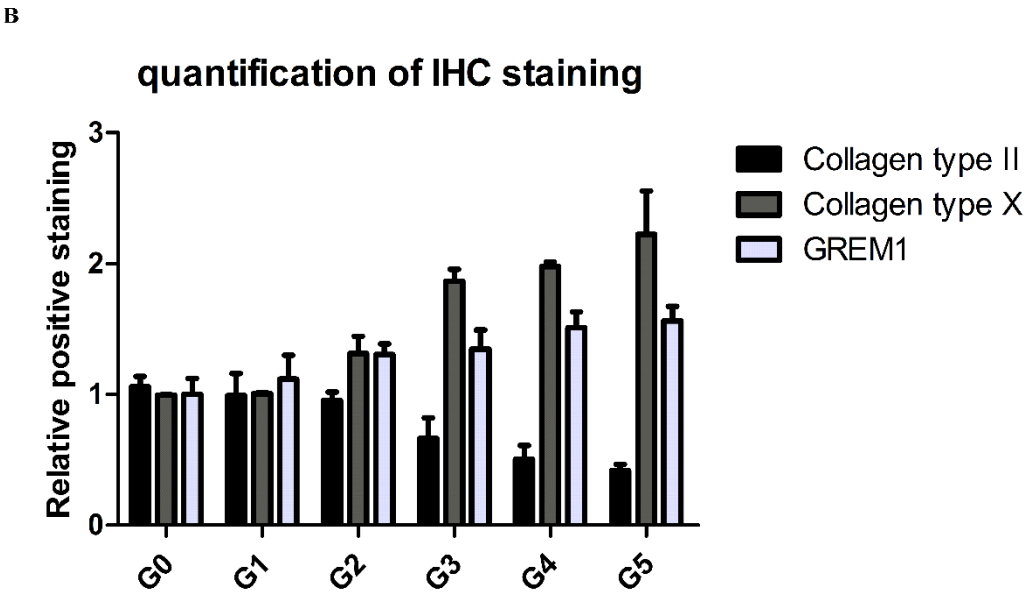

B
quantification of IHC staining
Relative positive staining
0
1
2
3
G0
G1
G2
G3
G4
G5
Collagen type II
Collagen type X
GREM1

Figure 2. The protein expression of collagen type II, collagen type X and GREM1 was visualized by IHC (scale bar 250µm). A. Representative pictures are shown. Images were taken using the Nanozoomer. G0, G1, G2, G3, G4, G5=Grade 0, Grade 1, Grade 2, Grade 3, Grade 4, Grade 5. **B.** Quantification of positive staining was performed by ImageJ software.

Gene expression profiles in cartilage at different stages

To characterize the gene expression during OA progression, qPCR was performed in cartilage specimens with grades 0, 1, 2, 3, 4 and 5. The expression of cartilage related markers is summarized in Table 1. For all three cartilage markers, *SOX9, ACAN* and *COL2A1*, gene expression gradually decreased with increasing severity of OA. This was especially obvious for the expression of *SOX9,* the master transcription factor for chondrocyte development [21, 22], which was reduced to below detection levels in grade 5 tissue. The expression of the chondrocyte dedifferentiation marker *COL1A1* gradually increased from grades 2-5. The ratio *COL2A1/COL1A1* was sharply decreased from grades 0-5.

Previously, we have shown that WNT antagonists DKK1 and FRZB, and the BMP antagonist GREM1 are regulators for cartilage homeostasis [10]. In our study, the expression of *DKK1* was significantly decreased at grade 3, 4 and 5, *FRZB* was linearly decreased from grade 1 to 4. The expression of *GREM1* slightly decreased between grades 0 and 2 and steeply increased between grades 4 and 5. This increase was further substantiated by an increase in GREM1 staining in IHC (Figure 2 and Supplemental figure 3).

In a subset of patients, OA is associated with hypertrophic differentiation of chondrocytes [23]. The expression of hypertrophy-related genes is summarized in table 2. The transcription factor linked to chondrocyte hypertrophy, *RUNX2,* was under detection level at grades 0 and 1, which was gradually increased from grade 2 with the severity of OA. *RUNX2* drives the expression of the terminal differentiation markers including *COL10A1* and *IHH,* which were increased 10- and 9- fold respectively at grade 5. *COL10A1*, which was under detection level at grades 0-3, was first detected at grade 4. The gene expression is in line with the IHC results (Figure 2 and Supplemental figure 4).

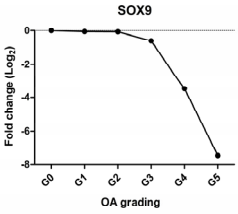
Since WNT and BMP signaling are indicated to be involved in cartilage pathophysiology [24], we measured the expression of *AXIN2* as a target gene of WNT signaling and *BMP2* as target gene of BMP signaling. *AXIN2* was not detectable in cartilage grades 0, 1, 2 and 3, however it started to be expressed in OA grade 4 and increased further at grade 5. The expression of *BMP2* was sharply increased at grade 2 and further increased at grade 3, and could not be detected at grades 4 and 5.

Correlation between gene expression and the severity of OA

The Pearson correlation method was applied to reveal a correlation between gene expression based on qPCR data and OA severity. The expression of *SOX9* (r = -0.927, p = 0.008), *ACAN* (r = -0.959, p = 0.002) and *COL2A1* (r = -0.960, p = 0.002) was negatively correlated with OA, while the expression of the chondrocyte dedifferentiation marker *COL1A1* (r = 0.963, p = 0.002) was positively correlated with OA grading (Table 1).

Previously we have shown that the expression of these *DKK1*, *FRZB* and *GREM1* is downregulated in OA [10]. We therefore looked at the correlation between *DKK1*, *FRZB* and *GREM1* expression and OA progression (Table 1). *DKK1* (r = -0.812, p = 0.05) and *FRZB* (r = -0.896, p = 0.016) mRNA expression levels were negatively correlated with grading of knee OA. Although high expression of GREM1 was observed in high grade of OA (grade 4 and grade 5) by qPCR and IHC, we found a moderate correlation of *GREM1* with OA grading, which was not significant, probably due to the relatively small sample size (r = 0.714, p = 0.111). However, the expression of *RUNX2* (r = 0.908, p = 0.012) and *IHH* (r = 0.961, p = 0.0018) showed a significantly positive correlation with OA grading (Table 2).

In addition, we found that *FRZB* positively correlated with cartilage marker *ACAN* (r = 0.944, p = 0.005) and *COL2A1* (r = 0.868, p = 0.03), *IHH* negatively correlated with all three cartilage markers *SOX9* (r = -0.968, p = 0.001), *ACAN* (r = -0.914, p = 0.011), and *COL2A1* (r = -0.914, p = 0.011), *FRZB* positively correlated with *DKK1* (r = 0.816, p = 0.05), and *GREM1* positively correlated with the hypertrophic markers *COL10A1* (r = 0.995, p = 0.000035), *IHH* (r = 0.829, p = 0.041), *RUNX2* (r = 0.997, p = 0.003), *AXIN2* (r = 0.933, p = 0.007). We did not observe a significant correlation between expression of the antagonists and the tested hypertrophic markers (Supplemental table 3).

Gene name	Protein Function	Gene expression trend	Correlation between gene expression and OA severity		Correlation significant
SOX9	Chondrogenic transcription factor for chondrogenesis		Pearson correlation coefficients (r)	P values	**
			-0.927	0.008	

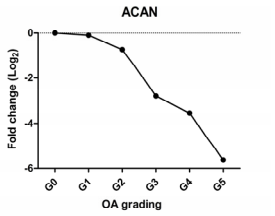
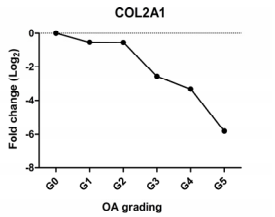
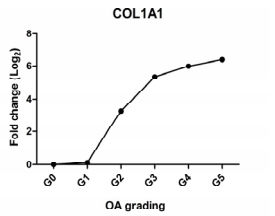
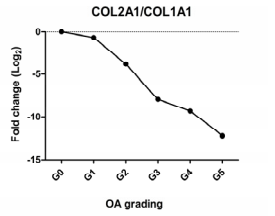
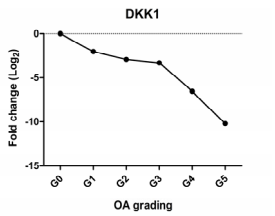
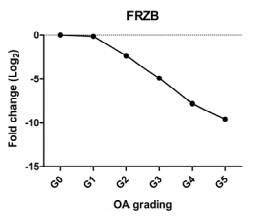
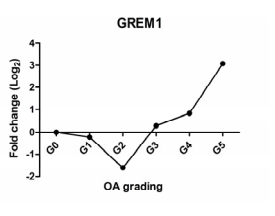
ACAN	Extracellular matrix protein, provides strength to cartilage		-0.959	0.002	**
COL2A1	Extracellular matrix protein, provides cartilaginous framework and tensile strength		-0.960	0.002	**
COL1A1	Provides cartilaginous framework, the marker of dedifferentiated chondrocyte		0.963	0.002	**
COl2A1/COL1A1	Reflects the replacement of collagen type II by collagen type I during OA		-0.868	0.025	*
DKK1	Blocks chondrocyte hypertrophy, promotes chondrogenesis		-0.812	0.050	*
FRZB	Inhibits chondrocyte hypertrophy, promotes chondrogenesis		-0.896	0.016	*
GREM1	Inhibits terminal chondrocyte differentiation and endochondral bone formation		0.714	0.111	ns

Table 1. Expression of cartilage related genes in OA cartilage and correlation with the severity of OA (n=12). RT-PCR was performed to assess gene expression. Pearson correlation was used to examine the correlation between gene expression and the severity of OA. $p < 0.05$ was considered statistically correlated. Ns: no correlation; *$p < 0.05$; **$p < 0.01$: significant correlation.

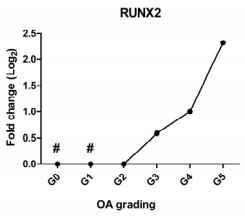
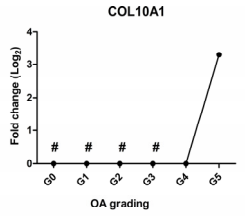
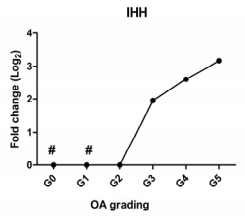
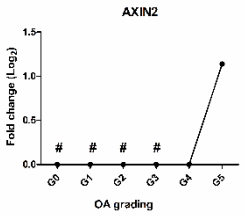
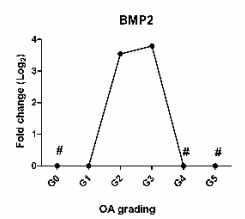
Gene name	Protein Function	Gene expression trend	Correlation between gene expression and OA severity		Correlation significant
RUNX2	Promotes chondrocyte hypertrophy and bone formation		0.908	0.012	*
COL10A1	The marker of hypertrophic chondrocytes, regulates matrix mineralization		0.705	0.118	ns
IHH	Promotes chondrocyte hypertrophy and bone formation		0.961	0.002	**
AXIN2	Induces chondrogenesis at low level while inhibits chondrogenic differentiation at high level		0.827	0.052	ns
BMP2	Stimulates chondrogenesis and increases cartilage matrix turnover		-0.007	0.990	ns

Table 2. Expression of hypertrophy-related genes in OA cartilage and correlation with the severity of OA (n=12). RT-PCR was performed to assess gene expression. Pearson correlation was used to examine the correlation between gene expression and the severity of OA. Y axis=0 is indicated by # means this gene under detection level. $p < 0.05$ was considered statistically correlated. Ns: no correlation; *$p < 0.05$; **$p < 0.01$: significant correlation.

Chondrocyte apoptosis associates with the severity of OA

It has been indicated that death of chondrocytes and loss of extracellular matrix are key features in cartilage degeneration during OA [25]. We therefore performed TUNEL staining for detecting DNA fragmentation in different stages of OA (Figure 3). It has been reported that chondrocyte apoptosis is observed in both the superficial and middle layer in cartilage [26]. For quantification, the number of apoptotic chondrocytes was counted in the superficial (SL) and middle layers (ML). The percentage of apoptotic chondrocytes at higher stages of OA (grades 4 and 5) was significantly higher than that in the lower stages of OA (grades 1 and 2), apoptotic cells were not observed at grade 0. There was a significant positive correlation between grading and apoptotic chondrocyte numbers in OA cartilage ($r = 0.894$, $p = 0.016$).

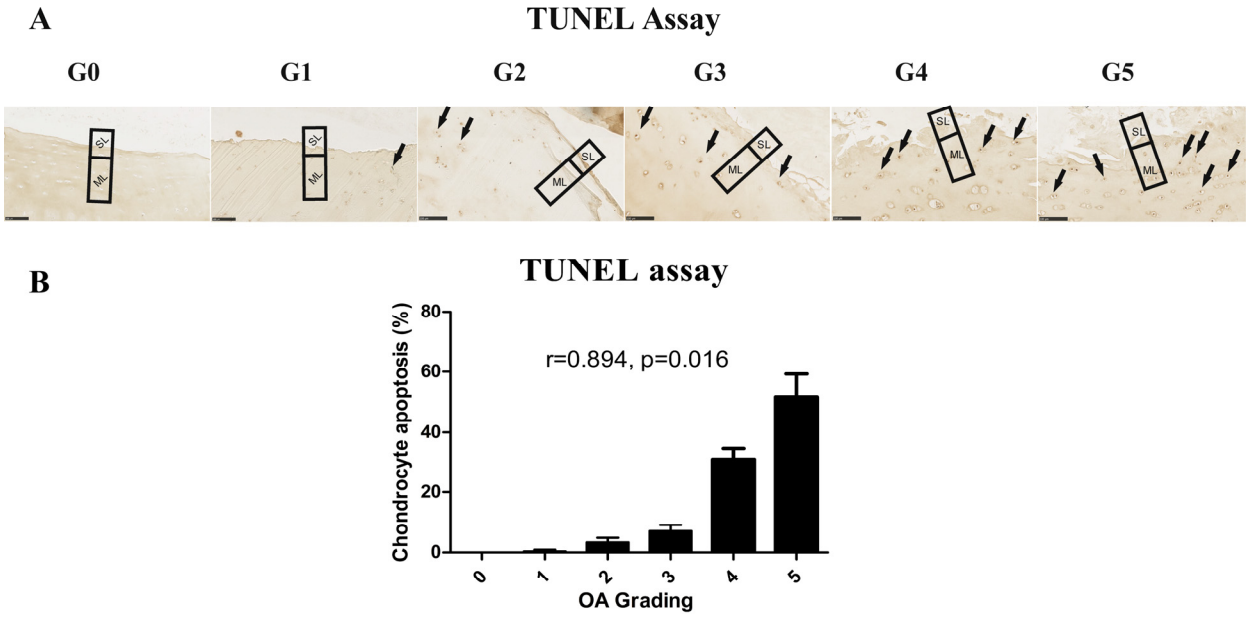


Figure 3. Correlation between histopathological grade and chondrocyte apoptosis. A. TUNEL assay was performed to identify chondrocyte apoptosis at different stages of OA (scale bar 100μm). Arrows indicate apoptotic cells. **B.** Apoptotic cells were counted in superficial layer (SL) and middle layer (ML) in cartilage sections for quantification. Apoptosis is quantified as percentage apoptotic cells of total cells counted.

Discussion

In this study, cartilage samples from patients were graded according to OARSI guidelines and varied from grades 0-5. Twelve candidate cartilage related genes were quantified by qPCR and/or immunohistochemistry.

It is important to discuss the fact that when we obtain cartilage samples from OA patients undergoing total joint replacement surgery, the articular cartilage in the joint from most of patients is almost gone. In addition, cartilage with multiple OA grades is found within single joints. RNA samples from one patient therefore are not enough for any genetic study as ours, so we pooled cartilage samples with the same OA grade (according to the OARSI guidelines) from different patients (Supplemental table 1). We therefore can only discuss the trend of the gene expression levels coupled to the OA grade of the cartilage. For the qPCR assays we have no information on the patient-to-patient variability, but results are an average of material of multiple patients. It is therefore key to note that the protein expression levels of COL2A1, COL10A1 and GREM1, as measured in IHC experiments and which were done for each patient, correspond to the trend described with the qPCR experiments.

Chondrocytes express specific markers, such as collagen type II and the chondrogenic master regulator, the transcription factor, SOX9 [22]. Damage to collagen type II and loss of other cartilage ECM components occur in OA [27, 28]. As expected, the gene expression of all three chondrocyte markers: *SOX9, ACAN* and *COL2A1,* was decreased with OA severity and negatively correlated with OA severity in line with previous reports [29]. Interestingly, while the expression of *ACAN* and *COL2A1* was sharply decreased between stages 2 and 5, the loss of *SOX9* was more gradual. This indicates the lack of a one to one relationship between *SOX9* and these cartilage markers despite the fact that these markers are direct target genes of the transcription factor *SOX9*. This may imply that OA is initiated by differential regulation of the signaling network that regulates SOX9 protein activity, and not with downregulation of *SOX9* at the transcriptional level. The loss of expression of COL2A1 also was confirmed by IHC, which was in accordance with previously described data [30, 31].

COL1A1 is a fibroblastic marker expressed in dedifferentiated chondrocytes [32, 33]. We found that *COL1A1* expression increased significantly in higher OA grades and that the ratio of *COL2A1/COL1A1* dropped strongly with OA severity. This expression trend is in agreement with previous reports that collagen I was strongly increased at end-stage OA as compared to normal and early-stage OA [28, 34], and that a shift of phenotypes towards fibroblasts was indicated by the drop in the *COL2A1/COL1A1* ratio in OA [35, 36].

Previously, we reported that three antagonists, DKK1, FRZB and GREM1, are key factors that maintain cartilage homeostasis by diminishing terminal hypertrophic differentiation in long-bone explant cultures and chondrogenically differentiating human mesenchymal stem cells [10]. DKK1 is associated with OA development and high levels of DKK1 have a protective function against cartilage degeneration [37-39]. FRZB-knockout mice display more severe OA cartilage degeneration in response to joint instability, enzymatic injury, or inflammation [40]. In addition, it was shown that the highest FRZB serum levels were associated with a modest reduction in risk of incident hip OA [37]. We found that the expression of *DKK1* and *FRZB* was lost with the increased severity of OA and that both of these two factors negatively correlated with OA grading. This is in line with the proposed protective roles of DKK1 and FRZB in articular cartilage, and that loss of these factors might lead to OA progression. We found down-regulation of *GREM1* in low grade of OA while the expression is up-regulation in advanced OA. IHC demonstrated that GREM1 was highly expressed in late stage of OA, which has been reported [7]. Our group previously demonstrated that *GREM1* mRNA expression was decreased in OA [23]. In this previous study the decrease in *GREM1* mRNA expression was measured between macroscopically healthy looking cartilage from an OA joint and OA cartilage without grading of samples. The samples from the previous study might belong to grades 0-2. However, in the current study we used histological grading of the samples from OA grade 0 to 5, thereby being more careful in specifying healthy and OA tissue. In our previous study we also found the increase in *GREM1* expression specifically after mechanical loading [23]. In the present study we did not identify whether the high grade OA cartilage was isolated from load-bearing regions, which may explain the high expression of GREM1 in end stage OA. However, we cannot exclude that the differences are due to joint-specific differences in gene expression.

Hypertrophy-like changes of chondrocytes have been reported in both human OA joints and experimental models of OA [41-43]. These changes play a crucial role in the OA disease progress since they result in protease-mediated cartilage degradation [44]. When chondrocytes in OA undergo hypertrophic differentiation, this changes their behavior, as indicated by (increased) expression of hypertrophic markers, matrix calcification, and an enlarged cellular volume [45]. In this study, the expression of three hypertrophic markers, *RUNX2, COL10A1* and *IHH,* gradually increased with the severity of OA. In addition, *RUNX2* and *IHH* positively correlated with OA severity. *COL10A1* was not expressed in healthy articular cartilage (grade 0) and in early stage of OA (grade 1, 2, and 3) but highly expressed in late stage OA (grade 4 and grade 5). These results are in line with another finding that collagen type X is consistently

found in advanced stages of OA and is absent in normal cartilage [46], indicating that hypertrophic differentiation might play a role in late stage of OA.

It has been shown that WNT [47, 48] and BMP [49] are involved in experimental and human OA [50]. In our study, the target gene of canonical WNT signaling, *AXIN2*, became highly detectable at the mRNA level in OA grades 4 and 5. Excessive activity of WNT signaling increases chondrocyte hypertrophy and expression of matrix-degrading enzymes [44]. The reduced expression level of the WNT antagonists *DKK1* and *FRZB* at the mRNA level might be related to this high activity of WNT signaling at late stages of OA, subsequently leading to high expression of hypertrophic markers, consequently contributing to OA. *BMP2* expression was below detection levels in normal cartilage (grade 0) and was low in OA grade 1 but strongly increased at grade 2 and further increased at grade 3 followed by undetectable levels in grade 4 and 5. Similar results are observed in previous studies showing that BMP2 mRNA expression and protein expression is low in normal cartilage, but is increased in the area directly around OA lesions and in OA cartilage [51, 52]. The regulation of BMP2 varies in acute OA and chronic OA. In chronic OA patients a reduced BMP2 expression is found [53]. The absence of *BMP2* expression in grade 4 and grade 5 in our study may indicate that the patients have chronic OA.

Chondrocytes are the only cell type present in cartilage and are responsible for the maintenance of the specialized ECM of the tissue [54]. During the development or progression of OA, chondrocytes change their function or undergo apoptosis or chondroptosis. It has been reported that in human OA articular cartilage, the main pathway of chondrocyte death is classical apoptosis [55], and that apoptosis mainly occurred in the superficial and middle layers in equine OA. In our study, the number of apoptotic cells was significantly increased in the superficial and middle layer in high grade OA, but not in low grade OA. Whether chondrocyte apoptosis is a cause or a result of cartilage degeneration in OA is debatable. Our results, however, show that apoptosis of chondrocytes correlates with cartilage degeneration and as such might play an important role in the pathogenesis of OA.

In conclusion we have shown that the expression of cartilage-related genes (*SOX9, ACAN, COL2A1, DKK1, FRZB*) was decreased while hypertrophy-or OA-related genes (*RUNX2, COL10A1, COL1A1, IHH, AXIN2*) was increased or could be detected during OA progression. Moreover, we see a sharp loss of the *SOX9* target genes while loss of *SOX9* mRNA expression is more gradual, suggesting that the loss of *COL2A1* and *ACAN* during OA might be also regulated by other additional mechanisms. We therefore conclude that the expression of cartilage-specific genes correlate negatively with the different stages of OA, and hypertrophy

genes correlate positively with OA severity. In addition, at high OA grades, apoptosis was induced. These differences in gene expression provide us with an OA stage specific gene expression profile that can be used for staging OA at the molecular level and ultimately to therapeutically targeting of chondrocyte hypertrophy by the WNT signaling antagonists DKK1 and FRZB.

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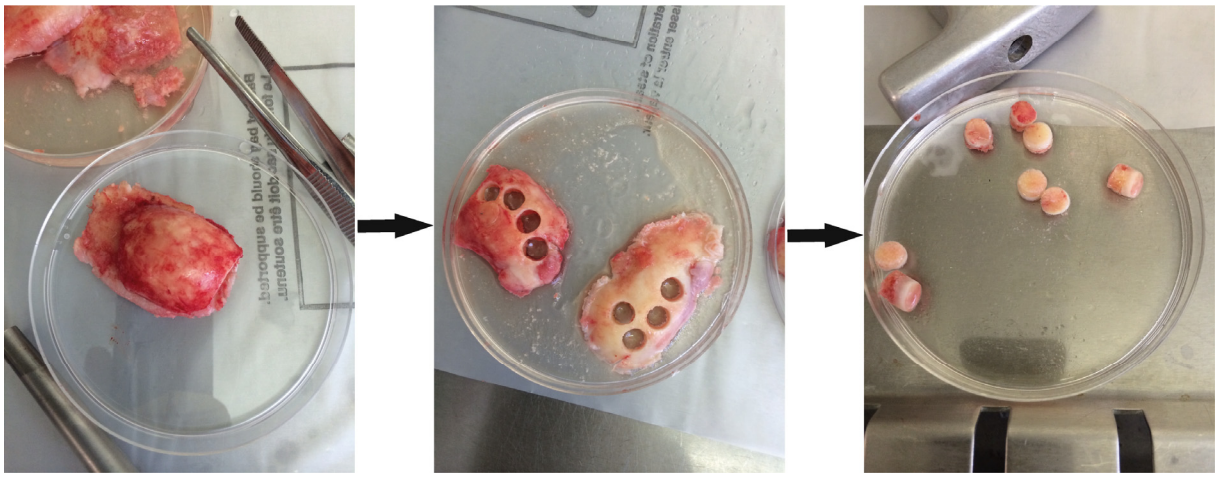
Supplemental data



Supplemental figure 1. Articular cartilage was obtained from patients. The cartilage samples with subchondral bone were punched aseptically using a steel punch, Doughnut-shaped cartilage cylinders were obtained. Subsequently the subchondral bone was removed.

Grade / Donor Information			0	1	2	3	4	5
Donor Number	Age	Gender	Pooled RNA Sample (n = 2)	Pooled RNA Sample (n = 2)	Pooled RNA Sample (n = 4)	Pooled RNA Sample (n = 4)	Pooled RNA Sample (n = 3)	Pooled RNA Sample (n = 3)
1	60	F	×	–	–	–	–	–
2	62	M	×	–	–	–	–	–
3	79	M	–	×	–	–	–	–
4	65	F	–	×	–	–	–	–
5	67	M	–	–	×	–	×	–
6	66	F	–	–	×	×	–	–
7	78	M	–	–	–	×	–	×

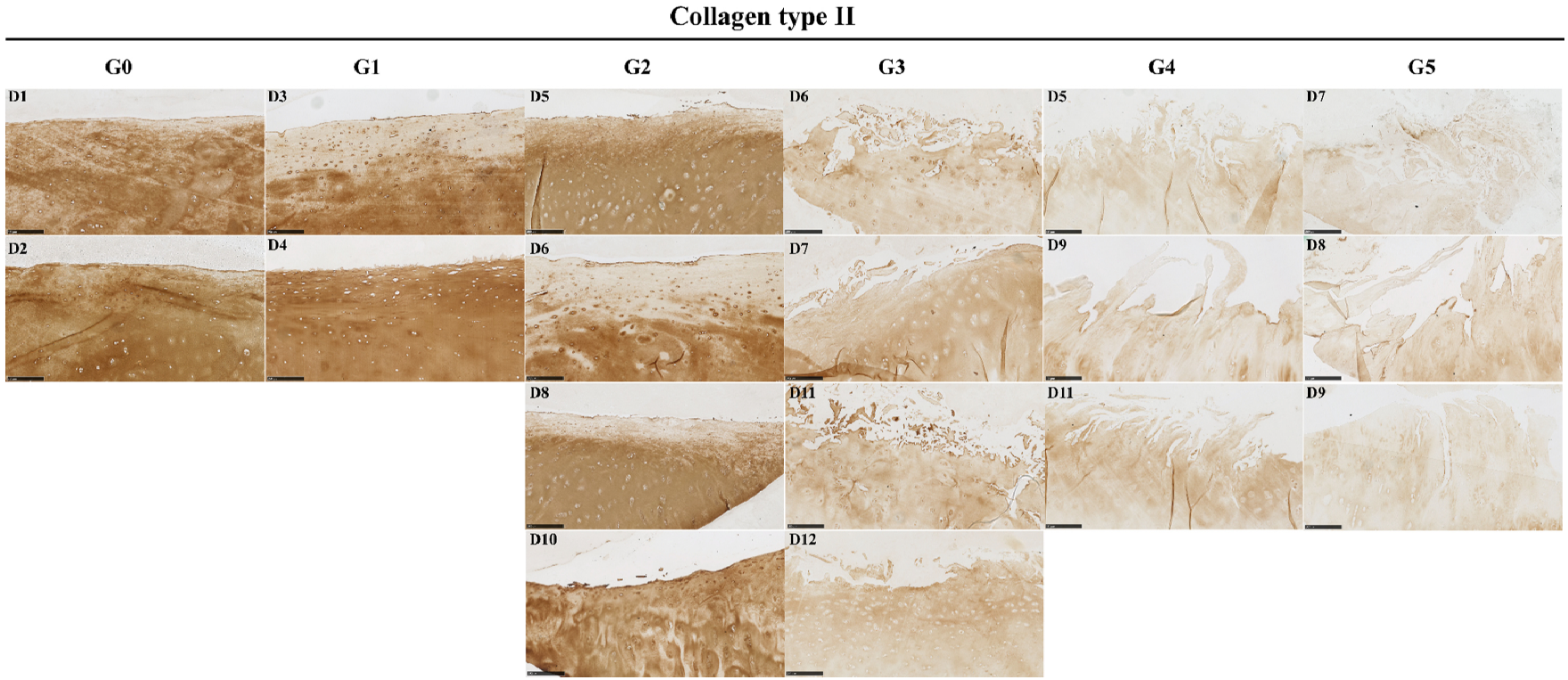
8	64	F	–	–	×	–	–	×
9	69	F	–	–	–	–	×	×
10	65	M	–	–	×	–	–	–
11	67	F	–	–	–	×	×	–
12	71	M	–	–	–	×	–	–

Supplemental table 1. Patient's information. Cartilage was obtained from 12 patients (68±6 years) with OA undergoing total knee replacement surgery. Patient's information including age, gender: male (M) and female (F) is indicated in this table. In addition, some cartilage specimens with different grades were collected from the same patient and cartilage specimens with same grade were pooled together for RNA isolation. Information is provided which samples were pooled for RNA extraction.

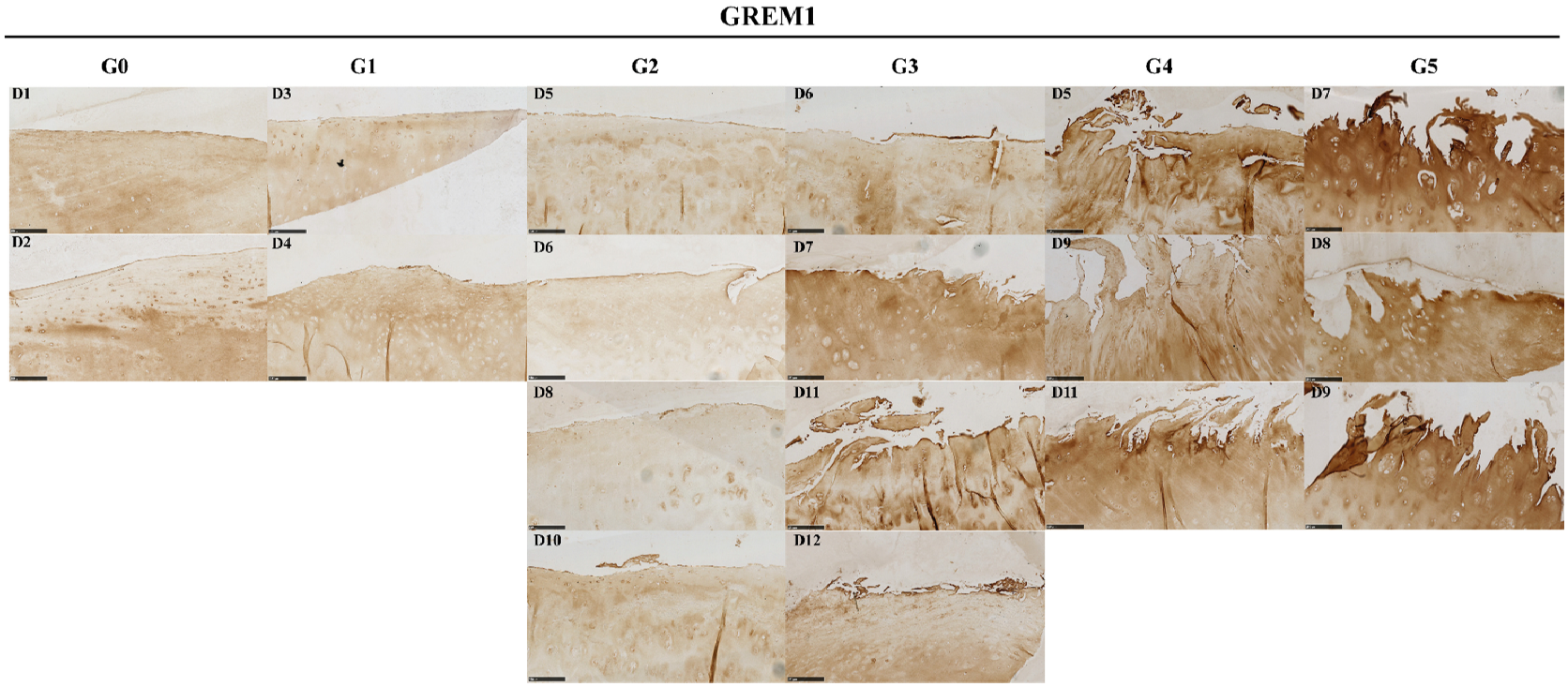
Gene Name	Primer Sequence	Product Size	Annealing Temperature
GAPDH	Forward primer: 5′ CGCTCTCTGCTCCTCCTGTT 3′ Reverse primer: 5′ CCATGGTGTCTGAGCGATGT 3′	81	60
SOX9	Forward primer: 5′ TGGGCAAGCTCTGGAGACTTC 3′ Reverse primer: 5′ ATCCGGGTGGTCCTTCTTGTG 3′	98	60
ACAN	Forward primer: 5′ TTCCCATCGTGCCTTTCCA 3′ Reverse primer: 5′ AACCAACGATTGCACTGCTCTT 3′	121	60
COL2A1	Forward primer: 5′ GGCGGGGAGAAGACGCAGAG 3′ Reverse primer: 5′ CGCAGCGAAACGGCAGGA 3′	129	60
RUNX2	Forward primer: 5′ GGAGTGGACGAGGCAAGAGTTT 3′ Reverse primer: 5′ AGCTTCTGTCTGTGCCTTCTGG 3′	133	60
COL10A1	Forward primer: 5′ GAACTCCCAGCACGCAGAAT 3′ Reverse primer: 5′ CCTGTGGGCATTTGGTATCG 3′	121	60
IHH	Forward primer: 5′ GCCATCTCCTCTGCCATGAA 3′ Reverse primer: 5′ TGCCAGCCTCAAGGTCTCTA 3′	153	60
DKK1	Forward primer: 5′ AGTACTGCGCTAGTCCCACC 3′ Reverse primer:5′ TCCTCAATTTCTCCTCGGAA 3′	172	60
FRZB	Forward primer: 5′ACGGGACACTGTCAACCTCT 3′	155	60

	Reverse primer: 5′ CGAGTCGATCCTTCCACTTC 3′		
GREM1	Forward primer: 5′ GTCACACTCAACTGCCCTGA 3′	375	60
	Reverse primer: 5′ GGTGAGGTGGGTTTCTGGTA 3′		
AIXN2	Forward primer: 5′ AGTGTGAGGTCCACGGAAAC 3′	103	60
	Reverse primer: 5′ CTGGTGCAAAGACATAGCCA 3′		
BMP2	Forward primer: 5′ GCTAGACCTGTATCGCAGGC 3′	74	60
	Reverse primer: 5′ TTTTCCCACTCGTTTCTGGT 3′		
COL1A1	Forward primer: 5′ GTCACCCACCGACCAAGAAACC 3′	121	60
	Reverse primer: 5′ AAGTCCAGGCTGTCCAGGGATG 3′		

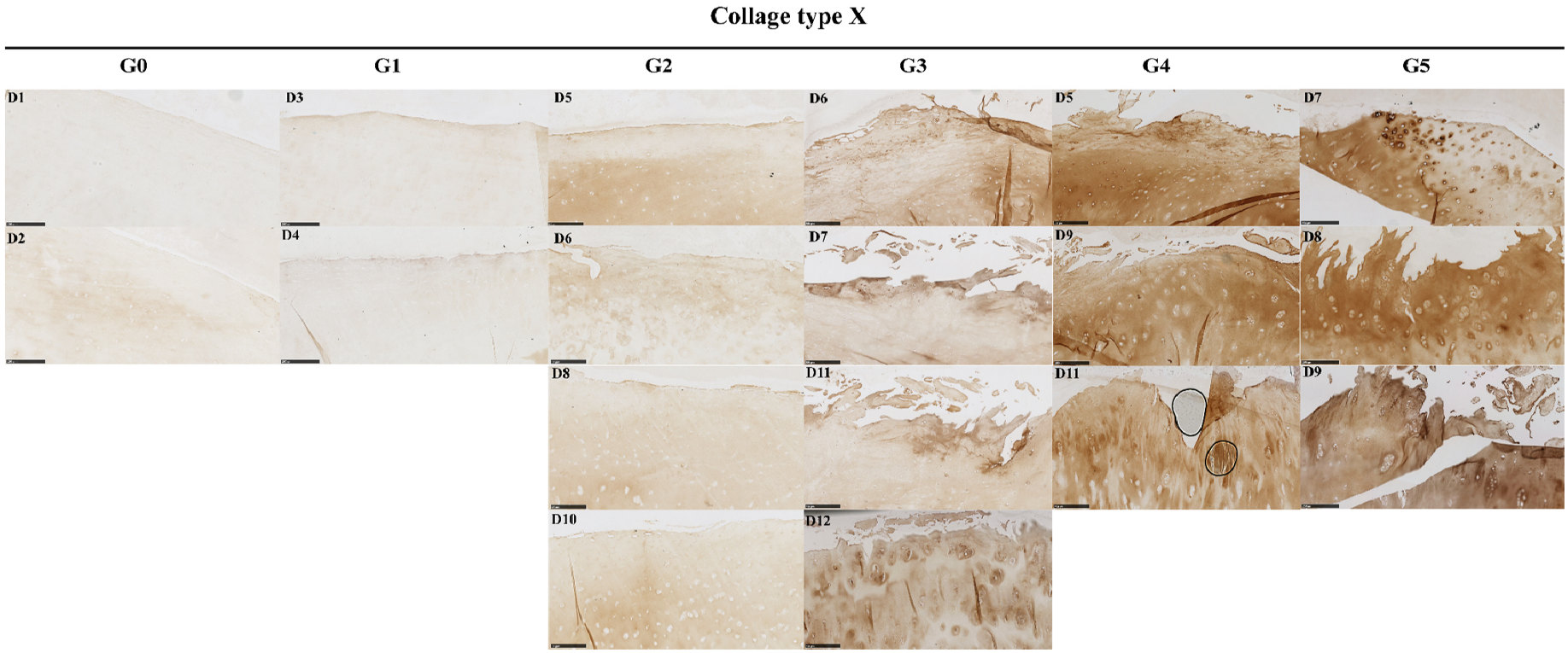
Supplemental table 2. Forward (F) and Reverse (R) primers used for quantitative RT-PCR.



Supplemental figure 2. The protein expression of collagen type II was detected by IHC in each donor (scale bar 250µm). Images were taken using the Nanozoomer. G0, G1, G2, G3, G4, G5=Grade 0, Grade 1, Grade 2, Grade 3, Grade 4, Grade 5; D1-D12=Donor 1-12.



Supplemental figure 3. The protein expression of GREM1 was detected by IHC in each donor (scale bar 250µm). Images were taken using the Nanozoomer. G0, G1, G2, G3, G4, G5=Grade 0, Grade 1, Grade 2, Grade 3, Grade 4, Grade 5; D1-D12=Donor 1-12.



Supplemental figure 4. The protein expression of collagen type X was detected by IHC in each donor (scale bar 250µm). Images were taken using the Nanozoomer. G0, G1, G2, G3, G4, G5=Grade 0, Grade 1, Grade 2, Grade 3, Grade 4, Grade 5; D1-D12=Donor 1-12.

R^2	*SOX9*	*ACAN*	*COL2A1*	*DKK1*	*FRZB*	*GREM1*	*COL10A1*	*IHH*	*RUNX2*	*AXIN2*
SOX9	–	–	–	–	–	–	–	–	–	–
ACAN	0.87 *	–	–	–	–	–	–	–	–	–
COL2A1	0.89 *	0.96	–	–	–	–	–	–	–	–
DKK1	0.61	0.76	0.82	–	–	–	–	–	–	–
FRZB	0.72	0.94 **	0.86 *	0.82 *	–	–	–	–	–	–
GREM1	−0.75	−0.57	−0.57	−0.34	−0.41	–	–	–	–	–
COL10A1	−0.72	−0.55	−0.55	−0.36	−0.42	0.99 ***	–	–	–	–
IHH	−0.97 **	−0.91 *	−0.91 *	−0.63	−0.78	0.83 *	0.81	–	–	–
RUNX2	−0.78	−0.65	−0.65	−0.77	−0.60	0.99 **	0.99 *	0.91	–	–
AXIN2	−0.91 *	−0.68	−0.68	−0.46	−0.54	0.93 **	0.93 *	0.91 *	0.95 *	–
BMP2	0.34	−0.16	−0.16	−0.28	−0.38	−0.39	−0.37	−0.19	−0.70	−0.48

Supplemental table 3. Pearson correlations of all tested genes. Correlation coefficient determined by Pearson's correlation analysis *P< 0.05, **P< 0.01, *** P< 0.001.

Chapter 4

Synovial fluid concentrations of Dickkopf-related protein 1 (DKK1), Frizzled-related protein (FRZB) and Gremlin 1 (GREM1) correlate to OA progression in patients with previous knee injuries

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Creep before you walk (循序渐进)

Abstract

Purpose: Investigate the concentrations of Dickkopf-related protein 1 (DKK1), Frizzled-related protein (FRZB) and Gremlin 1 (GREM1) in synovial fluid (SF) from knee injury (acute and chronic), osteoarthritis (OA) and healthy subjects; and correlate their expression with other markers. In a cross-sectional study SF was aspirated from patients with acute (n = 158) and chronic (n = 50) knee injury, OA (n = 22) and from subjects with healthy knee (n = 9). SF concentrations (conc.) of DKK1, FRZB and GREM1 were determined using enzyme-linked immunosorbent assay (ELISA). SF median conc. of DKK1 is 1.3 times lower in chronic injury group (CI) than in early acute injury (AI) ($p < 0.001$), 1.2 fold lower in OA than in injury group (AI + CI) ($p = 0.005$). FRZB conc. was 1.8 times lower in CI group than in reference group (Ref) ($p = 0.063$), 2.2 fold lower in CI than in AI ($p < 0,001$), and 1593 times lower in OA than in Ref ($p = 0.004$). GREM1 conc. was 1.2 times lower in CI than in AI ($p < 0.001$), however, 1.5 times higher in AI than in Ref ($p = 0.008$) and 1.2 times higher in OA than in CI ($p = 0.034$). DKK1, FRZB and GREM1 were negatively correlated to time after injury, and significantly decreased after injury with age. Additionally, GREM1 is negatively correlated with FRZB ($r_S = -0.364$, $p < 0.001$) while positively correlated with DKK1 ($r_S = 0.250$, $p = 0.002$). FRZB SF conc. positively correlated with chondrocytes markers (GAG, ARGS-aggrecan, COMP, and type II collagen epitope C2C), DKK1 and GREM1 positively correlated with IL-8. Taken together, Our findings indicate that the expression of DKK1, FRZB and GREM1 was changed during injury progression, and regulated by other factors. This suggests that DKK1, FRZB and GREM1 can be used potential biomarkers for study of the disease mechanisms in joint disorders, such as joint injury and posttraumatic OA.

Introduction

Osteoarthritis (OA) is the most common form of arthritis and it is characterized by progressive degradation of joint cartilage, bony sclerosis and changes in the synovium [1, 2]. Multiple risk factors have been implicated in the initiation and progression of OA, including mechanical injury, obesity, genetics and aging [3]. Of these, joint injury predisposes to the development of post-traumatic OA with high penetrance [4]. Mechanical injury induces damage to extracellular matrix directly or indirectly via upregulation of matrix-degrading enzymes and downregulation of biosynthetic activity of chondrocytes [5]. At the molecular level, mechanisms by which injury causes cartilage degradation and contributes to the gradual progression in OA are poorly understood. Furthermore, biomarkers that can be used to assess joint's health status, disease progression or response to therapy are still lacking despite numerous efforts to identify such biomarkers.

In synovial fluid samples from cross sectional and longitudinal cohorts of patients with knee injury and OA we have analyzed cartilage and bone markers as well as inflammatory markers studying their gradual changes over time [6-10].

The secreted wingless-type MMTV integration site (WNT) and the bone morphogenetic protein (BMP) signal pathways have been implicated as driving factors in OA development after injury [11]. Numerous studies have revealed a central role of WNT signaling in cartilage homeostasis. In healthy articular cartilage, moderate activity of WNT is essential for chondrocyte proliferation and maintenance of the cell phenotype in the superficial zone of articular cartilage [12]. In contrast, in animal models of OA aberrant canonical WNT-signaling has been implicated in disease development. Indeed, in human OA and in injured cartilage, increased activity of the canonical WNT pathway has been linked to loss of the chondrocyte phenotype and premature hypertrophic differentiation [13, 11, 14].

Although BMPs have a protective effect in articular cartilage [15, 16], they are also implicated in the development of OA [17]. BMP2 is drastically increased in OA chondrocytes and it has been shown that high BMP signaling is associated with the induction of chondrocyte hypertrophy [18].

Typical features of hypertrophic differentiation, such as expression of collagen type 10, cartilage matrix mineralization, invasion of blood vessels and apoptotic cell death, are characteristics of OA affected cartilage at least in a subset of patients [19]. Given the profound role of high levels of WNT [13] and BMP signaling [14] in the regulation of chondrocyte

hypertrophy it is not surprising that the joint expresses a number of WNT- and BMP-signaling antagonists that can control their signaling activity. Indeed, we have identified the WNT antagonists Dickkopf 1 homolog (DKK1) and Frizzled-related protein (FRZB) and the BMP antagonist Gremlin1 (GREM1) as critical regulators of cartilage homeostasis by preventing hypertrophic differentiation of articular chondrocytes [20]. These factors are secreted soluble proteins able to inhibit WNT and BMP-signaling directly or indirectly [21-23]. These antagonists are expressed in articular cartilage at the mRNA and protein level and their mRNA expression is inversely correlated with the severity of osteoarthritis [20, 24].

We hypothesize that the secreted WNT antagonists DKK1 and FRZB and BMP antagonist GREM1 are part of the endogenous mechanism that control WNT and BMP signaling thereby preventing healthy articular cartilage from entering into hypertrophic differentiation. Changes in the expression of these antagonists in the joint may disturb the balance between WNT and BMP signaling and hence may contribute to the loss of the stable articular cartilage phenotype contributing to OA development. We tested this hypothesis using samples from patients with knee injuries by measuring the synovial fluid concentrations of DKK1, FRZB and GREM1. Furthermore we performed correlation analyses with pro-inflammatory cytokines and cartilage degradation markers which were previously measured in the same cohort.

Materials and methods

Subjects

From a cross-sectional convenience cohort, 208 knee injured patients had synovial fluid aspirated from their knee. Of those, 104 subjects did not fulfill the inclusion criteria of a randomized controlled trial [6], and the remaining 104 subjects were studied in the course of previous cross-sectional investigations [25-27, 10] (Table 1). The knee injured subjects were organized by time between injury and synovial aspiration, and for our primary analysis stratified into two groups of recent injury (sampling 0-77 days, or 0-11 weeks after injury) and old injury (sampling 1-37 years after injury); for a secondary analysis of temporal patterns after knee injury, subjects were instead stratified by time after injury into 8 groups of 20 to 30 subjects each (Table 1).

From another convenience cohort we used synovial fluid from 22 subjects with OA (of which seven samples were collected during arthroplasty) and from 9 knee healthy reference subjects without a history of knee symptoms or knee injury, or with normal findings on clinical

examination (Table 1). These samples have been used in previous investigations [25-27, 10, 6, 28].

Synovial fluid was aspirated (without lavage) from each subject at one time point only, centrifuged at 3000x g for 10 minutes at 4ºC and the supernatants were stored at -80ºC. All subjects consented to take part in this study by informed consent, which was approved by the regional ethical review board at Lund University.

Main diagnostic groups	**Sub groups of injury**	**Time between injury and sampling**	**n (% women)**	**Age in years, median (range)**
Reference subjects	-	-	9 (33)	30 (17-58)
Injury, all	-	0-36.9 years	208 (25)	29 (13-70)
	Recent injury	0-77 days	158 (23)	25 (13-64)
	Recent injury, sub stratification	*0 days*	*26 (19)*	*33 (14-46)*
		1 day	*28 (21)*	*24 (13-57)*
		2-3 days	*29 (31)*	*26 (16-54)*
		4-7 days	*30 (37)*	*25 (14-64)*
		8-22 days	*25 (16)*	*22 (15-57)*
		23-77 days	*20 (5)*	*29 (17-49)*
	Old injury	1-36.9 years	50 (30)	35 (18-70)
	Old injury, sub stratification	*1-2.5 years*	*27 (37)*	*32 (18-61)*
		2.8-36.9 years	*23 (22)*	*43 (18-70)*
Osteoarthritis	-	-	22 (41)	64 (39-86)

Table 1. Characteristics of the study subjects. The knee injury groups are presented with stratification based on time between injury and sample aspiration. The clinical diagnosis of the injured subjects was: isolated cruciate ligament injuries (ACL n = 24 or PCL n = 4), ACL injury with meniscus tear (n = 28),

ACL injury with meniscus tear and other ligament injuries (n = 31), ACL injury with other ligament injuries (n = 32), isolated meniscus tear (n = 53), meniscus tear with other ligament injuries (n = 6), patellar dislocation with or without soft tissue injuries (n = 13) other types of injuries (medial or lateral collateral ligament tears n = 5, PCL tear with meniscus or ligament injuries n = 1, give-way n = 2), no signs of soft tissue injury (n = 9).

Analysis of DKK1, FRZB and GREM1 in synovial fluid

Synovial fluid concentrations of DKK1 (R&D system), FRZB (R&D system) and GREM1 (Bio-connect diagnostics) were determined using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer.

Samples were diluted in reagent diluent at a ratio of 1:2 for DKK1 and FRZB analysis, and 1:24 for GREM1 analysis. The technical performance of the ELISAs in synovial fluid was conducted as described previously [7] (Table 2). The lower limit of detection (LLOD) and upper limit of detection (ULOD) were established based on the performance of the standard curve on eight plates, by analyzing which was the highest and lowest concentrations the standards had a back-calculated relative error within 20% of the nominal concentration and had a coefficient of variation (CV) between duplicates below 20% [29]. Randomly selected knee synovial fluid samples were used to assess dilution linearity (n = 3) and spiking recovery (n = 4). Details on the technical performance of the DKK1, FRZB and GREM1 ELISA in synovial fluid in terms of dilution linearity and spiking recovery, intra- and inter-assay CV and the influence of thawing and freezing of samples is presented in Table 2. Each of the assay characteristics was within the acceptable range for reliable concentration measurements in complex bodily fluids [6].

Analyte			**FRZB**	**DKK1**	**GREM1**
Concentration, pg/ml		LLOD	7.77	7.77	57
		ULOD	16000	8000	16000
Recovery, mean (range) %					
	Dilution (n = 3)	***Ratio***			
		1:2	87 (78-96)	97 (77-115)	Na
		1:4	93 (72-120)	93 (85-112)	Na
		1:8	91 (71-113)	87 (76-106)	Na
		1:24	Na	Na	114 (97-131)
		1:48	Na	Na	83 (68-97)
		1:96	Na	Na	98 (82-119)
	Spiking (n = 3)	***Concentration***			
		High	92 (83-110)	90 (87-94)	102 (81-124)
		Middle	90 (84-102)	85 (80-92)	107 (94-121)
		Low	104 (86-122)	84 (80-90)	97 (84-115)
	Freeze-thaw (n = 3)	***Cycle***			
		1st	81 (73-89)	103 (93-121)	107 (93-121)
		2nd	83 (79-88)	98 (79-117)	100 (81-135)
CV, mean %		***Assay***			
		Intra (n=5)	5.2	6.6	8.3
		Inter (n=4)	6.2	7.6	9.2

Table 2. Technical performance of the DKK1, FRZB and GREM1 immunoassays using synovial fluid. To calculate dilution linearity randomly selected synovial fluid samples (n = 3) were prepared in three different dilutions and analyzed. Results are expressed as % recovery: 100 x [(concentration at a specific dilution) divided by (original concentration divided by the dilution times)]. Spiking recovery was calculated from randomly selected synovial fluid samples (n = 3) which were spiked using high, middle or low concentrations of standards. Spiking recovery is expressed in % as recovery: 100x [(concentration of a sample spiked with a specific amount of standard or another sample) divided by (the individual concentration of sample plus the individual concentration from standard or another sample)]. To analyze freeze-thawing effects on DKK1, FRZB and GREM1 concentrations randomly selected synovial fluid samples (n = 3) were freeze/thawed two cycles. Recovery after freeze-thaw cycles is expressed in relation to the amount obtained the first time the sample was thawed. Intra and inter DKK1, FRZB and GREM1 assay coefficient of variations (CV) was calculated using randomly selected synovial fluid samples (n = 3) loading 5 repeats (intra) on a single plate or loading duplicates on 4 plates (inter). Lower limit of detection (LLOD) and upper limit of detection (ULOD) for the DKK1 FRZB and GREM1 assays were estimated. Na = not applicable due to either to low or to high dilution ratio.

Other biomarkers and cytokines

A subset of synovial fluid samples from the recent injury group (n = 104 to 136) was analyzed in the course of previous investigations [6, 7, 28, 25] for the following biomarkers: sulfated glycosaminoglycan (sGAG), ARGS neoepitope of aggrecan (ARGS-aggrecan from aggrecanase cleavage at the TEGE392↓393ARGS site), osteocalcin, secreted protein acidic and rich in cysteine (SPARC), cartilage oligomeric matrix protein (COMP), type II collagen epitope C2C, osteopontin, interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α.

Statistics

According to Shapiro-Wilk tests, synovial fluid FRZB, DKK1 and GREM1 concentrations were not normally distributed in all diagnostic groups and therefore when groups were compared non-parametric analysis was conducted. Between-groups comparisons were made using Mann-Whitney rank sum tests, and for correlation analysis Spearman's rank correlation (r_S) analysis were used. Pearson Chi-square test was used for comparison of genders between subject groups, and for comparison of age Students t-test was used. All these tests were 2-tailed. Samples with concentrations below the lower limit of detection (LLOD) were imputed and given a value equal to half the value of LLOD. The synovial fluid concentrations of FRZB, DKK1 and GREM1 and the amount of samples with imputed values (between 0 and 64% depending on groups) in the different subject groups are shown as supplementary data (Table S1). In the analysis on group levels the imputed values were included. In the correlation analysis, samples with and without (measured values only) imputed values were analyzed, but significance was considered only when both analyses showed correlation, and the presented data herein (r_S and p-values) include imputed values. The IBM Statistical Package for Social Sciences (SPSS, version 21) was used for statistical analysis and P-values less than 0.05 was considered statistically significant. We did not compensate for multiple testing due to the exploratory nature of the study.

Results

Concentration of FRZB, DKK1 and GREM1 in synovial fluid of different patient groups

The median synovial fluid concentrations of FRZB, DKK1 and GREM1 in synovial fluid of the 4 different populations (reference, recent injury, old injury and OA) are depicted in the left panels of Figure 1. The right panels show the median expression as function of time after injury in the injury group only. Supplementary table S1 summarizes the numerical values of the measurements as well as the p-values of the Mann-Whitney U test comparing the injury (or OA) groups versus the reference group. The median synovial fluid concentration of FRZB was 1.7 times higher in the recent injury group compared to the old injury group ($p = 0.009$), and the median FRZB concentration in OA patients was 1600-fold lower compared to the median concentration in the reference group (Figure 1A, Table S1). In the secondary analysis with subjects stratified in eight groups, we note a delayed and transient increase of FRZB levels, peaking after approximately 3 weeks after injury, although these fluctuations were all within levels found in the reference group (Figure 1B, Table S1).

The median synovial fluid concentration of DKK1 was 1.3 times higher in the recent injury group compared to the old injury group ($p < 0.001$), and the DKK1 concentration in the OA group was no different from concentrations observed in the reference group (Figure 1A, Table S1). The secondary analysis with injury sub-stratified in eight groups suggested that the DKK1 concentrations peaked within the first day after injury, with a subsequent gradual decrease towards levels comparable to those of the reference group, although without statistically significant differences compared to the reference group at any time point (Figure 1B, Table S1).

For GREM1, the median synovial fluid concentration was 1.2 times higher in the recent injury group compared to the old injury group ($p < 0.001$) and 1.5 times higher compared to the reference group ($p = 0.007$; Figure 1A, Table S1). The secondary analysis showed that GREM1 values peaked at the first day after injury and were approximately 2-fold higher compared to the reference group for up to a week after injury; thereafter values gradually decreased towards levels comparable of those found in the reference group (Figure 1B, Table S1). The OA group had slightly higher concentrations of GREM1 compared to the reference group (Figure 1A, Table S1) although this was not statistically significant ($p = 0.094$).

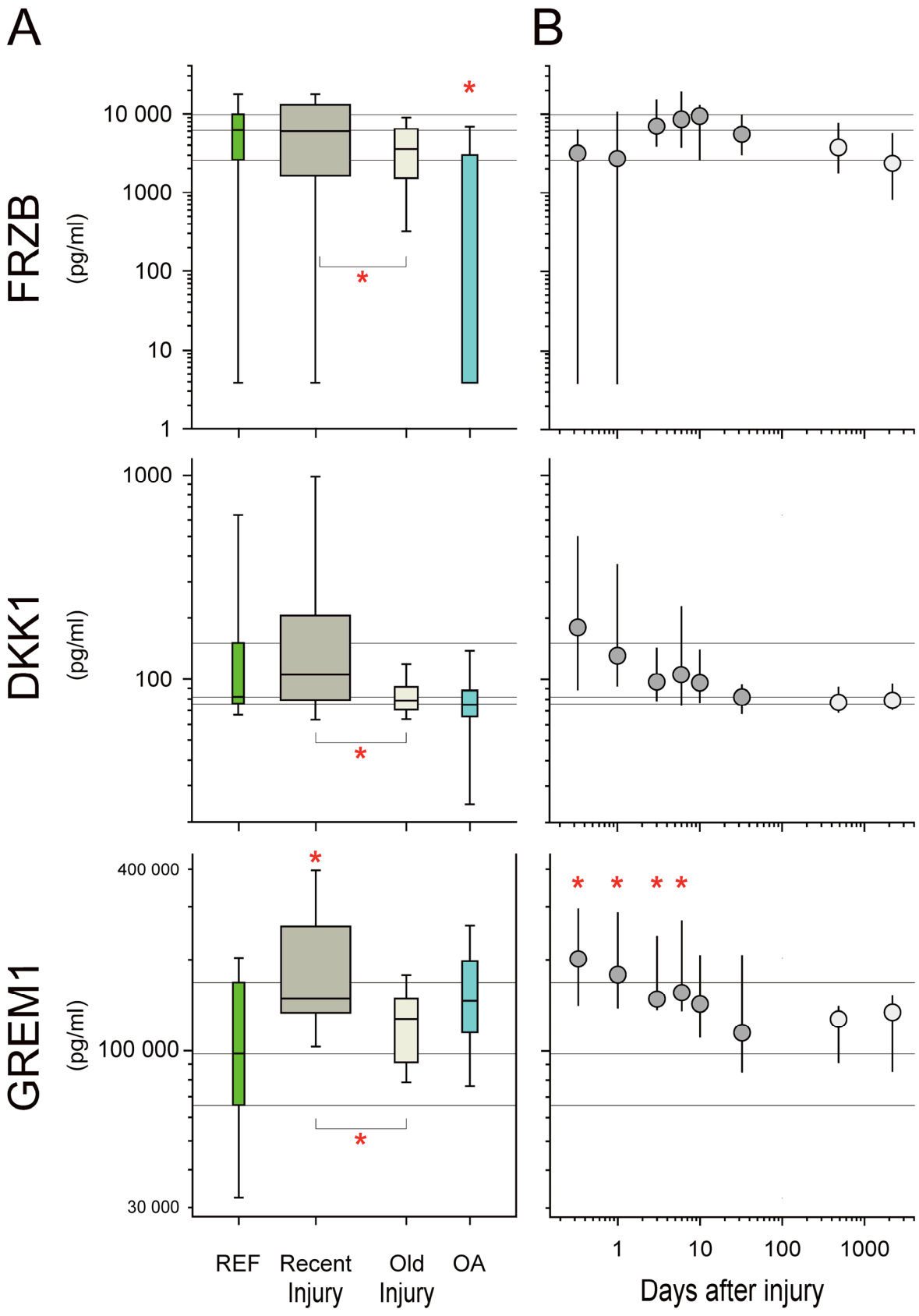

A
B
FRZB
(pg/ml)
10 000
1000
100
10
1
DKK1
(pg/ml)
1000
100
GREM1
(pg/ml)
400 000
100 000
30 000
*
REF
Recent Injury
Old Injury
OA
1
10
100
1000
Days after injury

Figure 1. Synovial fluid concentrations of FRZB, DKK1 and GREM1. (A) Box plots with subjects ordered by the diagnostic groups reference (REF; green), Recent injury (dark grey), Old injury (grey), and osteoarthritis (OA; blue). Boxes show the quartiles (median, 25th and 75th percentiles) with error bars and whiskers for the 10th and 90th percentiles, with box width indicating the relative group size. The quartiles of the reference group are extended as thin horizontal lines in both panels for comparison. (B) Knee-injured ordered by days after injury in sub-groups of 20-30 subjects (Table 1). Quartiles are plotted as filled circles (medians) and error bars (25th and 75th percentiles). Fill colour of circles indicate the origin of the sub-group; Recent injury (dark grey) or Old injury (grey). Group differences by Mann-Whitney U test versus reference (A and B) or between the Recent and Old injury groups (A) is indicated by red asterisks, and is presented as supplementary data in Table S1.

Concentration of DKK1 and GREM1 in synovial fluid after injury is age dependent

There was no difference in gender between the diagnostic groups (data not shown), although the OA patients were older than the subjects in the rest of the groups ($p < 0.001$), and the old injury patients were older than the patients in the recent injury group ($p < 0.001$; Table 1).

For the knee injury group, we next evaluated the biomarker levels in relation to the age at which the aspirate was taken (Figure 2). The synovial fluid levels of FRZB did not change with age; in contrast, the levels of DKK1 and GREM1 negatively correlated with age (Figure 2). To exclude that age related differences between patient groups could be the reason for the observed differences in biomarker concentrations between the recent and old injury groups, we randomly selected patients from both groups generating two new groups (selected recent injury group $n = 80$, selected old injury group $n = 45$), which were similar in age (data not shown). These two selected injury groups had significant differences in levels of FRZB ($p = 0.022$), DKK1 ($p = 0.001$) and GREM1 ($p = 0.001$). This suggests that the differences in biomarker concentrations between the recent and old injury groups seen in Figure 1 were not due to differences in age between the groups.

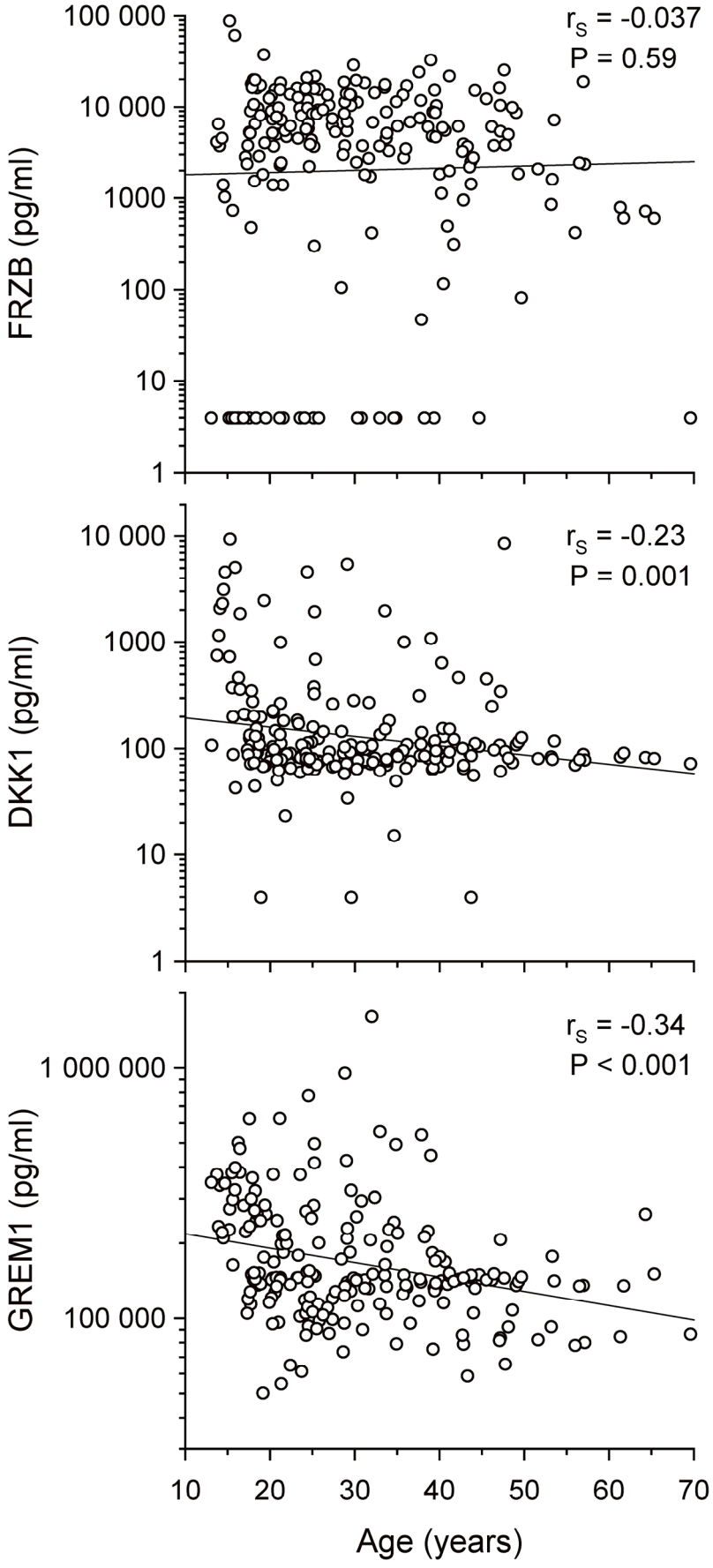

FRZB (pg/ml)
100 000
10 000
1000
100
10
1
r_S = -0.037
P = 0.59
DKK1 (pg/ml)
10 000
1000
100
10
1
r_S = -0.23
P = 0.001
GREM1 (pg/ml)
1 000 000
100 000
r_S = -0.34
P < 0.001
10
20
30
40
50
60
70
Age (years)

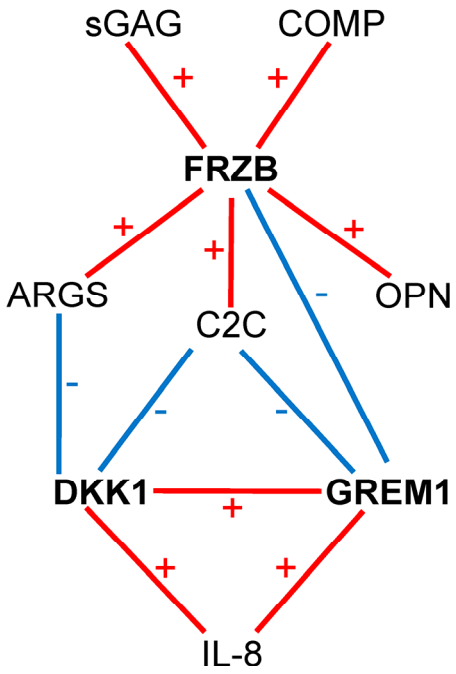
Figure 2. Bi-variate scatter plots of FRZB, DKK1 and GREM1 versus age in all knee-injured subjects. Regression line and correlation with age (Spearman's rho; rS) is indicated for each biomarker.

Correlation between DKK1, FRZB and GREM1 and other markers in the recent injury group

We performed correlation analysis between the expression of DKK1, FRZB and GREM1 (Figure 3). As shown in the table at the bottom of Figure 3, the expression of FRZB and GREM1 was weakly negatively correlated (-0364; $p < 0{,}001$). GREM1 and DKK1 expression was weakly positively correlated (0,250; $p = 0.002$). No correlation was found between DKK1 and FRZB expression.

Previously, we have measured a number of cytokines and bone and cartilage turn over markers in synovial fluid of patients from the early injury group [6, 7, 28, 25]. Remarkably, FRZB positively correlated with bone and cartilage turnover markers sulfated GAG (sGAG; moderate), ARGS neoepitope of aggrecan from aggrecanase cleavage at the TEGE392↓393ARGS site (ARGS; moderate), Osteopontin (OPN; weak), Cartilage Oligomeric Protein (COMP; weak), and the C2C type II collagen epitope (weak) (Figure 3). In contrast, weak negative correlations were found for DKK1 with COMP and C2C and GREM1 and C2C. Both DKK1 and GREM1 were weakly positively correlated with the pro-inflammatory cytokine IL-8. No correlations were found between the expression of the antagonists and IL-1β, IL-6, TNF-α, osteocalcin and secreted protein acidic and rich in cysteine (SPARC).

Fig. 3



	n	FRZB, r_S (p value)	GREM1, r_S (p value)	DKK1, r_S (p value)
FRZB	158	-	**-0.364 (<0.001)**	0.025 (0.759)
GREM1	158	**-0.364 (<0.001)**	-	**0.250 (0.002)**
IL-1β	104	-0.161 (0.104)	0.073 (0.459)	0.179 (0.068)
IL-6	104	-0.158 (0.110)	0.017 (0.860)	0.148 (0.133)
IL-8	104	-0.173 (0.079)	**0.238 (0.015)**	**0.265 (0.007)**
TNF	104	-0.018 (0.859)	0.159 (0.106)	0.135 (0.71)
sGAG	136	**0.490 (<0.001)**	-0.006 (0.944)	0.052 (0.546)
ARGS	103	**0.516 (<0.001)**	-0.133 (0.182)	**-0.281 (0.004)**
COMP	104	**0.367 (<0.001)**	-0.029 (0.773)	-0.167 (0.091)
C2C	123	**0.393 (<0.001)**	**-0.313 (<0.001)**	**-0.268 (0.003)**
Osteocalcin	104	0.038 (0.705)	0.182 (0.064)	0.153 (0.120)
SPARC	104	-0.002 (0.982)	0.161 (0.102)	0.134 (0.176)
Osteopontin	104	**0.379 (<0.001)**	-0.062 (0.533)	-0.143 (0.148)

Figure 3. Correlation between DKK1, FRZB, GREM1 and other biomarkers. Correlation, using Spearman rho (rS), between synovial fluid biomarkers was analyzed in samples from the recent injury group. Red = positive correlation, blue = negative correlation. Significant correlations are bolded. FRZB, frizzled-related protein; GREM1, Gremlin 1; DKK1, Dickkopf-related protein; IL, interleukin; TNF, tumor necrosis factor α; sGAG, sulfated glycosaminoglycan; ARGS, ARGS-aggrecan; COMP, cartilage oligomeric matrix protein; C2C, type II collagen epitope C2C; SPARC, secreted protein acidic and rich in cysteine; OPN, osteopontin.

Discussion

Biomarker measurements in synovial fluid may be better predictive for the health status of a joint than measurements in serum or urine due to the dilution effect of biomarkers in these bodily fluids. In addition, many of the potential biomarkers, such as pro-inflammatory cytokines and matrix metalloproteinases, are expressed by multiple tissues in the body each contributing to the expression of these markers in serum. Finally, OA is usually limited to one or a few joints in particular in post-traumatic OA and measurements in synovial fluid may provide direct information on the traumatized joints. Biomarker measurements in serum may reflect the health status of the more than 170 joints in the body and appears therefore more suited for assessing biomarkers in systemic joint diseases such as rheumatoid arthritis.

To our knowledge, this is the first investigation of expression level of signaling natural antagonists (DKK1 and FRZB for WNT, GREM1 for BMP) and their correlation with other cartilage and bone markers and pro-inflammatory cytokines in SF collected from recent (0-77 days from injury) to old (1-37 years after injury) knee injuries.

The concentrations of DKK1, FRZB and GREM1 were lower in the old injury group as compared to levels found in the recent injury group, which suggested that DKK1, FRZB and GREM1 were gradually decreased with injury progression. This is supported by our recent finding that expression of DKK1 and FRZB decrease in cartilage during OA progression (Chapter 3). Possibly as a result of the decreased expression of the WNT and BMP antagonists, WNT and BMP signaling was enhanced during the period of old knee injury as was supported by the interesting observation that WNT16 and β-catenin was strongly upregulated while WNT antagonist FRZB was strongly decreased after cartilage explant injury [13].

In this study, the DKK1 concentrations were much lower compared to levels for FRZB and GREM1 in most of samples. The average median was 86.8pg/ml, which is quite similar to the data from another study showing that DKK1 levels in synovial fluid (58.6pg/ml) were significantly lower than in paired plasma samples (396.0pg/ml) [30]. DKK1 concentrations peaked within the first day after injury. Since high DKK1 has a protective function against cartilage degeneration and DKK1 is associated with OA development [31, 32, 30]. It might be that high expression of DKK1 upon early injury is self-protection response.

The functional importance of the FRZB in joint homeostasis is emphasized by the observation that a single nucleotide polymorphism causing loss of function of the FRZB and gene product is associated with hip OA in humans [33, 34]. FRZB-knockout mice have more

severe OA cartilage deterioration in response to instability, enzymatic injury, or inflammation [35]. In addition, another study shows that FRZB plays an important role in determining hip shape and modifying the relationship between hip shape and OA in older Caucasian women [36]. Serum FRZB level has been studied in early rheumatoid arthritis and hip OA [37, 38]. For example, it has been reported that the highest FRZB serum levels associated with a modest reduction in risk of incident hip OA [31]. FRZB protein levels in human synovial fluid have remain largely uninvestigated. Our results showed that the FRZB concentration in SF was significantly decreased in the OA group as compared to the Ref control, are in line with these previous studies. In addition, we found FRZB expression was completely lost in few OA patients. We found the level of FRZB in synovial fluid is extremely high compared to the level of DKK1. It has been shown other joint tissues such as bone, synovium and ligaments also secret FRZB and DKK1 [35, 39, 40]. High concentration of FRZB suggesting other joint tissues that secret FRZB but not DKK1 contribute to high level of FRZB. For example, it has been shown that skeletal muscle also secrete FRZB protein [41].

GREM1 is a BMP signal transduction antagonist. BMP plays an important role in the development and in homeostasis of articular cartilage. Injury induces BMP2 expression in human cartilage [42, 13], and allelic polymorphisms of BMP2 are associated with OA, suggesting its role in joint homeostasis [43]. We found that the GREM1 concentration was higher in the samples from the recent injury group compared to the reference group. GREM1 expression was also statistically significantly higher in OA than in old injury. So it seems that GREM1 expression increased in both the AI and OA group due to abrupt injury, then decreased with the time of the injury, and slightly increased again with the increased severity of OA. Considering the role of BMP in the joint, it is reasonable to assume that high activity of BMP signaling results in the up-regulation of its antagonists GREM1, which establishes a negative feedback relieving BMP signaling.

Although there is multiple evidence showing crosstalk between WNT and BMP signaling in many biological processes [44-46], there are no studies showing that the WNT and BMP antagonists work cooperatively in modulating signaling amplitudes in the cell. In this study, we found that GREM1 negatively correlated with FRZB while its expression positively correlated with DKK1. Gazzerro et al found that GREM1 is able to inhibit WNT signaling via an unknown indirect mechanisms [23]. The positive correlation between GREM1 and DKK1 might explain that GREM1 inhibits WNT signaling via upregulating DKK1.

Here, we demonstrated that synovial fluid concentrations of DKK1, FRZB and GREM1 were decreased due to the time after injury. It has been reported that joint defects in young people

have the potential for intrinsic repair [47, 48]. The transiently high level of DKK1, FRZB and GREM1 protein in synovial fluid early following injury may be a self-protective response to injury. It has been demonstrated that the WNT and BMP pathways are transiently activated early following acute injury in human articular cartilage and other organ systems [11, 49]. Insufficient expression of these natural antagonists may fail to confront the excessive WNT and BMP signaling and thus result in repair failure, which may contribute to evolution towards posttraumatic OA.

Interestingly, we found for the knee injury groups that the concentrations of DKK1 and GREM1 in synovial fluid are significantly decreased with age, besides largely dependent on the different disease backgrounds, which suggested that the prevalent risk of knee disease increased with age due to the gradual elevation of WNT and BMP signaling due to the loss of DKK1 and GREM1. Considering the prevalence of OA in older adults worldwide [50, 51], we hypothesize that the changes of WNT and BMP signaling in older subjects might contribute to the susceptibility to OA.

COMP fragments are primarily found in cartilage and released into the synovial fluid [52], but are also found in other tissues such as the synovium and tendon [53, 54]. It is well established that COMP can be used as a marker of cartilage turnover [55], and elevated serum levels of COMP are associated with ongoing joint destruction in rheumatoid arthritis [56, 57]. In addition, raised synovial fluid concentrations of COMP have been found in both rheumatoid arthritis and OA [58], indicating that COMP can be used as a biochemical biomarker of cartilage destruction. The release of COMP seems to be increased in joint trauma and in early stage osteoarthritis [59]. The presence of type II collagen degradation products (C2C) has been demonstrated to be a sensitive and specific biomarker for subtle osteoarthritic changes in experimentally induced stifle OA models in dogs [60, 61]. It was shown that the C2C concentration in synovial fluid is correlated to the severity of joint injury in race horses [62]. In this study, we found FRZB to be positively correlated with the cartilage makers GAG and ARGS-aggrecan and matrix turnover markers COMP, and type II collagen epitope C2C, this positive correlation suggest that the low level of FRZB is accompanied by low level of these markers. It has been reported that the expression of GAG, ARGS-aggrecan and COMP is decreased with time from injury [63], which matches to the previously described protective role of FRZB on joints. In contrast, DKK1 negatively correlated with ARGS-aggrecan, and type II collagen epitope C2C. GREM1 is also negatively correlated with the type II collagen epitope C2C, this suggested that low expression

of DKK1 and GREM1 is accompanied with high expression of C2C. The increased levels of C2C after injury have been reported [64]. Interestingly, both DKK1 and GREM1 positively correlated with IL-8. It has been shown that DKK1 functions as an anti-inflammatory factor by inhibiting multiple signaling pathways in pericytes and myofibroblasts [65]. In addition, expression of DKK1 correlated with inflammatory cytokine levels and DKK1 was shown to mediate IL-1β promotion of chondrocyte apoptosis [66]. The positive correlation between GREM1 and IL18 is in line with a report that GREM1 is highly expressed in rheumatoid arthritis and its level correlated with proinflammatory factors [67]. GREM1 is positively correlated with DKK1, which suggest that DKK1 and GREM1 may work cooperatively as anti-inflammatory modulators, especially in OA.

This study has certain limitations. The first limitation is the relatively small sample size of reference and OA group. This small sample size may the reason that we did not find a significant difference between some groups. Secondly, due to individual differences, samples from different patients showed large variability, which reduces the power to detect differences between groups. However, thanks to our small sample size we now have better insight into the robust differences between the groups that were included, enabling the identification of combinations of correlated biomarkers that really stand out between these groups.

Taking together, our data shows that synovial fluid concentrations of DKK1, FRZB and GREM1 were changed in OA and/or injured knee joint and that their levels associated with sGAG, ARGS-aggrecan, C2C type II collagen, COMP, osteopontin, and IL-8. In addition, the down-regulation of DKK1, FRZB and GREM1 with time after injury and age suggests that self-healing failed. In addition, this study suggests that changes in these molecular factors may trigger a repair response at early stages after injury. However the failure to persistently express these factors in the later stage may lead to the development of posttraumatic joint diseases, including OA. Therefore we suggest that DKK1, FRZB and GREM1 can be used as potential biomarkers for studying the disease mechanisms in joint diseases and that these factors should be exploited as effective medicine to prevent OA after knee injury.

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Supplemental data

DKK1

Groups	SF (pg/ml)	Norm	P value	n	n < LLOD (%)
Ref	82.0 (75.9, 150.4)	1	-	9	0 (0)
Recent knee injuries	105.1 (79.1, 205.2)	1.3	0.526	158	3 (2)
0 days	*180.5 (95.3, 540.1)*	*2.2*	*0.136*	*26*	*1 (4)*
1 day	*130.8 (93.1, 364.5)*	*1.6*	*0.108*	*28*	*0 (0)*
2-3 days	*96.8 (78.7, 141.5)*	*1.2*	*0.757*	*29*	*1 (3)*
4-7 days	*105.7 (74.9, 228.2)*	*1.3*	*0.539*	*30*	*1 (3)*
8-22 days	*96.2 (77.5, 138.7)*	*1.2*	*0.848*	*25*	*0 (0)*
23-77 days	*84.6 (68.3, 93.7)*	*1.0*	*0.183*	*20*	*0 (0)*
Old knee injuries	78.6 (70.7, 91,4)	1.0	0.102	50	0 (0)
1-2.5 years	*77.2 (69.2, 91.2)*	*0.9*	*0.067*	*27*	*0 (0)*
2.8-36.9 years	*79.0 (72.2, 94.5)*	*1.0*	*0.263*	*23*	*0 (0)*
OA	75,1 (65,4, 88,0)	0.9	0.116	22	1 (5)

FRZB

Groups	SF (ng/ml)	Norm	P value	n	n < LLOD (%)
Ref	6.20 (2.61, 9.91)	1	-	9	1 (11)
Recent knee injuries	5.99 (1.63, 13.01)	1.0	0.930	158	24 (24)
0 days	*3.19 (0.004, 6.17)*	*0.5*	*0.157*	*26*	*9 (35)*
1 day	*2.73 (0.004, 10.46)*	*0.4*	*0.333*	*28*	*8 (29)*
2-3 days	*7.03 (3.98, 14.92)*	*1.1*	*0.441*	*29*	*3 (10)*
4-7 days	*8.61 (3.84, 19.0)*	*1.4*	*0.230*	*30*	*1 (3)*
8-22 days	*9.48 (2.59, 12.71)*	*1.5*	*0.470*	*25*	*1 (4)*
23-77 days	*5.52 (3.10, 9.63)*	*0.9*	*1.000*	*20*	*2 (10)*
Old knee injuries	3.58 (1.52, 6.46)	0.6	0.165	50	3 (6)
1-2.5 years	*3.78 (1.80, 7.59)*	*0.6*	*0.316*	*27*	*1 (4)*
2.8-36.9 years	*2.37 (0.83, 5.55)*	*0.4*	*0.112*	*23*	*2 (9)*
OA	0.004 (0.004, 3.0)	0.0006	**0.003**	22	14 (64)

GREM1

Groups	SF (ng/ml)	Norm	P value	n	n < LLOD (%)
Ref	112.2 (65.7, 168.1)	1	-	9	0 (0)
Recent knee injuries	148.2 (133.0, 258.6)	1.5	**0.007**	158	0 (0)
0 days	*201.3 (141.1, 294.0)*	*2.1*	***0.003***	*26*	*0 (0)*
1 day	*178.3 (138.5, 285.9)*	*1.8*	***0.004***	*28*	*0 (0)*
2-3 days	*148.0 (136.6, 238.0)*	*1.5*	***0.012***	*29*	*0 (0)*
4-7 days	*155.5 (135.8, 269.2)*	*1.6*	***0.009***	*30*	*0 (0)*
8-22 days	*142.4 (111.2, 206.0)*	*1.5*	*0.086*	*25*	*0 (0)*
23-77 days	*114.4 (85.2, 206.0)*	*1.2*	*0.274*	*20*	*0 (0)*
Old knee injuries	127.0 (91.2, 148.1)	1.3	0.461	50	0 (0)
1-2.5 years	*126.9 (91.5, 139.4)*	*1.1*	*0.590*	*27*	*0 (0)*
2.8-36.9 years	*133.7 (85.7, 151.1)*	*1.2*	*0.409*	*23*	*0 (0)*
OA	160.0 (114.4, 198.2)	1.5	0.094	22	0 (0)

Supplementary Table S1. Concentrations of DKK1, FRZB and GREM1 in synovial fluid. Concentrations in synovial fluid (SF) expressed as median values (25^{th}, 75^{th} percentiles) were measured

from: recent knee injured subjects (0-77 days) with sub-stratified groups (in italics), subjects with old knee injuries (1-36.9 years) with sub-stratified groups (in italics), OA patients and knee-healthy subjects (reference). Median values were normalized (Norm) against the reference group. P values, Mann-Whitney U tests of injury versus reference group. Diagnostic groups were according to Table 1. Significance, $P<0.05$ marked bolded. n = total amount of samples analyzed. n < LLOD = amount of the analyzed samples that were below lower limit of detection.

Chapter 5

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

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Knowledge is power (知识就是力量)

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 MMP-1, -3, and -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. IκB-α was able to counteract the effect of TCF4 on NF-κB target gene expression. 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.

Reagents

Recombinant human IL-1β and recombinant human Wnt3A were obtained from R&D Systems.

Plasmid constructs and viral transduction

Adenoviruses expressing null control, GFP, human TCF4, LEF1 and IκB-α (Vector Biolabs) were used to infect human chondrocytes at an MOI (multiplicity of infection) of 100. Protein or RNA samples were harvested 72 hours after adenoviral transduction unless otherwise specified.

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). Protein or RNA samples were harvested 72 hours after lentiviral transduction.

Reporter assay

Human chondrocytes were infected with Cignal™ lentiviruses containing TCF/LEF or NF-κB responsive elements and 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) 72 hours after lentiviral transduction. 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) 72 hours after lentiviral transduction. Data were normalized for transduction efficiency by measuring luciferase DNA contents in chondrocytes using qPCR.

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). 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 blot

Immunoprecitaion was performed using the Dynabeads Co-Immunoprecipitation kit (Invitrogen). Total cell proteins for Western blot were collected using RIPA buffer (Cell Signaling) supplemented with the Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). Antibodies used for immunoprecipitation and Western blot were anti-β-catenin (BD Biosciences), pro-MMP1 (R&D Systems), pro-MMP3 (R&D Systems), pro-MMP13 (R&D Systems), GAPDH (Sigma-Aldrich), NF-κB p50(C-19) (Santa Cruz), NF-κB p65(A) (Santa Cruz), IκB-α (Santa Cruz), IκB-β (Santa Cruz), FLAG (Origene), TCF4 (Cell Signaling), 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). Isolated proteins were incubated with 4-

aminophenylmercuric acetate (APMA) for 24 hours to activate all pro-MMPs. MMP substrates were then incubated with activated proteins for 30 minutes and fluorescence signals were measured. 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 were expressed as the mean ± SD and analyzed by two-tailed student's t-tests. $P < 0.05$ was considered statistically significant.

Results

Expression of TCF/LEF family members in human articular cartilage

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, -3 and -13 mRNA expression was increased by TCF4 overexpression. In contrast, LEF1 overexpression significantly decreased the mRNA expression of MMP-1, -3 and -13 (Fig. 1A). Overexpression of two TCF3 variants showed the same effect on MMP expression as LEF1 (Fig. 1B). Knockdown of TCF4 significantly decreased MMP1 and MMP13 mRNA expression but failed to change MMP3 expression (Fig.

1C). Knockdown of LEF1 slightly but significantly upregulated MMP1 mRNA expression only (Fig. 1C).

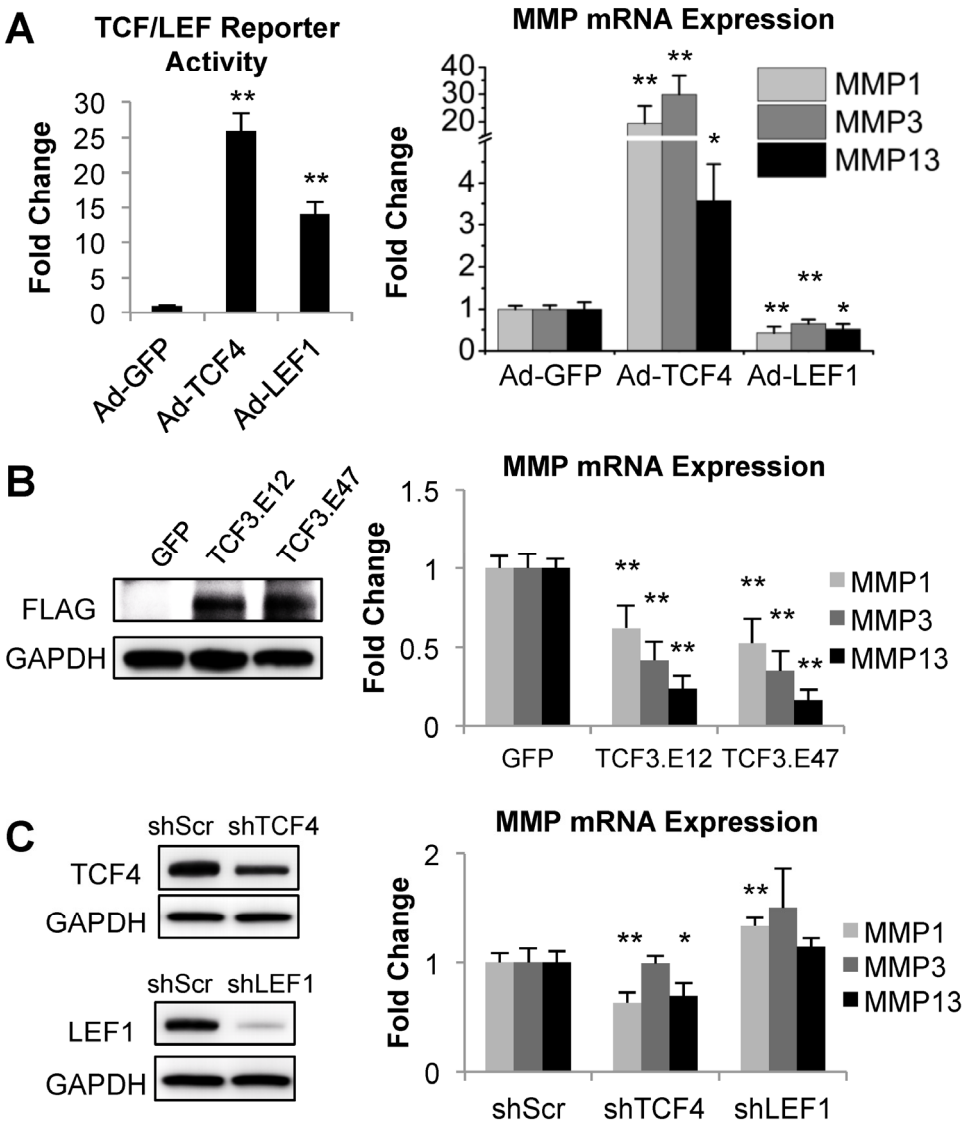

Figure 1. Effects of TCF/LEF members on MMP mRNA expression. A. Human primary chondrocytes were infected with adenoviruses expressing GFP, TCF4 and LEF1. Activation of the canonical WNT pathway by TCF4 or LEF1 overexpression was validated by a luciferase reporter assay. *p<0.05, **p<0.01, n = 3 donors (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).

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, -3 and -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).

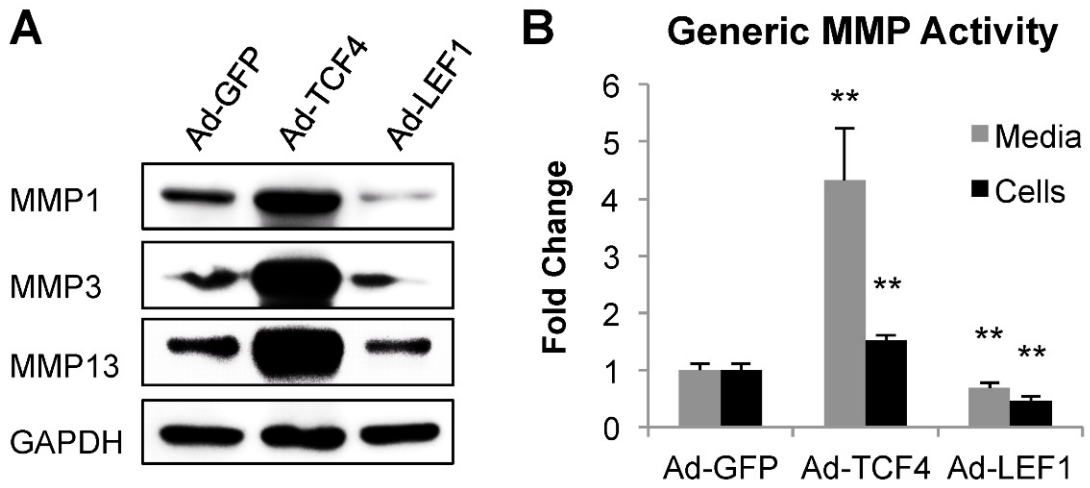

Figure 2. Effects of TCF4 and LEF1 on MMP protein expression and activity. Human chondrocytes were infected with adenoviruses expressing GFP, TCF4 and LEF1. A. MMP protein levels were detected by Western blot. B. Generic MMP activity in culture media and cell lysates was measured using a generic MMP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

TCF4 potentiates NF-κB signaling in human chondrocytes

Previously, we have shown that knockdown of TCF4 in mouse cells abolished WNT/β-catenin-induced MMP expression indicating that at least in animal models MMPs are direct target genes of TCF/LEF transcription factors (9). To determine whether MMPs are direct

target genes of TCF4 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 1144 bp 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 knock down of TCF4 which significantly decreased MMP1 and MMP13 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 chondrocytes (9). Therefore, we tested if TCF4 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 LEF1 (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 LEF1 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.

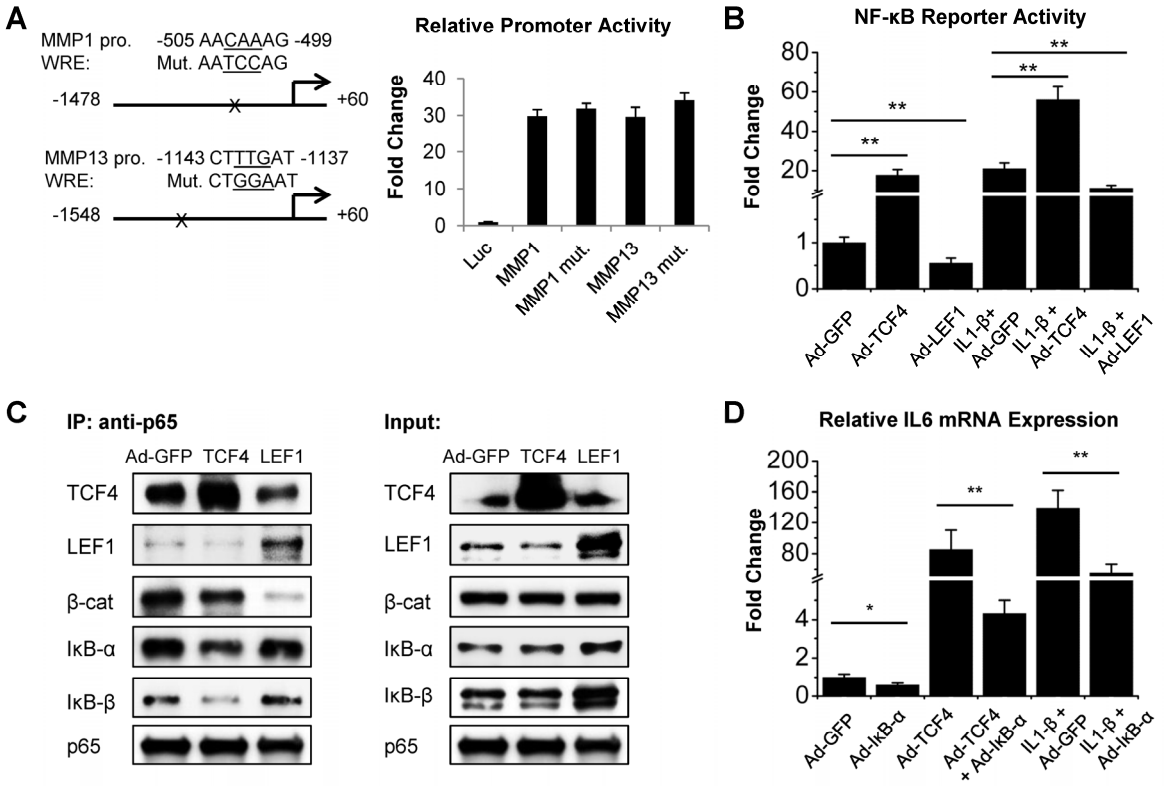

Figure 3. TCF4 potentiates NF-κB signaling in human chondrocytes. A. Schematic representation of the MMP1 and -13 promoter reporter constructs is shown. The sequence of the putative WRE and the inactivating mutants are given. Basel activities of MMP1 and MMP13 promoters and promoters containing mutant 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. Cells were treated with 5 ng/ml IL-1β for 24 hours before measurement of luciferase activity. **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 or IκB-α as indicated, treated with 5 ng/ml IL-1β for 24 hours before collection of RNA samples. mRNA expression was measured by qPCR. *p<0.05, **p<0.01, n = 3 donors.

We next evaluated whether TCF4 and LEF1 could also influence the expression of other NF-κB target genes such as IL6 and SERPINA1. IL6 mRNA expression was significantly upregulated by TCF4 overexpression but repressed by LEF1 overexpression (Fig. S1). However, expression of SERPINA1 was not significantly affected by either TCF4 or LEF1 overexpression (Fig. S1). This indicated that a subset of NF-κB target genes might be

selectively regulated by the interaction between NF-κB and TCF4 or LEF1 transcription factors. In addition, an evaluation of NF-κB target gene expression by TCF4 or LEF1 overexpression over time was also performed (Fig. S2). TCF4 and LEF1 showed significant effects from 24 hours when the protein levels started to increase, suggesting a fast and direct effect of TCF/LEF on NF-κB activity. Although the expression of IL-1β was also changed, as a target gene of NF-κB (16), the initial upregulation of NF-κB target gene may be dependent on the interaction of TCF4 and NF-κB. The increased IL-1β may form a positive feedback to enhance the action of NF-κB signaling as previously shown (16).

In agreement with the effect of TCF4 on the binding of IκB-α to NF-κB, overexpression IκB-α counteracts the induction of expression of target genes including IL6 (Fig. 3D) and MMPs (Fig. S3) by TCF4 overexpression as well as IL-1β treatment. It suggests that TCF4's enhancing effect on NF-κB activity may be at least partly dependent on its inhibitory effect on the binding of IκB-α to NF-κB. TCF4-induced IL1B expression was also almost eliminated by IκB-α overexpression (Fig. S3) indicating that the upregulation of IL1B expression by TCF4 is very likely to depend on its direct potentiating effect on NF-κB. Taken together, these data suggest that the effect of TCF4 is rather dependent on its direct action on p65 activity than upregulation of IL1B expression.

TCF4 induces human chondrocyte apoptosis

Overexpression of TCF4 but not of LEF1 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).

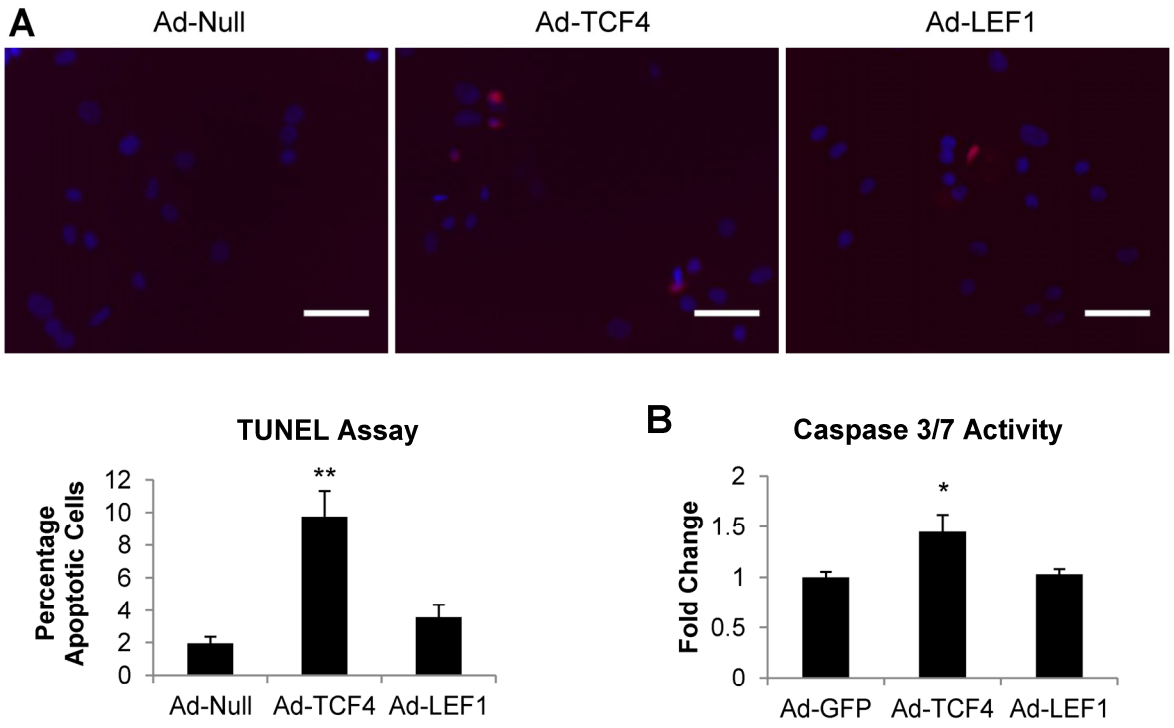

Figure 4. TCF4 induces human chondrocyte apoptosis. A. Human chondrocytes were infected with adenoviruses expressing null control, TCF4 and LEF1. Chondrocyte apoptosis was detected 4 days after adenoviral transduction using TUNEL staining (Red: TUNEL; Blue: Nuclei. Scale bar = 20 μm). Representative 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 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. In agreement with this, MMP1 and MMP13 mRNA expression was higher in OA cartilage than in healthy cartilage, although MMP3 expression did not show any difference (Fig. 5). It suggests 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. We further explored if TCF4 expression was regulated by WNT or NF-κB pathways. Neither Wnt3A or IL-1β treatment influenced TCF4 mRNA expression in human articular chondrocytes (Fig. S4). In contrast, LEF1 expression was significantly upregulated by both stimuli (Fig. S4), in agreement with previous reports (17, 18). It implies

that other factors in the osteoarthritic environment may contribute to the increased TCF4 expression.

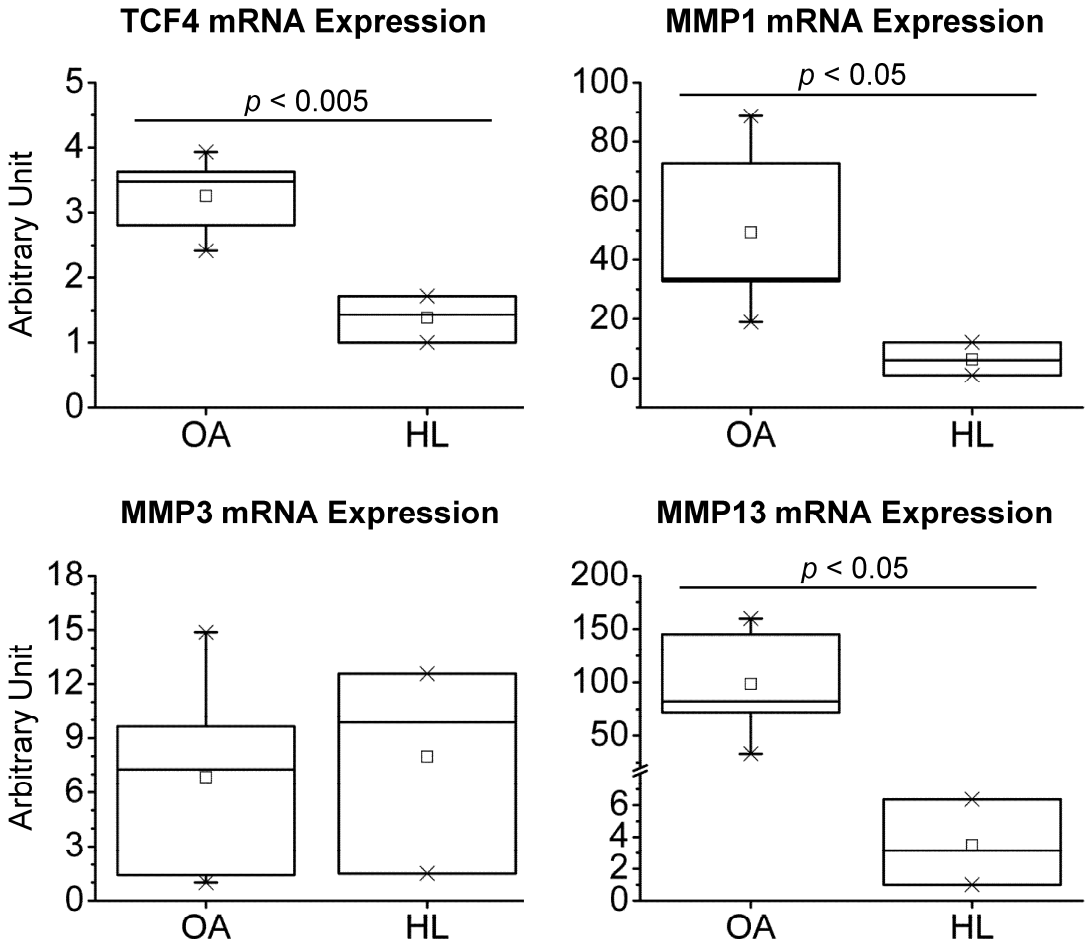

Figure 5. TCF4 mRNA expression is upregulated in OA cartilage. Human articular cartilage RNA samples were isolated from 5 OA donors and 3 healthy (HL) donors. TCF4 and MMP mRNA expression was measured by qPCR.

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, -3 and -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-1β-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 (19-21). 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 (22-25). We demonstrated that TCF4 was a strong activator of MMP-1, -3 and -13 mRNA expression. Knockdown of TCF4 led to a decrease in the basal transcription of MMP1 and MMP13 but not of MMP3. This might be due to the usage and/or compensation of other regulatory pathways in MMP3 transcription. Despite the fact that we identified consensus WREs in the promoter regions of the MMP1 and MMP13 genes mutation analysis of these WREs in MMP1 and -13 promoters failed to show their involvement in promoter regulation. 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, -3 and -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-IP 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 of NF-κB 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 (26-28). 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 (29). In addition, the C-terminal binding protein (CtBP) binds to TCF4 but not to LEF1 (30). 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, 31). In agreement with this, IκB-α overexpression was found to counteract the positive effect of TCF4 on NF-κB target gene expression. This also supports that TCF4 probably functions through interaction with NF-κB to regulate NF-κB target gene expression. However it remains elusive if integration of TCF4 into the transcription complex of NF-κB is required for TCF4's regulation of NF-κB target gene expression.

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 or the inhibiting effect of LEF1 on NF-κB-mediated gene transcription was not limited to MMPs but was also found for other established target genes such as IL-6. Remarkably, TCF4 or LEF1 couldn't affect the expression of the NF-κB target gene SERPINA1. This might be explained by the involvement of different co-factors which might not be affected by TCF/LEF (31).

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.

In addition to its pro-catabolic effects, it has been suggested that NF-κB may play a role in chondrocyte apoptosis (32-34). 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 (33, 34). 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.

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Supplemental data

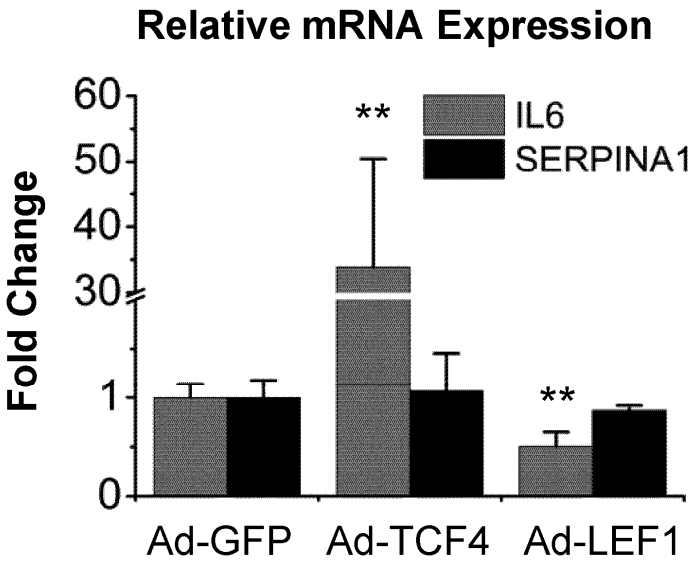

Figure S1. Effects of TCF/LEF members on IL6 and SERPINA1 mRNA expression. Human chondrocytes were infected with adenoviruses expressing GFP, TCF4 and LEF1. IL6 and SERPINA1 mRNA expression was measured by qPCR. **p<0.01, n = 4 donors.

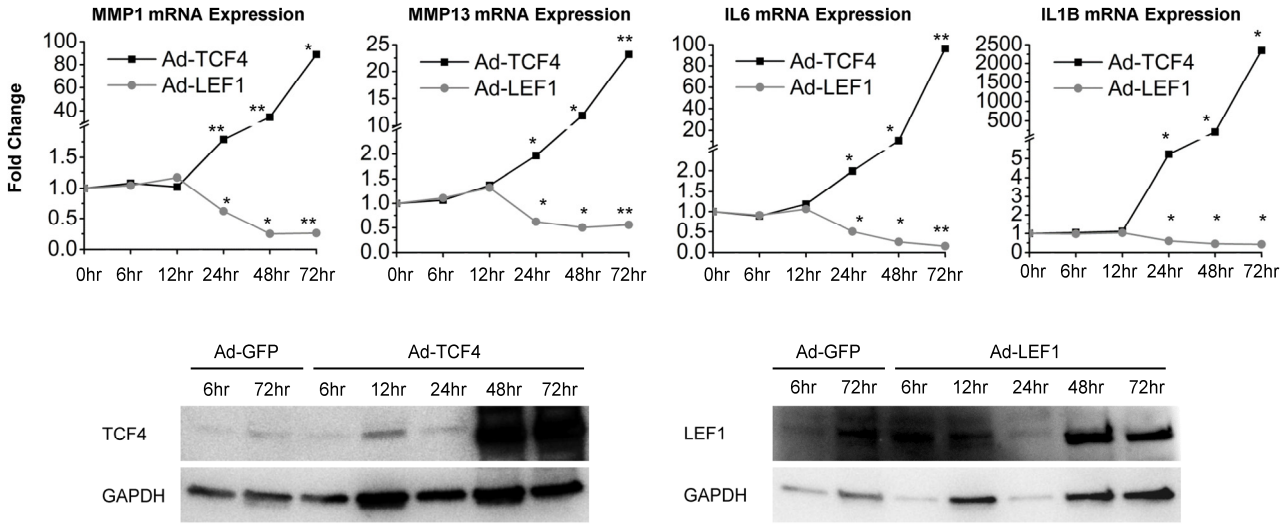

Figure S2. Overtime evaluation of TCF4 or LEF1 overexpression-mediated gene expression. Human chondrocytes were infected with adenoviruses expressing GFP, TCF4 or LEF1 for indicated

time. mRNA expression was measured by qPCR. *p<0.05, **p<0.01, n = 3 donors. Protein expression of TCF4 and LEF1 was detected by immunoblotting.

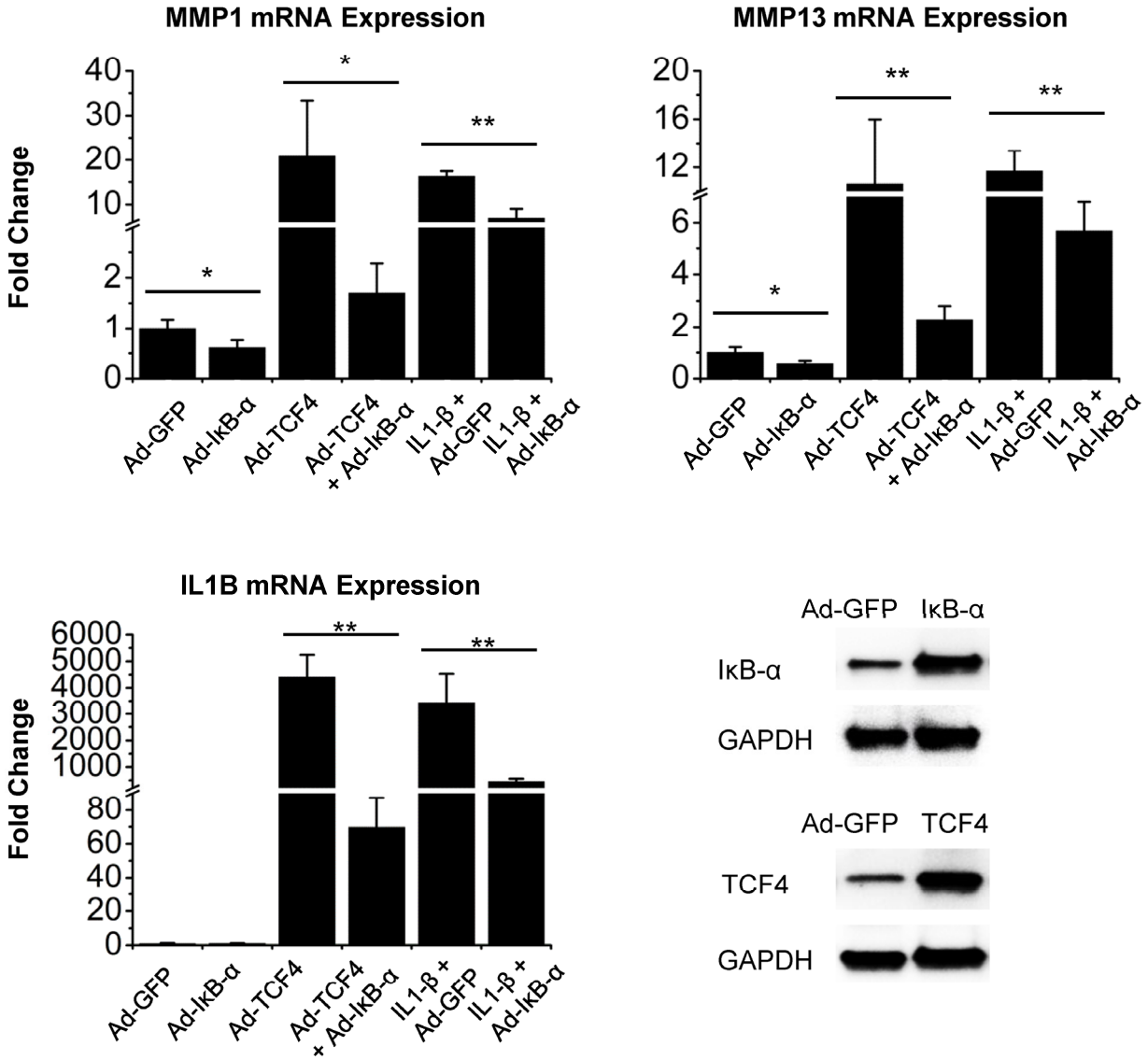

Figure S3. Effects of IκB-α overexpression on TCF4-induced gene expression. Human chondrocytes were infected with adenoviruses expressing GFP, TCF4 or IκB-α as indicated, and treated with or without 5 ng/ml IL-1β for 24 hours before collection of RNA and protein samples. mRNA expression was measured by qPCR. *p<0.05, **p<0.01, n = 3 donors. Protein expression of TCF4 and IκB-α was detected by immunoblotting.

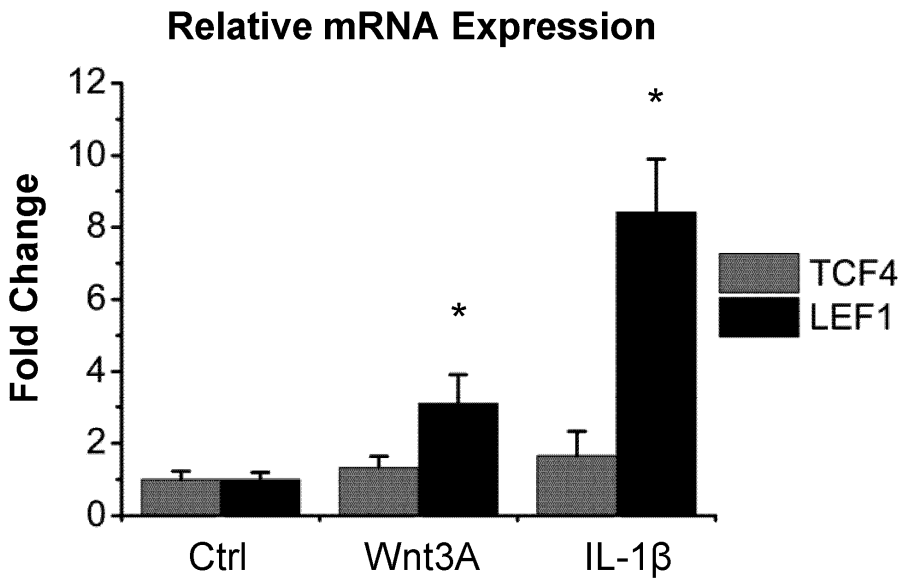

Figure S4. Effects of Wnt3A and IL-1β on TCF4 and LEF1 mRNA expression. Human chondrocytes were treated with 100 ng/ml Wnt3A and 5 ng/ml IL-1β for 24 hours. TCF4 and LEF1 mRNA expression was measured by qPCR. *$p<0.05$, n = 3 donors.

Chapter 6

Endogenous DKK1 and FRZB regulate chondrogenesis and hypertrophy in 3D cultures of human chondrocytes and human mesenchymal stem cells

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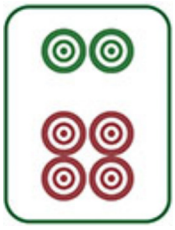

Practice makes perfect (熟能生巧)

Abstract

Hypertrophic differentiation occurs during *in vitro* chondrogenesis of mesenchymal stem cells (MSCs), decreasing the quality of the cartilage construct. Previously we identified WNT pathway antagonists Dickkopf 1 homolog (DKK1) and frizzled-related protein (FRZB) as key factors in blocking hypertrophic differentiation of hMSCs. In this study, we investigated the role of endogenously expressed DKK1 and FRZB in chondrogenesis of hMSC, chondrocyte redifferentiation and in preventing cell hypertrophy using three relevant human cell based systems, isolated hMSCs, isolated primary human chondrocytes (hChs), and co-cultures of hMSCs with hChs for which we specifically designed neutralizing nano-antibodies. We selected and tested variable domain of single chain heavy chain only antibodies (VHH) for their ability to neutralize the function of DKK1 or FRZB. In the presence of DKK1 and FRZB neutralizing VHH, glycosaminoglycan (GAG) and Collagen type II staining was significantly reduced in mono-cultured hMSCs and mono-cultured chondrocytes. Furthermore, in co-cultures, cells in pellets showed hypertrophic differentiation. In conclusion, endogenous expression of the WNT antagonists DKK1 and FRZB is necessary for multiple steps during chondrogenesis: firstly DKK1 and FRZB are indispensable for the initial steps of chondrogenic differentiation of hMSCs, secondly they are necessary for chondrocyte redifferentiation, and finally in preventing hypertrophic differentiation of articular chondrocytes.

Introduction

Articular cartilage repair is a challenge due to very limited capacity of self-repair after damage. Autologous chondrocyte implantation (ACI) has become the golden standard treatment for large-size cartilage defects [1]. However, ACI leads to donor-site morbidity and is dependent on 2D expansion of isolated chondrocytes, which are prone to undergo dedifferentiation during proliferation [2]. To overcome this drawback, a (partial) replacement of chondrocytes with mesenchymal stem cells (MSCs) is an option. Although the application of MSCs in cartilage repair is promising, the use of MSCs in cartilage engineering still has some drawbacks. One of the problems with using MSCs for cartilage regeneration is the expression of hypertrophic markers during *in vitro* chondrogenesis followed by formation of transient calcifying cartilage [3]. So maintaining the chondrogenic phenotype during *in vitro* expansion and avoiding hypertrophy of chondrogenically differentiating MSCs, remain a big challenge in these cell-based strategies [4]. Successful chondrogenesis of hMSCs is regulated by various signaling pathways, including: FGF, TGF-β, WNT/β-catenin, Notch and hypoxia [5]. Articular chondrocytes produce a stable, non-mineralizing cartilage [6] and co-culture of MSCs and articular cartilage reduces hypertrophy [7] and enhances functional properties of engineered cartilage [8].

WNT/β-catenin signaling plays a pivotal role in articular cartilage [9], where enhanced signaling activity results in cartilage destruction via chondrocyte hypertrophic differentiation and subsequent development of an OA-like phenotype [10]. Decreased WNT activity also results in cartilage destruction via the induction of apoptosis [11]. Therefore, maintaining strict control over WNT/β-catenin signaling in articular cartilage is of the highest importance. We previously found that the WNT antagonists DKK1 and FRZB are highly enriched in articular cartilage as compared to epiphyseal growth plate cartilage [12]. These antagonists constitute a key part of the natural mechanism that balances WNT/β-catenin signaling activity. DKK1 antagonizes WNT signaling pathway by binding to the Frizzled co-receptors low-density lipoprotein receptor–related protein 5 (LRP-5) and LRP-6 [13, 14]. Secreted Frizzled-related proteins (sFRP) antagonize WNT signaling through interactions with WNTs and/or through the formation of non-functional complexes with the Frizzled receptors [15-17], resulting in the inhibition of both canonical and noncanonical pathways [18]. DKK1 increases hMSCs proliferation and promotes entry into the cell cycle *in vitro* [19]. In addition, we have previously shown that in chondrogenically differentiating hMSC pellet cultures and in explanted mouse

tibiae, exogenous addition of DKK1 or FRZB prevented terminal hypertrophic differentiation of chondrocytes, supporting their role in articular cartilage homeostasis [12].

WNT pathway activity repressed the expression of cartilage-specific extracellular matrix (ECM) molecules and might be involved in chondrocyte dedifferentiation during *in vitro* expansion of primary human chondrocytes [20, 21]. Healthy articular chondrocytes keep their differentiated state and do not undergo dedifferentiation suggesting that endogenous WNT/β-catenin signaling is blocked by an unknown mechanism. DKK1 and FRZB are natural antagonists and are highly expressed by healthy human chondrocytes [12]. We previously found that when cultured under prolonged passaging, chondrocytes lose their phenotype and that this is accompanied by changes in expression of DKK1 and FRZB [22]. This indicates that the combined endogenous expression of DKK1 and FRZB plays a role in chondrocyte dedifferentiation.

So the correct regulation of the WNT signaling plays an important role in the maintenance of the chondrogenic potential as well as in suppression of endochondral ossification [23]. However, the mechanism by which WNT signaling is regulated *in vivo* is as yet unknown. It has been reported that FRZB knockout mice have no cartilage phenotypic changes, but are more susceptible to OA in mechnical injury [24]. DKK1 knockout mice shows no change in cartilage degeneration, only osteophyte formation is increased upon mechanical loading [24]. This suggests the functional redundancy of DKK1 and FRZB because both of them are capable of inhibiting the WNT/β-catenin pathway, the one can compensate for the loss of the other. We propose that the combined endogenous expression of DKK1 and FRZB plays a determining role in both the chondrogenesis of hMSCs and prevention of hypertrophic differentiation *in vivo*.

There is ample evidence that the correct modulation of WNT activity is necessary and sufficient for the formation and maintenance of healthy articular cartilage. However, the exact mechanisms regulating the amplitude of WNT activity in articular cartilage *in vivo* are as yet unknown and are the subject of this project. We hypothesized that endogenous expression of DKK1 and FRZB is both necessary and sufficient for articular cartilage formation and prevention of hypertrophic differentiation. Specifically, we hypothesized that simultaneously blocking DKK1 and FRZB would negatively influence the chondrogenic potential of hMSCs and dedifferentiated chondrocytes, and that blocking endogenous DKK1 and FRZB leads to hypertrophic differentiation. To prove our hypotheses, we have evaluated the combined role of endogenous levels of DKK1 and FRZB by blocking their activity using neutralizing VHH

antibodies in three relevant human cell based systems: isolated hMSCs, isolated primary human chondrocytes (hChs), and co-cultures of hMSCs with hChs.

Materials and Methods

Selection of anti-DKK1 VHH from immune VHH library

Two llamas were immunized with recombinant human DKK1 (R&D systems) as described in [25]. The immuninization protocol was approved by the Utrecht University ethical committee (DEC: 2007.III.01.013). Total RNA was extracted from peripheral blood lymphocytes, cDNA was synthesized. VHH were subsequently cloned in phagemid pUR8100, which was derived from pHEN [26] described by [27]. VHH binding to DKK1 were selected by panning in 2 rounds of selection as described [25]. Screening of the selected VHH for binding to DKK1 led to the identification of 14 different VHH. The amino acid sequences of the VHH show different clones from different families (Supplemental Figure 1), of which 2 (G5 and H7) were recurrent and therefore used for further analysis.

To test the affinity of the anti-DKK1 VHH, sequences were subcloned into the expression plasmid pMEK219 containing C terminal Myc and His tags. VHH were subsequently produced from *E. coli* by induction of the lac promoter with 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) [28, 29] and were purified from the periplasmic fraction via the C terminal His-tag by cobalt affinity chromatography (TALON His-Tag Purification Resin, ClonTech). The size and purity of the VHH were assessed by SDS-PAGE [30, 31] .

Purified VHH were tested by enzyme-linked immunosorbent assay (ELISA) assay. Plates were coated with DKK1 (60 nM) and blocked with 4% skimmed milk in PBS (MPBS), then incubated with a concentration range of VHH (0–7μM). Unbound VHH were washed with PBS-Tween and bound VHH were detected by incubation with mAb anti-myc (9E10) and a HRP-conjugated anti-mouse.

To assess the biological activity of the anti-DKK1 VHH, KS483-4C3 mouse progenitor cells were used as a model for osteogenic differentiation [32]. Cells were seeded at 10000 cells/cm^2 (day 0). At day 4, cells were cultured for another 3 days with ascorbic acid (50 μg/ml; Sigma Aldrich) and stimulated with BMP6 (100 ng/ml; R&D Systems) in the presence or absence of DKK1 (300 ng/ml; R&D Systems) with a concentration series of VHH G5 or H7 (0–70 nM). At day 7, alkaline phosphatase (ALP) activity was evaluated by CDPStar kit (Roche). Luminescence was measured using Vector Microplate Luminometer (Promega). The

luminescence units were corrected for DNA content. DNA concentration was determined using the CyQuant Cell Proliferation Assay (Invitrogen).

Selection of anti-FRZB from a non-immunized llama VHH library

VHH binding to FRZB (R&D systems) were selected from non-immunized llama VHH-phage display library [33], kindly provided by BAC B.V. (Thermofisher, Leiden, the Netherlands) in two panning rounds [33]. Selection and screening was as described for the anti-DKK1 VHH, with the exception of applying more phages for the 1st round of selection [33]. Screening of the FRZB binders lead to identification of 5 VHH candidates. The amino acid sequences of the VHH were indicated in supplemental Figure 2. Anti-FRZB VHH was cloned in the expression plasmid pMEK222 containing C terminal FLAG and His tags. Production and purification of the VHH was as described for the anti-DKK1 VHH. Apparent affinity of the purified FRZB VHH was measured with ELISA as described for anti-DKK1, with the exception of detecting bound VHH with mAb M1 directed against FLAG instead of mAb 9E10.

Cell culture and expansion

Human primary chondrocytes were obtained from relatively healthy looking full thickness cartilage, dissected from knee biopsies of three patients (mean±SD age 60±3 years) undergoing total knee replacement, as described previously [34]. To isolate cells, the cartilage was digested in chondrocyte proliferation medium containing collagenase type II (0.15% Worthington, NJ, US) for 20–22 h. Subsequently the hChs were expanded at a density of 3000 cells/cm^2 in chondrocyte proliferation medium until the monolayer reached 80% confluency. Chondrocyte proliferation medium consisted of DMEM supplemented with 10% fetal bovine serum (FBS), 1×non-essential amino acids, 0.2 mM ascorbic acid 2-phosphate (AsAP), 0.4 mM proline, 100 U/ml penicillin and 100 µg/ml streptomycin. The hChs were used in passage two unless otherwise stated. The hMSCs were isolated from human bone marrow aspirates as described previously [34] and cultured in MSCs proliferation medium (α-MEM supplemented with 10% FBS, 1% L-glutamax, 0.2 mM ascorbic acid, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 ng/ml bFGF).

Pellet cultures and chondrogenic differentiation

In order to get enough cell pellets for analysis, micro patterned agarose chips were used in this experiment. Micro patterned agarose chips were prepared at a concentration of 4% w/v by replica molding as described previously [35]. PDMS stamps were used to routinely replicate

the microstructures. For mono-cultures, 250,000 cells of hChs or hMSCs were seeded into 1585 microwells of micro patterned agarose chips. This resulted in roughly 160 cells/pellet.

For co-cultures 250,000 cells were seeded at a ratio of hMSC/hChs = 80/20. Cells were suspended in chondrogenic differentiation medium (DMEM supplemented with 50 µg/mL ITS-premix, 50 µg/mL of AsAP, 100 µg/mL of sodium pyruvate, 10 ng/mL of TGFβ3, 10^{-7} M of dexamethasone (DEX), 100 U/mL of penicillin and 100µg/ml of streptomycin, and pipetted into the agarose wells. Subsequently the agarose chips were centrifuged for 5 min at 500×g to form pellets. Cell pellets were incubated in the microwells at 37°C in a humid atmosphere with 5% CO_2 for a period of 4 weeks before analysis. The medium was supplemented with 5 µg/mL of VHH anti-DKK1 (D-H7) and VHH anti-FRZB (TSF-1F7). Non-specific goat IgG (5 µg/mL) was used as a negative control. The medium, with antibody supplements, was refreshed twice per week.

Total RNA extraction and qPCR

Cell pellets were pooled and RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel, Duren, Germany). The concentration and purity of RNA samples were determined using the Nanodrop 2000 (Thermo scientific, Wilmington, USA). Total mRNA was reverse-transcribed into cDNA using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). qPCR was performed using the SYBR Green sensimix (Bioline). PCR Reactions were carried out using the Bio-Rad CFX96 (Bio-Rad, Hercules, CA) under the following conditions: cDNA was denatured for 5 min at 95°C, followed by 39 cycles consisting of 15s at 95°C, 15s at 60°C and 30s at 72°C. For each reaction a melting curve was generated to test primer dimer formation and non-specific priming. Primers sequences are listed in supplemental table 1.

Histological analysis

Cell pellets were collected after 4 weeks incubation and fixed with 10 % phosphate buffered formalin (pH = 7) for 15 min at room temperature, dehydrated with graded ethanol and embedded in paraffin using routine procedures. 5µm thick sections were cut using a microtome (Shandon, France). Before staining the slides were de-paraffinized in xylene and rehydrated with graded ethanol.

Alcian blue and Safranin O staining

Slides were either stained for sulfated glycosaminoglycans (GAG) with a 0.5% w/v solution of Alcian blue (pH=1, adjusted with HCl) for 30 min. The samples were then counterstained with nuclear fast red (0.1%w/v of nuclear fast red in 5% aluminum sulfate) for 5 min to visualize nuclei [36], or stained for sulfated GAG with a 0.1% solution of Safranin O for 5 min (Sigma Aldrich, St. Louis, Missouri, USA).

Alizarin red S staining

Slides were stained with a 2% w/v solution of Alizarin red S (pH=4.2, adjusted with ammonium hydroxide) to assess the presence of mineralization. Light microscopy was used to visualize red mineralized nodules as described [36].

Immunohistochemistry (IHC)

Immunohistochemical staining of collagen type II was performed using 5µm sections. Slides were de-paraffinized in xylene and rehydrated with graded ethanol. Samples were pre-incubated with 5µg/mL proteinase K (Sigma Aldrich) for 10 min at RT followed by 1 mg/mL hyaluronidase (Sigma Aldrich) for 40 min at 37 °C. Samples were blocked in 1.5% normal goat blocking serum in PBS for 1 hour. Rabbit polyclonal collagen type II antibody (Abcam, ab34712) was diluted 1:200 in PBS containing 1.5% blocking serum (Santa Cruz Biotechnology) and incubated overnight at 4 °C. Non-immune controls underwent the same procedure without primary antibody incubation. The target protein was detected by incubation in rabbit ABC staining system (sc-2018; Santa Cruz) according to the manufacturer's protocol and imaged using a Nanozoomer (Iwata City, Japan).

Immunofluorescent staining (IF)

Immunofluorescent staining of collagen type II and collagen type X was performed using 5µm sections. Slides were de-paraffinized in xylene and rehydrated with graded ethanol. Samples were pre-incubated with 5µg/mL proteinase K (Sigma Aldrich) for 10 min at RT followed by 1 mg/mL hyaluronidase (Sigma Aldrich) for 40 min at 37 °C. Samples were blocked in 5% BSA in PBS for 1 hour, then incubated with rabbit anti-collagen II antibody (ab34712, Abcam), which was diluted 1:100 in 5% BSA in PBS and mouse collagen type X (2031501005, Quartett LifeSpan), which was diluted 1:50 for overnight at 4°. Cells were rinsed with PBST for 3 times, 5min/time. Then Alexa®Fluor 546-labelled goat anti-rabbit or anti-mouse antibody in 5% BSA

in PBS was added and incubated for 2 hours at RT. Samples were rinsed with PBS and added mounting medium with DAPI. Slides were viewed by BD pathway confocal microscopy.

Apoptosis assay

Apoptosis was detected in paraffin-embedded pellets using The DeadEnd™ colorimetric TUNEL assay (Promega) following the manufacturer's procedure. Apoptotic nuclei stained dark brown.

Statistical analysis

For the experiments using primary human cells (hMSCs and hChs), samples were obtained from three hChs donors and two hMSCs donors. Each experiment was performed in triplicate. Statistical differences between two groups were analyzed by two-tailed student's t-tests or one-way ANOVA. $P < 0.05$ was considered statistically significant and indicated with an asterisk. Data are expressed as the mean ± SD.

Results

The camelidae family express a special class of antibodies that are devoid of light chains [37]. These antibodies are called single domain antibodies engineered from heavy chain camelid antibodies (VHH), which are formed by two protein chains. Compared with conventional antibodies, using VHH has several advantages including: small size (15 kDa), high binding specificity and affinity [38], simple and easy to clone and subsequently to genetically modify, they can be produced in bacterial and yeast [38, 39]. Moreover, VHH antibodies easily penetrate into 3D cell pellets. Selection of functional VHH is of the utmost importance to determining the quality of the data and is not trivial. We therefore describe the selection of the VHH against DKK1 and the VHH against FRZB in the sections below.

Selection of anti-DKK1 VHH

Anti-DKK1 VHH were selected from llama VHH-phage display libraries immunized with recombinant DKK1. The phage display library is constructed from RNA of PBMCs isolated from the immunized llama. Selection led to the identification two VHH, G5 and H7, which display apparent affinities (Kd) of 5.9 x 10-8 M and 1.1 x 10-7 M respectively (Figure 1A). The ability of the two VHH to modulate DKK1 biological activity was tested *in vitro* in murine KS483-4C3 mesenchymal precursor cells, which were induced to express Alkaline Phosphatase (ALP) after treatment with BMP6. BMP induced ALP expression is mediated via canonical WNT-signaling [10]. Indeed co-treatment of cells with BMP6 and DKK1 effectively abrogated ALP induction (Figure 1B). This inhibition could be reversed by the addition of VHH G5 or H7 in a dose dependent manner (Figure 1C and 1D). Both VHH showed effective neutralization of DKK1 in this bioassay.

Selection of anti-FRZB VHH

VHH against FRZB were selected from a non-immune llama VHH-phage display library. Two rounds of panning selection led to the selection of 8 candidate VHH. After subcloning into pMEK222 and sequence determination, 5 VHH with different sequences were successfully expressed and purified. ELISA was used to rank the different VHH according to the apparent affinities (Figure 1 E).

Functional analysis of anti DKK1 and anti FRZB VHH

Functional assays were performed to choose the most efficient VHH out of 2 DKK1 VHH and 5 FRZB VHH in blocking the function of DKK1 or FRZB in human primary chondrocytes, respectively. We found that H7 was most efficient in blocking DKK1 and TSF-1F7 was most effective for blocking FRZB (Supplemental Figure 3 A and B). Although TSF-1F7 has the lowest affinity to FRZB based on ELISA, it has the best neutralizing efficiency in cells compared to the other four FRZB VHH. This might be due to specific binding of TSF-1F7 to the biological domain of endogenous FRZB thereby blocking its function, while the other four FRZB VHHs bind other FRZB domains.

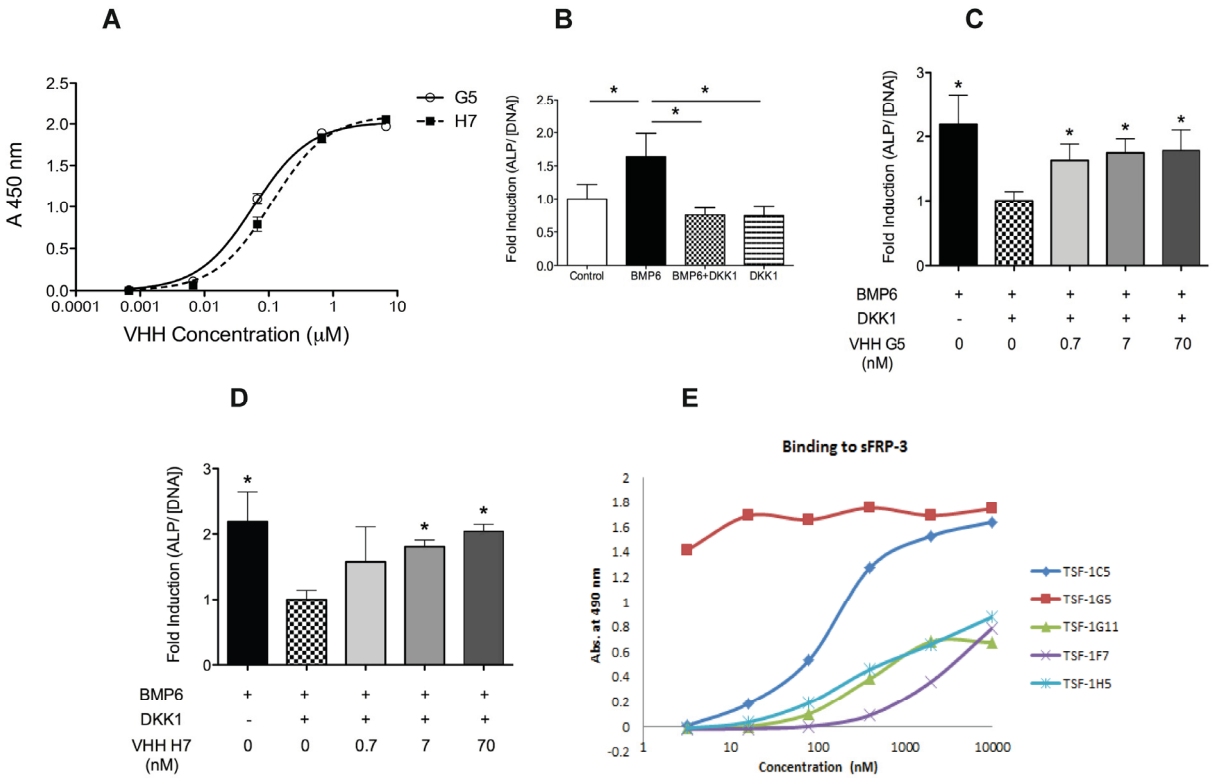

Figure 1. Selection and analysis of anti-DKK1 and anti-FRZB VHH, (A) VHH bind to DKK1 and neutralize DKK1 activity. Concentration range of VHH G5 and H7 was incubated in wells coated with DKK1 (60 nM) or PBS (negative control). After several washes, bound VHH were detected with a mouse anti-VHH serum and a Donkey anti-rabbit antibody coupled to a peroxidase. The amount of converted HRP (Absorbance at 450 nm; A 450 nm) is proportional to the amount of bound VHH. Error bars represent standard deviation (N=3). (B) Addition of BMP6 stimulates ALP expression in KS483-4C3 cells. The co-incubation of BMP6 with DKK1 reversed the ALP expression back to basal levels.

The addition of DKK1 to the standard cell culture did not show any significant difference in ALP expression when compared with control. The ALP activity is normalized by the total DNA content of KS483 cells after 7 days of culture, and expressed as fold induction relative to control. (C, D) KS483-4C3 cells were stimulated with BMP6, DKK1 and VHH G5 (C) or VHH H7 (D) in a concentration range of 0 – 70 nM. Co-incubation of VHH with DKK1 and BMP6 reversed DKK1 mediated inhibition of BMP6 induced ALP activity in a dose-dependent manner demonstrating effective neutralization of DKK1 activity by the VHH G5 and H7. ALP activity was measured and expressed as relative enzyme activity corrected for DNA, and expressed as fold induction relative to BMP6/ DKK1/ VHH (+/+/-). * $p< 0.05$. (N=3). (E) The apparent affinities of different FRZB VHH. Plates were coated with FRZB (60 nM) and blocked with 4% skimmed milk in PBS (MPBS), then incubated with a concentration range of different VHH (0-7 μM). Unbound VHH were washed with PBS-Tween and bound VHH were detected by incubation with mAb M1 and a HRP-conjugated anti-mouse.

VHH against DKK1 and FRZB function as neutralizing antibodies as measured by restored WNT signaling

Dose-response experiments were performed to determine the optimum concentration of the VHH in blocking DKK1 and FRZB. A concentration of 5μg/mL of neutralizing VHH completely reversed the blocking effect of DKK1 or FRZB on WNT3A induced *AXIN2* mRNA expression as determined by qPCR analysis (Figure 2A and B). This concentration of 5μg/mL was used in all subsequent experiments. At the same concentration, a non-specific IgG control did not have any effect on the expression of the examined marker genes (Figure 2C, D and E).

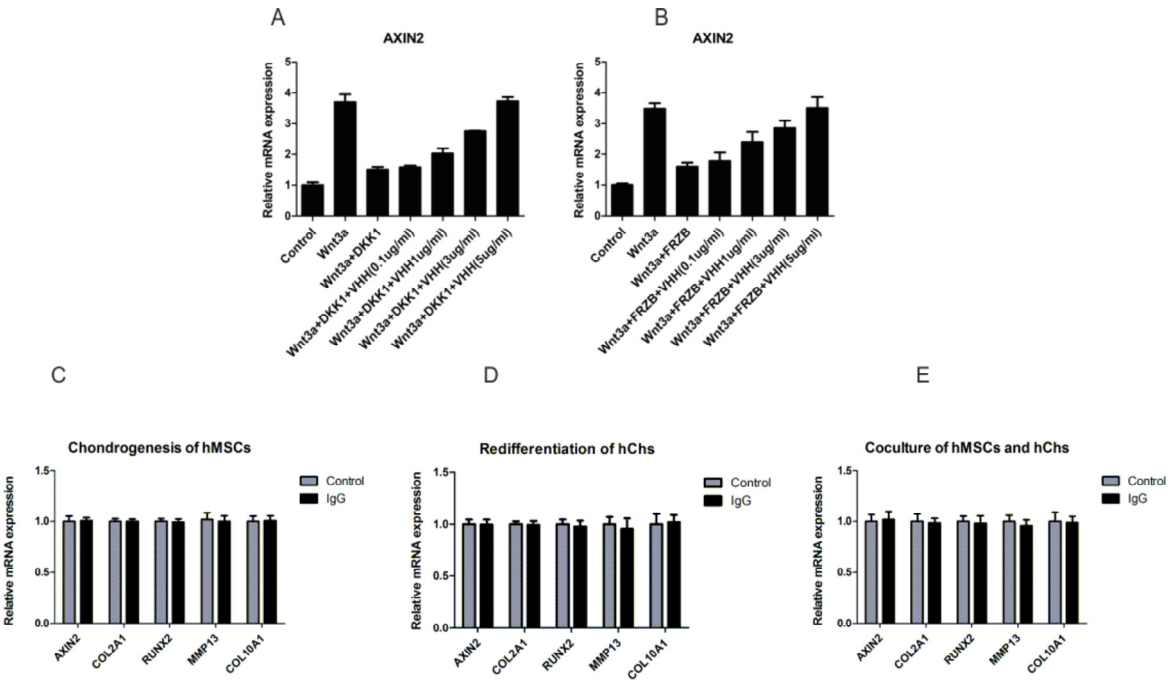

Figure 2. VHHs against DKK1 (A) or FRZB (B) can be used for restoring WNT signaling in human chondrocytes. A concentration of 5 µg / ml DKK1 or FRZB neutralizing antibodies restores WNT activation to its original levels (A and B). The control IgG antibody, did not interfere with gene expression in hMSCs (C), hChs (D) and co-cultures of hChs and hMSCs (E) of the investigated genes as compared to control cells receiving no treatment.

Blocking DKK1 and FRZB inhibits both chondrogenesis of hMSCs and chondrocyte redifferentiation

We have found that the endogenous expression levels of DKK1 and FRZB in hMSCs are low (Supplemental Figure 4), while their expression increases during hMSCs chondrogenesis (Supplemental Figure 5). This suggests that DKK1 and FRZB are involved in chondrogenesis of hMSCs. To prove this, neutralizing VHH were added to block DKK1 and FRZB in monoculture hMSCs pellets during chondrogenic induction. The effect of anti-DKK1 and anti-FRZB treatment on chondrogenesis of hMSCs was measured after four weeks. We found a significant loss of GAG deposition and the expression of Collagen type II in pellets with DKK1 and FRZB blockade as compared to the controls. Immunofluorescence further confirmed IHC result showing that the loss of collagen type II (Figure 3A).

We hypothesized that endogenous expression of DKK1 and FRZB is essential for maintaining the chondrocyte phenotype and that DKK1 and FRZB are necessary for

chondrocyte re-differentiation. To test this hypothesis, cells were dedifferentiated during expansion culturing for 2 passages after which pellet cultures were made to initiate re-differentiation in chondrogenic differentiation medium containing TGFβ and DEX. Neutralizing VHH against DKK1 and FRZB were added to the pellet cultures. The effect of anti-DKK1 and anti-FRZB treatment on chondrocyte redifferentiation was measured after four weeks. Based on Alcian blue staining, successful re-differentiation of hChs took at least three weeks in 3D cultures (Supplemental Figure 6). Alcian blue and safranin O showed that GAG deposition was absent in pellets in which DKK1 and FRZB were blocked (Figure 3C). In addition, blocking DKK1 and FRZB resulted in reduced amounts of collagen type II protein deposition, measured by immunohistochemistry and immunofluorescence (Figure 3B).

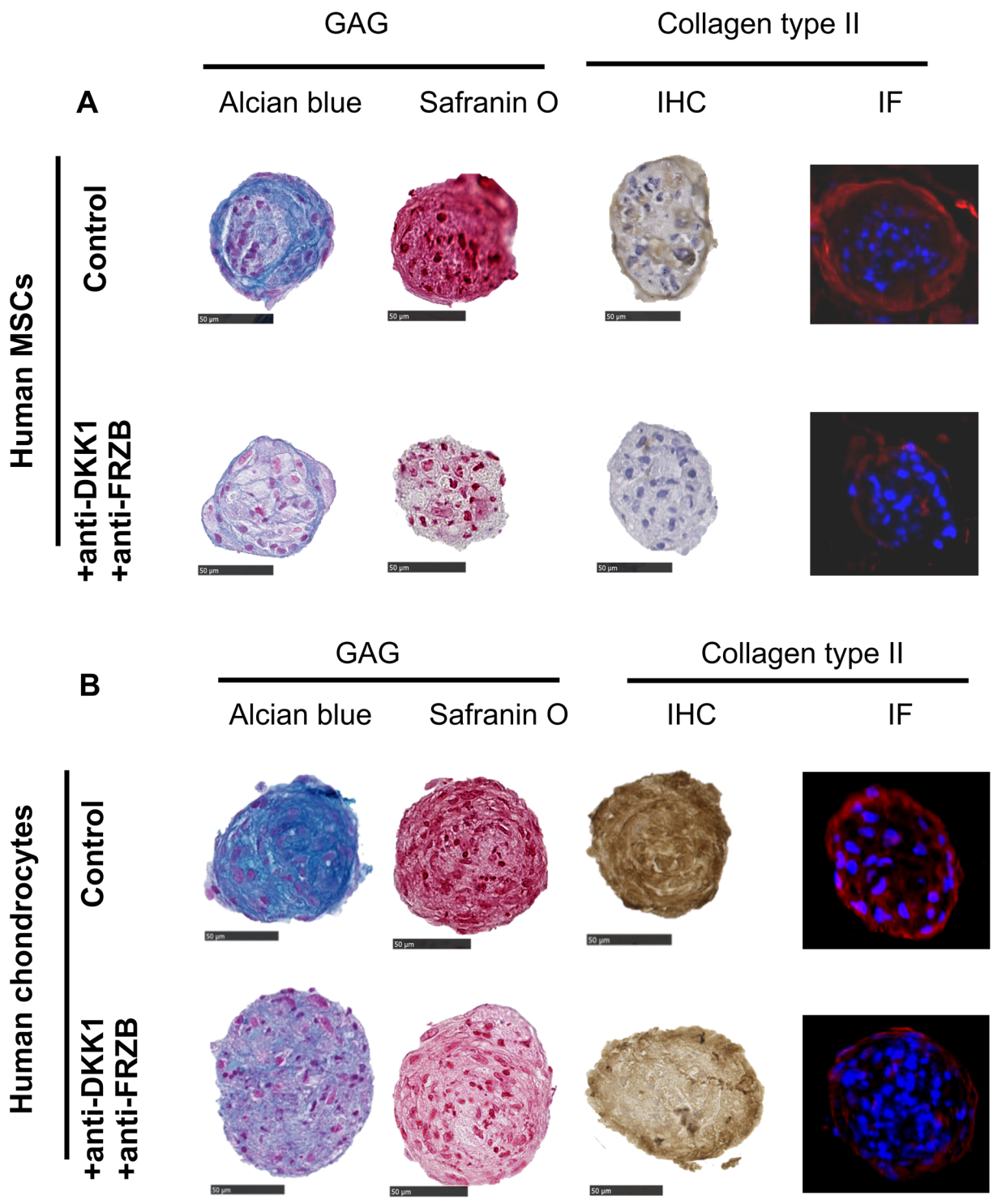

Figure 3. Blocking DKK1 and FRZB inhibits both chondrogenic differentiation of hMSCs and chondrocyte re-differentiation. Histological analysis of GAGs using Alcian blue and safranin O staining. Collagen type II was measured by immunohistochemistry and immunofluorescence staining on mid-sagittal sections of the chondrogenically differentiating hMSCs (A) and the chondrocyte (B) micromasses.

Blocking DKK1 and FRZB results in expression of hypertrophic markers and cellular hypertrophy in pellet co-cultures

To investigate if endogenous expression of DKK1 and FRZB prevents hypertrophy, neutralizing VHH against DKK1 and FRZB were added to co-culture pellets of hMSCs and hChs. Co-culture pellets were cultured for 4 weeks in chondrogenic differentiation medium. Gene expression in the co-cultures was measured by qPCR. Control experiments using a non-specific IgG did not affect gene expression (refer to figure 2E). Results showed a 1.5-fold increase in mRNA levels of *AXIN2* after the addition of the neutralizing VHH, suggesting the activation of WNT signaling after blocking of DKK1 and FRZB (Figure 4A). Furthermore, blocking DKK1 and FRZB resulted in upregulation of the hypertrophic marker *COL10A1* (Figure 4B), and the master regulator of bone formation *RUNX2*. *MMP13* expression was marginally increased, albeit significant in t-test.

Alcian blue and safranin O staining indicated that co-culturing of hMSCs and hChs in chondrogenic differentiation medium containing neutralizing VHH showed no obvious difference in GAG production (Figure 4C). IHC and IF results showed that collagen type II expression had no difference between control and cultures treated with neutralizing VHH (Figure 4D). *In vivo* and *in vitro*, during the process of hypertrophic differentiation, cells enlarge, terminally differentiate, mineralize, and ultimately undergo apoptosis [40, 41]. To elucidate the effects of blocking DKK1 and FRZB on (matrix) mineralization and apoptosis, we used Alizarin red S staining to evaluate mineralization and a TUNEL assay to detect the apoptosis of cells. Alizarin red S staining indicated that loss of DKK1 and FRZB increased matrix mineralization, the positive staining was especially present in the center of the pellets (Figure 4E). The TUNEL assay showed that apoptotic cells were present in both the control and the experimental group (Figure 4E). The number of apoptotic cells was increased in the VHH-treated group. In addition, the co-culture pellets treated with neutralizing VHH highly expressed collagen type X (Figure 4F) and were less compact and more sensitive to tissue handling.

Together, these data suggest that blocking DKK1 and FRZB increases hypertrophic differentiation by increasing hypertrophic marker expression, promotion of cartilage mineralization, and subsequent apoptosis in these cultures.

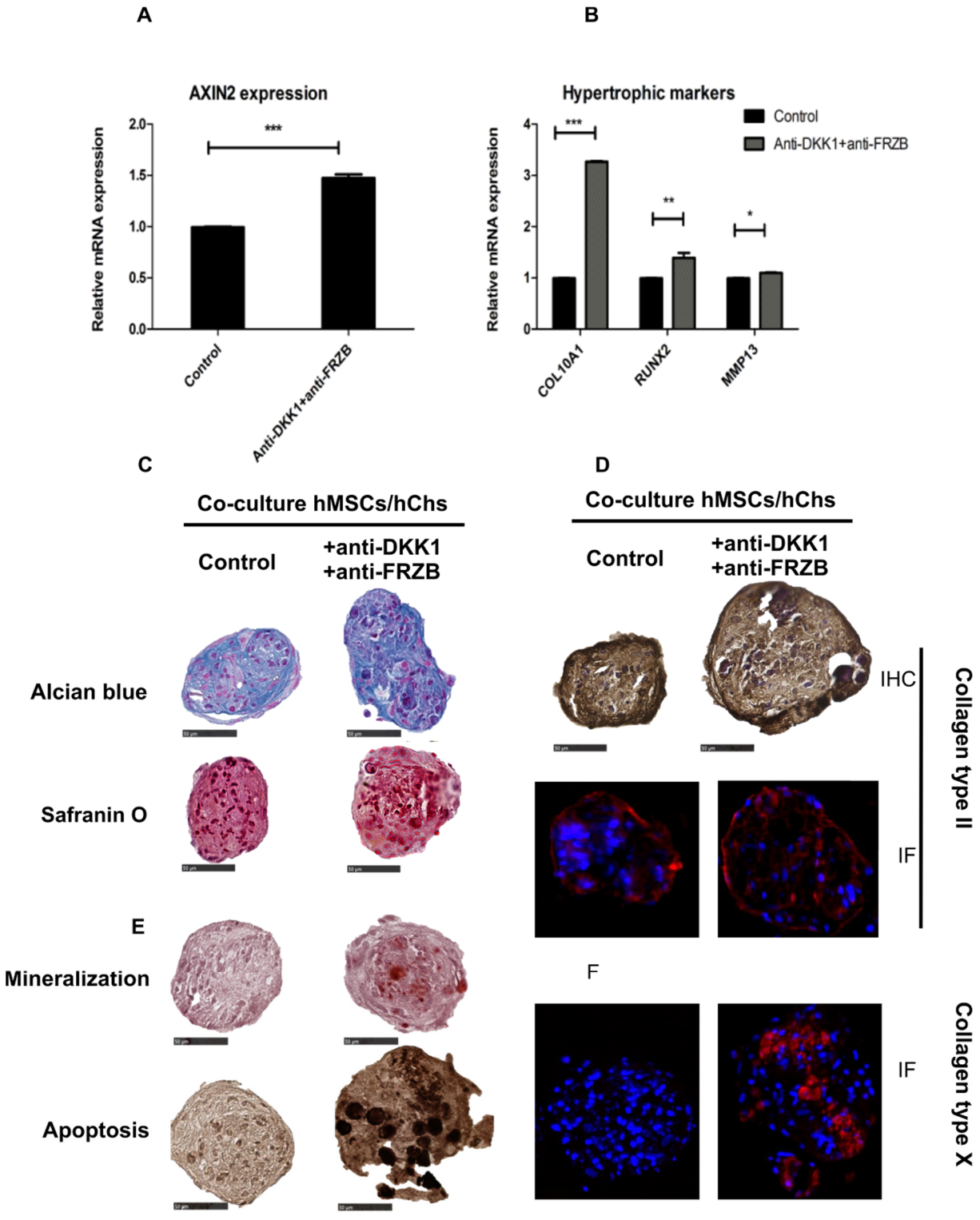

Figure 4. The effect of neutralizing antibodies against DKK1 and FRZB on 3D co-culture pellets of hMSCs and hChs after 4 weeks of culture. Gene expression was determined by quantitative real-time polymerase chain reaction analysis (A and B). The expression of target *gene* was standardized to *GAPDH* expression. Bars show the mean ± SD of triplicate cultures. *=P <0.05; **=p< 0.01 versus control. C: Paraffin sections of co-culture pellets stained for glycosaminoglycan by Alcian blue and

Safranin O. D: The expression of type II collagen was visualized by IHC and immunofluorescence (scale bar 50 μm). E: Alizarin red for matrix mineralization (visible as dark red regions) and TUNEL assay for apoptosis (apoptotic nuclei were stained dark brown). F. Collagen type X was measured by immunofluorescence. Data is based on 8 samples. For each experiment a representative section is shown.

Discussion

In this paper we proved that i) endogenous expression levels of DKK1 and FRZB are necessary for chondrogenesis of hMSCs, ii) endogenous levels of DKK1 and FRZB play a stimulating role in chondrocyte redifferentiation, and iii) endogenous DKK1 and FRZB are able to inhibit hypertrophic differentiation in a coculture system.

Chondrogenesis is defined by a series of steps that can be described as MSC condensation, cell proliferation and differentiation into chondroprogenitors followed by secretion of ECM components and further maturation into chondrocytes [40] (Figure 5). WNT/β-catenin signaling plays an important role in multiple steps [42] particularly in the initiation of chondrogenesis and inducing hypertrophic differentiation. Both *in vivo* and *in vitro* experiments reveal that low levels of β-catenin activity is required for the initiation of chondrogenesis in the first phase [43, 44] while high level of β-catenin activity block the express of cartilage marker by inhibiting the activity of SOX9 [42]. However, the endogenous mechanism regulating these processes *in vivo* has not been described. We hypothesized that endogenous expression of DKK1 and FRZB is sufficient to block or repress WNT signaling resulting in initiation of chondrogenesis and prevention of hypertrophic differentiation. Our results show that inhibition of DKK1 and FRZB during chondrogenesis of hMSCs resulted in loss of GAG deposition and Collagen type II expression, implying that these factors are indeed necessary for chondrogenic induction of hMSCs.

Primary human chondrocytes are enzymatically released from the cartilage matrix for autologous cell-based therapeutic interventions (ACI) [1] and research purposes. However, long expansion time and multiple passaging in monolayer culture, which are generally required to get enough amounts of chondrocytes, lead to dedifferentiation of the isolated chondrocytes. Ryu et al has reported that β-catenin expression levels are low in normal chondrocytes and significantly increased as cells undergo dedifferentiation in serial monolayer cultures. They found that overexpression of β-catenin using a β-catenin activator leads to dedifferentiation of

chondrocytes and that transcriptional activation of β-catenin is sufficient to cause the loss of chondrocyte phenotype in rabbit cells [45]. In addition, chondrocytes from *APC* knockout mice, which display activation of canonical WNT/β-catenin signaling, show loss of normal phenotype and dedifferentiation, further proving the role of β-catenin signaling in chondrocyte dedifferentiation [44]. We proved our hypothesis that DKK1 and FRZB are able to prevent chondrocytes dedifferentiation by blocking WNT/β-catenin signaling. Our results showed that antibody-based inhibition of DKK1 and FRZB led to a significant decrease in GAG deposition and the loss of collagen type II, indicating that removal of DKK1 and FRZB releases the break on WNT signaling resulting in impaired redifferentiation. We have previously shown that the expression of DKK1 increased (marginally) while FRZB expression decreased during the dedifferentiation process [22]. During dedifferentiation, the small increase in DKK1 might not be sufficient to compensate for the loss of FRZB, which not only blocks canonical but also non-canonical WNT pathways. This is in contrast to DKK1 that only blocks canonical WNT signaling. Loss of FRZB during the dedifferentiation could therefore switch the fine balance between the canonical and the non-canonical WNT pathways. Our data suggest that endogenous expression of DKK1 and FRZB prevents chondrocyte dedifferentiation and that combined blocking of these factors during chondrogenesis inhibits redifferentiation.

Loss-of-function and gain-of-function studies indicate that WNT/β-catenin signaling is involved in endochondral ossification. Overexpression of WNT/β-catenin signaling induces chondrocyte hypertrophy [46, 42] while blocking WNT signaling by using a WNT inhibitor during the last steps of chondrogenic differentiation prevents hypertrophic differentiation of the cells [23]. We proved our hypothesis that the endogenous WNT inhibitors DKK1 and FRZB play a determining role in preventing terminal chondrocyte differentiation by regulating WNT/β-catenin signaling. In contrast to our mono-culture experiments, we found that blocking of DKK1 and FRZB in co-culture pellets of hMSCs and hChs did not inhibit cartilage specific matrix formation and collagen type II production. The discrepancy between mono-cultures and co-cultures suggests that in co-cultures other mechanisms are operational that compensate for the loss of DKK1 and FRZB. We have previously shown that MSCs in co-cultures secrete trophic factors that enhance cartilage formation, and that these factors are not expressed in monocultures of either MSCs or chondrocytes [47]. This implies that in addition to DKK1 and FRZB expression, alternative factors that are specifically present in co-cultures compensate for the loss of DKK1 and FRZB, by directly or indirectly regulating WNT activity.

Since we observed GAG and collagen II expression in the co-cultures, indicative of cartilage

formation, we used the co-cultures to study the role of DKK1 and FRZB on terminal chondrocyte differentiation. We observed an increase in hypertrophic markers and matrix mineralization. Matrix mineralization is normally observed in endochondral bone formation [48]. It has been shown that premature induction of hypertrophy during *in vitro* chondrogenesis of hMSCs correlates with calcification after ectopic transplantion in SCID mice [49]. Both *in vivo* and *in vitro* studies have concluded that hypertrophic chondrocytes do not only express hypertrophic markers, but also undergo apoptosis [50, 49, 41]. Similarly, our results showed that the number of apoptotic cells increased in co-culture pellets with neutralizing VHH.

In conclusion, as far as we know, our study is the first one to show novel evidence on the combined role of endogenously produced DKK1 and FRZB in at least three stages of chondrocyte differentiation: i) firstly, in the initial stage, during onset of chondrogenesis in MSCs, ii) in redifferentiating chondrocytes, and iii) during the hypertrophic differentiation phase (figure 5). This study may help us to further improve the clinical cell-based cartilage repair strategies.

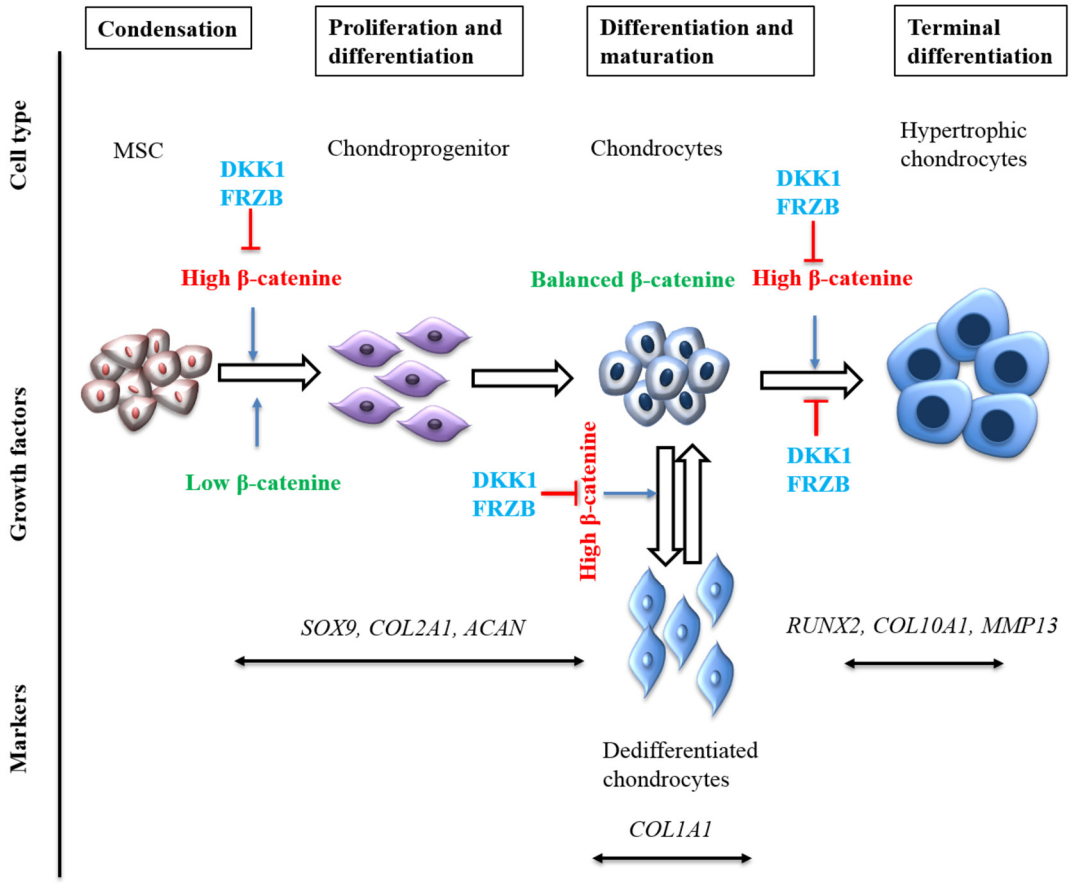

Figure 5. Endogenously produced WNT antagonists DKK1 and FRZB determine chondrogenesis and chondrocyte terminal differentiation. Chondrogenesis is defined by a series of processes. In the first step of chondrogenesis, DKK1 and FRZB promote chondrogenesis and prevent chondrocyte dedifferentiation through inhibiting WNT/β-catenin signaling. In the second step, DKK1 and FRZB inhibit chondrocyte terminal differentiation by blocking WNT/β-catenin signaling. The expression of typical markers of the various stages of chondrogenesis are indicated at the bottom.

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Supplemental data

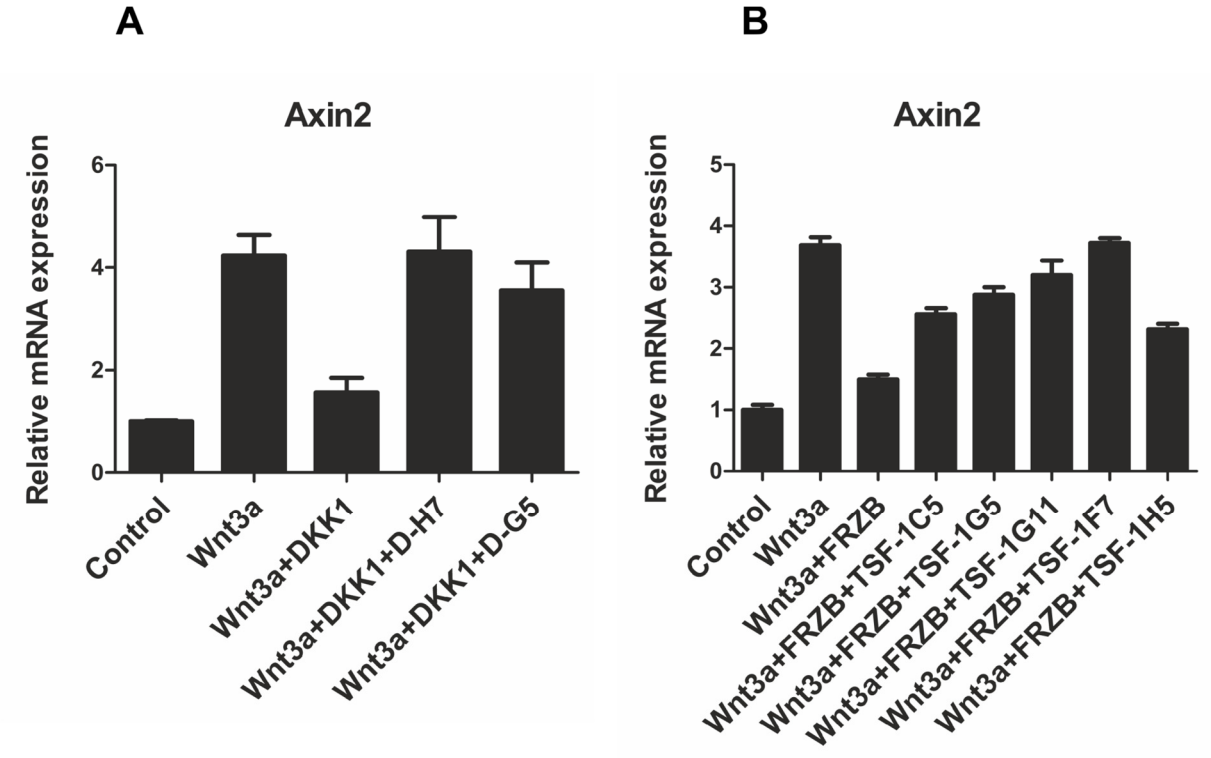
VHH	FR1	CDR1	FR2	CDR2
A7	EVQLVESGGGLVQAGGSLRLSCAAS	GSIVTFNP	MGWYRQAPGNQ--RELVASI	TSGG-GA
B7	EVQLVESGGGLVQAGGSLRLACAAS	GRTFSNYR	MGWFRQAPGQE--REFVAAI	SGSGSFT
B12	EVQLVESGGGLVQPGGSLRLSCAAS	GFTFSSYA	MSWVRQAPGKG--LEWVSAI	NSGGGST
C9	EVQLVESGGGLVQPGGSLRLSCVVS	GFTISNYG	MSWVRQAPGKGPEWEWVSAI	NSGGDST
D4	EVQLVESGGGLVQAGDSLRLSCAAS	GRSISLYA	MAWFRQAAGKE--REFVAAI	NWSGGST
G5	**EVQLVESGGGLVQAGGSLRLSCAAS**	**GRALSRSP**	**MAWFRQAPGKE--REFVVHW**	**ISG--ST**
H7	**EVQLVESGGGLVQAGGSLRLSCAAS**	**G---STGA**	**MAWFRQAPGKE--RDLVASI**	**SRSGVST**

VHH	FR3	CDR3	FR4
A7	NYVDSVKGRFTISVDSAKNTVYLQMNSLKPEDTAVYYCNA	D---------IFSSSRLSWDNY	WGQGTQVTVSS
B7	YYADSVKGRSTISRDNAKNTVYLQMNSLKPEDTAVYYCGA	G------VHLGAATSYTRYD-F	WGQGTQVTVSS
B12	SYADSVKGRFTISRDNAKNTLYLQMNSLKPEDTAVYRCAK	----YYEADP----AKNEYD-Y	WGQGTQVTVSS
C9	RYADSVKGRFTISRDNAKNTLYLQMNSLKPEDAAVYFCTR	EKTAYYCSGSGCYDPRYEFD-Y	WGRGTQVTVSS
D4	RYADSVKGRFSISRDTAKNTVYLTMNSLKPEDTAVYYCAT	DSS----TTVVFYSSSNSLR-Y	WGQGTQVTVSS
G5	**YYADSVKGRFTTSRDNAENTVYLQMNSLKPEDTAVYYCAA**	**GFA---PDTPSIFTSPRTYY-Y**	**WGQGTQVTVSS**
H7	**YYADSVKVRFTISRDNAKNTVFLQMNNLKPEDTGVYYCAA**	**G--------PTFRQSRATYT-D**	**WGQGTQVTVSS**

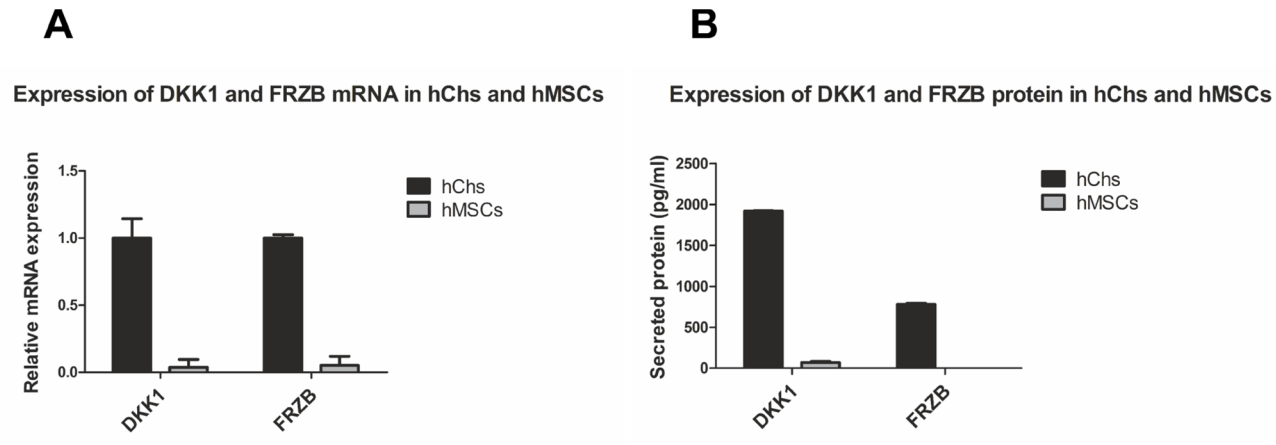
Supplemental Figure 1. Amino acid sequence alignment of VHH targeting DKK1 derived from a llama immunized library. Sequencing of the 14 isolated clones resulted in the identification of 7 unique clones. The sequence numbering is according to Chothia [51] where according to whom the different framework residues (FR) and complementary-determining region (CDR) are identified. The sequences of G5 and H7 are highlighted in bold.

VHH	Sequence
TSF-1C5	QVQLQESGGGLVQAGGSLRLSCVASGRTFSSVAMGWFRQAPGKQRELVAGISRGGTTN YLDSVKERFTISRDNAKNIVYLQMNNLKPEDTAVYYCNAVDFLDRTNYWGRGTQVTVS SEPKTPKPQPAAASGSLEQKLISEEDLNGAAHHHHHHGAA
TSF-1G5	QVQLVESGGDLVQPGGSLRLSCAASGIIFSLNAVGWYRQAPGKQRELVARIMGGGSTDY ADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNARPVLTRLDYWGQGNQVTVSS AAASGSLEQKLISEEDLNGAAHHHHHHGAA
TSF-1H5	QVQLVESGGGLVQPGGSLRLSCAASGSIFSINTMGWYRQAPGKPRELVATITRGAMINYA DSVQGRFTISRDNAKNTVYLQMNSLIPEDTAVYYCNYKGLQRTSLGIIRFDSWGQGTRVT VSSAAASGSLEQKLISEEDLNGAAHHHHHHGAA
TSF-1F7	**QVQLQESGGGLVQPGGSLRLSCAAPGIIFSINDMGWYRQAPGKQRELVAAIAGGGR TNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNAKSPWNTIPYWGHGT QVTVSSEPKTPKPQPAAASGSLEQKLISEEDLNGAAHHHHHHGAA**
TSF-1G11	QVQLQDSGGGLVQPGGSLRLSCAASGFTFSSSAMSWVRQAPGKGLEWVSSINKGGGITT YADSVKGRFTISRDNAKNTLYLQMNSLKPEDTAVYYCAKSRFYGSGLGAADYRGQGTQ VTVSSEPKTPKPQPAAASGSLEQKLISEEDLNGAAHHHHHHGAA

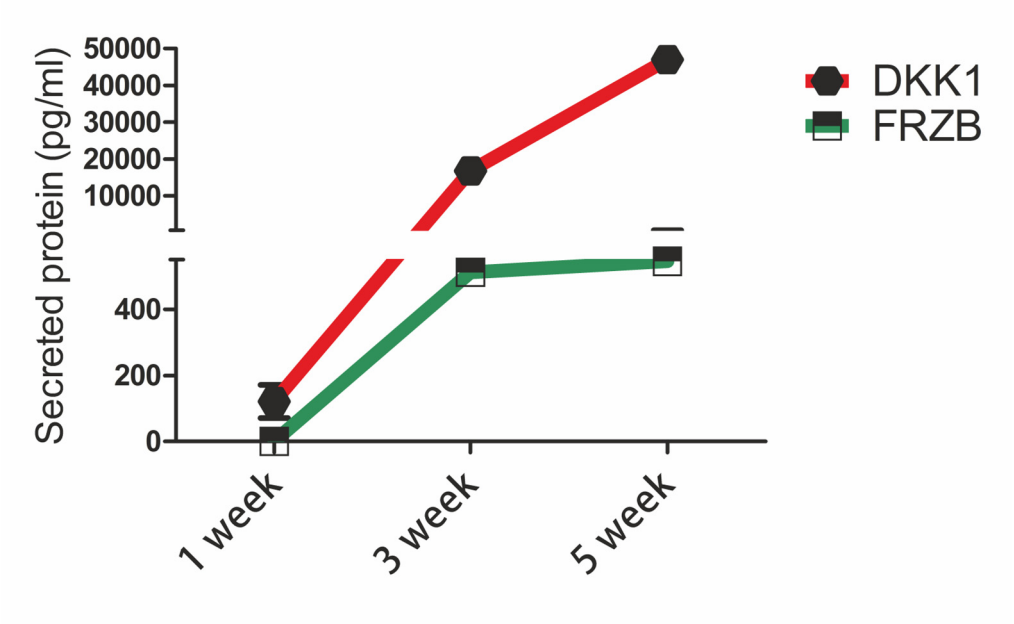
Supplemental Figure 2. Amino acid sequence alignment of VHH targeting FRZB derived from non-immune VHH library of BAC BV. Sequencing of the 8 isolated clones resulted in the identification of 5 unique clones against sFRP-3. The sequences of TSF-1F7 are highlighted in bold.



Supplemental Figure 3. Selection of most efficient DKK1 and FRZB neutralizing VHH. Human articular chondrocytes were stimulated with Wnt3a or Wnt3a plus DKK1/FRZB, or Wnt3a plus DKK1/FRZB combination with different neutralizing VHH, qPCR was used to measure the WNT target gene AXIN2 expression.

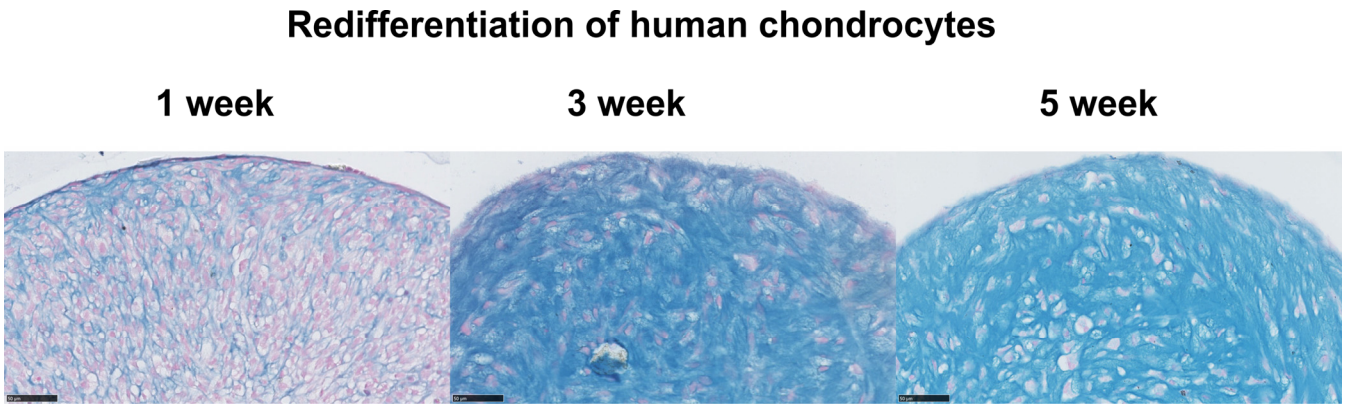


Supplemental Figure 4. Expression of DKK1 and FRZB in human chondrocytes (hChs) and hMSCs. mRNA levels were determined by quantitative real-time polymerase chain reaction analysis. The expression of *DKK1* and *FRZB* was standardized to *GAPDH* expression (A). Additionally, secretion of DKK1 and FRZB was analyzed using ELISA (B). Bars show the mean ± SD.



Supplemental Figure 5. The protein expression of DKK1 and FRZB during chondrogenesis of hMSCs. Human MSCs were cultured in chondrogenic differentiation medium containing

dexamethasone and TGFβ. Culture medium was collected at indicated time point. DKK1 and FRZB protein levels were analyzed using ELISA.



Supplemental Figure 6. Alcian blue staining of 3D pellet cultures shows clear GAG production after 3 weeks. After 5 weeks of differentiation, cartilage characteristics are clearly visible: chondrocytes are present in lacunae surrounded by GAGs.

Gene Name	Primer Sequence	Product size	Annealing temperature
GAPDH	Forward: 5'CGCTCTCTGCTCCTCCTGTT 3' Reverse: 5'CCATGGTGTCTGAGCGATGT 3'	81	60
AIXN2	Forward: 5' AGTGTGAGGTCCACGGAAAC 3' Reverse: 5' CTGGTGCAAAGACATAGCCA 3'	103	60
RUNX2	Forward: 5' GGAGTGGACGAGGCAAGAGTTT 3' Reverse: 5' AGCTTCTGTCTGTGCCTTCTGG 3'	133	60
MMP13	Forward: 5' AAGGAGCATGGCGACTTCT 3' Reverse: 5' TGGCCCAGGAGGAAAAGC 3'	72	60
COL10A1	Forward: 5' GAACTCCCAGCACGCAGAAT 3' Reverse: 5' CCTGTGGGCATTTGGTATCG 3'	121	60
DKK1	Forward: 5' AGTACTGCGCTAGTCCCACC 3' Reverse: 5' TCCTCAATTTCTCCTCGGAA 3'	172	60
FRZB	Forward: 5'ACGGGACACTGTCAACCTCT 3' Reverse: 5'CGAGTCGATCCTTCCACTTC 3'	155	60

Supplemental table 1. Forward (F) and Reverse (R) primers used for quantitative RT-PCR is listed in this table.

Chapter 7

Interleukin 1 beta (IL1β) reactivates WNT signaling in human chondrocytes by reducing DKK1 and FRZB expression via iNOS

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Success belongs to the persevering (坚持就是胜利)

Abstract

Osteoarthritis (OA) is a complex disease characterized by progressive cartilage degeneration and mild signs of inflammation. Both the Interleukin 1 beta (IL1β) and the WNT signaling pathways are involved in OA. Recent data suggests that there is a direct or indirect crosstalk between IL1β and WNT signaling. Revealing this link is expected to yield valuable mechanistic insights in OA development, which could aid the development of osteoarthritic drugs. In this study we aim to identify the possible mechanism by which IL1β influences cartilage degeneration by upregulation of WNT signaling. In human OA cartilage we observed a loss of DKK1 and FRZB expression, while the expression of IL1β and inflammatory mediator inducible nitric oxide synthase (iNOS) was highly increased as compared to preserved cartilage specimens. IL1β treatment of chondrocytes isolated from preserved human cartilage decreased expression of DKK1 and FRZB through upregulation of iNOS, thereby activating the transcription of WNT target genes. This effect could be reversed by treatment with iNOS inhibitor 1400W, which restored DKK1 and FRZB expression and their inhibitory effect on WNT signaling. In addition, 1400W also inhibited both MMP expression and cytokine-induced apoptosis. Microarray analysis revealed that in addition to the proinflammatory pathway, many pathways are involved in the molecular response of human primary chondrocytes to IL1β stimulation. These findings provide evidence that IL1β might contribute to OA by unleashing WNT signaling and activating inflammatory pathways.

Introduction

Osteoarthritis (OA) is the most common form of arthritis affecting the whole joint. OA is characterized by progressive degeneration of articular cartilage, mild signs of inflammation, and typical bone changes [1, 2]. The mechanisms underlying the pathogenesis of OA are still largely unknown. Accumulating evidence has strongly linked WNT activity to the onset and development of OA. Indeed, alterations of WNTs and WNT-related proteins have been found in human OA. For example, the WNT antagonists DKK1 and FRZB have been repeatedly linked with OA. Multiple whole genome studies indicated that single nucleotide polymorphisms (SNPs) in the WNT antagonist FRZB, causing loss of the function of FRZB, is related with hip OA [3, 4]. Another study showed that the highest FRZB serum levels associated with a modest reduction in risk of incident hip OA [5]. FRZB-knockout mice have more severe OA cartilage deterioration in response to instability, enzymatic injury, or inflammation [6]. It has been reported that high DKK1 has a protective function against cartilage degeneration and that DKK1 is associated with OA development [5, 7, 8] and elevated circulating serum levels of DKK1 are associated with reduced progression of radiographic hip osteoarthritis (RHOA) in elderly women [5]. Overexpression of DKK1 in the articular joint of mice decreased OA severity [9]. In previous work, we showed that the exogenous addition of high concentrations of the WNT antagonists DKK1 and FRZB prevent hypertrophic differentiation of chondrogenically differentiating mesenchymal stem cells Leijten et al. [10]. In addition we showed that the loss of DKK1 and FRZB expression is observed in OA [11]. In a more detailed study, we showed that the expression of these antagonists negatively correlated with grading of knee OA [12]. In addition, we showed that the expression of *DKK1* and *FRZB* mRNAs in human chondrocytes isolated from OA patients decreased after exposure of the cells to IL1β [13]

IL1β is a key pro-inflammatory cytokine that drives OA progression by inducing the expression of cartilage degrading enzymes such as matrix metalloproteinases (MMPs) [14, 15]. Injection of recombinant IL1β results in an inflammatory response of joint in equine model [16] and local injection of IL1β into mouse knee joints results in an OA-like phenotype [17]. Pro-inflammatory cytokines stimulate synthesis and release of nitric oxide (NO) and prostaglandin-E2 (PGE2), which are increased during the degenerative process, contributing to the joint pathology [18, 19]. NO is a pro-inflammatory mediator and catabolic factor that is associated with the pathogenesis of OA [20-22]. The role of NO in joint disorders has been well studied. For example, NO is highly expressed in OA chondrocytes [23-25] and cartilage [26]. NO

inhibits both synthesis of proteoglycan and collagen [27], activates MMPs, mediates chondrocyte apoptosis and promotes inflammatory responses. All of these effects contribute to the catabolic activities of NO in cartilage [28]. Furthermore, *in vivo* animal experiments have shown that inhibition of iNOS decreases the expression of catabolic factors [29] and iNOS knockout mice show resistance to experimental OA [30]. The association of the increased levels of catabolic enzymes and inflammatory mediators and the increased levels of cytokines like IL-1β and TNF-α in synovial fluid and joint tissue has been well-established. For example, a positive correlation between levels of MMP1, MMP3 and IL6 is observed in OA synovial fluid [31] and the dual expression of inflammatory factors and MMP are observed in the same chondrocytes in OA specimens by immunohistochemistry [32].

Despite the important roles of WNT and IL1β signaling in OA, it has remained largely unknown how these two pathways can cross communicate in chondrocytes and thus affect OA development. Crosstalk between WNT and IL1β signaling has been reported for distinct cells and tissues. For example, our group has previously shown that WNT/β-catenin inhibits IL1β induced MMP expression in human articular cartilage [13]. In addition, we have shown that the WNT/β-catenin regulated transcription factor TCF4 can bind to the NF-κB and thereby enhances NF-κB activity [33].

Other groups have reported that, in colon cancer cells, IL1β activates WNT signaling by inducing phosphorylation of GSK3β, thereby stabilizing β-catenin and enhancing TCF-dependent gene activation [34]. In turn, increased WNT signaling activity induced IL1β expression in alveolar epithelial cells [35].

It was recently shown that that IL1β induced upregulation of NO in cancer cells is responsible for a strong decrease in DKK1 expression, which in turn results in the upregulation of WNT/β-catenin signaling [36]. These data suggest that there is direct and / or indirect crosstalk between IL1β and WNT signaling. Insight into the exact interaction of these pathways is expected to yield valuable mechanistic insights in OA and could aid the development of osteoarthritic drugs. Here we tested our hypothesis that IL1β plays a role in initiating OA by increasing WNT/β-catenin activity via iNOS by reducing DKK1 and FRZB expression in human chondrocytes.

Materials and Methods

Human cartilage tissue

The collection and use of human cartilage was approved by a local medical ethical committee (METC). Cartilage was obtained from 11 patients with OA undergoing total knee replacement surgery. Preserved cartilage samples were isolated from macroscopically intact areas and OA cartilage specimens were isolated from areas that affected by OA. Cartilage samples were collected into 10 mL tubes and washed twice with PBS. For RNA isolation, subchondral bone was removed from the cartilage, and samples were cut into small pieces (1-2 mm) and quickly snap frozen into liquid nitrogen (LN2). The frozen samples were stored at -80° C.

For histology, cartilage samples were fixed using 10% phosphate buffered formalin (pH =7, Sigma Aldrich, St. Louis, Missouri, USA) overnight at 4 °C, decalcified for 4 weeks in 12,5% (w/v) EDTA solution containing 0.5% phosphate buffered formalin (pH 8.0), dehydrated using graded ethanol, and embedded in paraffin.

RNA isolation and qPCR from human cartilage

Cartilage pieces were transferred in a pre-cooled Cryo-Cup Grinder for crushing. The obtained cartilage powder was collected into 50 mL tubes and samples were weighed. One ml TRIzol reagent per 50-100 mg sample was added. Total RNA was isolated from the lysate according the manufacturer's protocol (TRIzol, ThermoFisher scientific, USA). The precipitated RNA was dissolved in RNase-free water and subsequently treated with RNase-free DNase I (Invitrogen life technologies, USA). The concentration of RNA was measured using the Nanodrop 2000. cDNA was obtained from 1µg of RNA with a cDNA synthesis kit (BIO-RAD, Hercules, CA). QPCR was performed using the SYBR Green sensimix (Bioline, London, UK). PCR Reactions were carried out using the Bio-Rad CFX96 (Bio-Rad, Hercules, CA) following standard protocol. For each reaction a melting curve was generated to test primer dimer formation and non-specific priming. GAPDH was validated as a reliable housekeeping gene and used for gene expression normalization. Primers sequences are listed in supplemental table 1.

Immunohistochemistry (IHC)

Immunohistochemical staining of DKK1, FRZB, and β-catenin was performed on 5µm tissue sections, which were pre-incubated with 5µg/mL proteinase K (Sigma Aldrich, Missouri, USA)

for 10 min in Tris-EDTA (TE) buffer at room temperature (RT) followed by incubation with 1mg/mL hyaluronidase dissolved in PBS (Sigma Aldrich) for 40 min at 37 °C. Rabbit polyclonal DKK1 (sc-25516, Santa Cruz biotechnology, Texas, USA), rabbit polyclonal FRZB (from sc-13941, Santa Cruz biotechnology, Texas, USA), rabbit polyclonal iNOS (sc-651, Santa Cruz biotechnology, Texas, USA), and rabbit polyclonal β-catenin (LS-C203657, LifeSpan Biosciences) were diluted 1:500 in 5% BSA in PBS and incubated overnight at 4°C. Non-immune controls underwent the same procedure without primary antibody incubation. The biotinylated secondary antibody was diluted 1:500 in 5% BSA in PBS and incubated for 30 minutes at RT. HRP-Streptavidin was added and incubated for 30 minutes at RT. For visualization, DAB substrate kit was used (ab64238, Abcam).

Human primary chondrocyte isolation and cell culture

Human primary articular chondrocytes (hChs) were isolated from macroscopically healthy looking areas of OA cartilage from patients undergoing total knee replacement and cultured in DMEM (Gibco) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml Penicillin, 100 mg/ml Streptomycin, 0.4mM proline, 0.2mM ascorbic acid diphosphate and 1% nonessential amino acids and used at passage two.

Recombinant proteins and reagents

Recombinant human IL-1β was obtained from R&D Systems. The inhibitor of nitric oxide, 1400W, was purchased from Cayman chemical (Michigan, USA).

RNA isolation and real-time quantitative polymerase chain reaction (qPCR) from chondrocytes

Medium was removed, cells were washed twice with PBS and lysed using lysis buffer. Total RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel), the purity and concentration of RNA samples were measured by Nanodrop 2000. cDNA was synthesized from total RNA with the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR (qPCR) was performed with the CFX96 real-time PCR detection system (Bio-Rad). GAPDH was used as internal control. Mean fold change of gene expression was transformed to log2， Then the value of log2 transformation was plotted.

Enzyme-linked immunosorbent assay (ELISA)

Cell culture medium was collected. Secreted DKK1 and FRZB protein concentrations were determined by ELISA following the manufacturer's instructions (cat numbers: DY1906 for DKK1; DY192 for FRZB, R&D systems).

Western Blotting

Total cell proteins were collected in RIPA buffer (Cell Signaling) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). The specific antibodies used for Western blot analysis including: Anti-iNOS (sc-651, Santa Cruz biotechnology, Texas, USA), anti-GAPDH (G8795, Sigma-Aldrich), anti-β-catenin (LS-C203657, LifeSpan Biosciences).

NO production assay

Cell supernatant was collected and quantified for nitrite using the Griess reaction as described [37].

Immunofluorescent staining (IF)

hChs were seeded at a density of 10^4 cells/cm^2 on coverslips and cultured for 24 hours in the presence or absence of 10ng/ml of IL1β treatment. Samples were washed three times with PBS, fixed with 10% formalin for 30 minutes, permeablized with 0.5% triton X-100 in PBS for 15 min at RT. Samples were blocked in 1.5% of BSA in PBST for 1 hour, then incubated with specific primary antibody against DKK1, FRZB, iNOS (Santa Cruz biotechnology, Texas, USA) or β-catenin (LifeSpan Biosciences) overnight at 4°. Cells were rinsed with PBS for 3 times, 5 min/time. Then Alexa®Fluor 546-labelled goat anti-rabbit or anti-mouse antibody in 1.5% of BSA in PBST was added and incubated for 2 hours at RT. Cells were rinsed with PBS and added mounting medium with DAPI. Slides were imaged using a BD pathway confocal microscope. Quantification of immunofluorescence was performed by CellProfiler 2.2.0 software. Immunofluorescence images were loaded to CellProfiler, then adjusted the pipeline to identify cells properly in the images, measurement of fluorescence intensity for each cell was performed. Mean intensity of all cells in images was calculated and presented pixels/cell. The intensity of fluorescence of experimental group was normalized to control group. Relative fluorescent intensity was presented in graphs.

Apoptosis assay

Human chondrocytes were exposed to 10 ng/ml of both recombinant human IL-1β and TNFα or 100uM iNOS inhibitor 1400W for 48 h. Apoptosis of human chondrocytes was detected using the DeadEnd colorimetric TUNEL assay (Promega, Madison, USA) following the manufacturer's procedure. Apoptotic nuclei were stained dark brown.

Whole genome gene expression microarray analysis

Microarray analysis was performed in human chondrocytes from three donors. Chondrocytes (passage 2) were stimulated with 10 ng/ml of IL1β for 48 hours. The Ambion Illumina total prep 96 kit was used to generate biotinylated cDNA from the RNA samples. 750ng of obtained cDNA of each sample was hybridized onto the Illumina HumanHT-12 V3 expression BeadChips. Samples were scanned using the Illumina iScan array scanner. Gene expression profiling was performed using Illumina's Genome studio v. 2010.3 software with the default settings advised by Illumina. Data was normalized by applying quantile normalization on the raw fluorescence intensity values. Different gene expression was analysed using the commercial software package Genespring, version 11.5.1 (Agilent technologies). Genes with at least a two-fold change being significantly differentially expressed according to student's t test using a cut-off rate of p=0.05 were selected. 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. Search tool for the retrieval of interacting genes/proteins (STRING9.0 http://string-db.org) was used to investigate the predicated gene-gene interactions network [38].

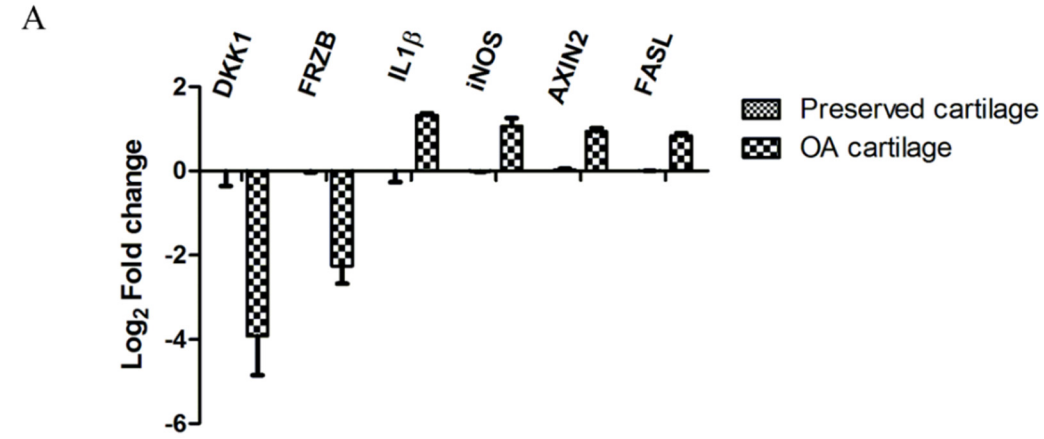
Statistical analysis

Statistical analysis was performed using student's t-test or one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

The expression of DKK1 and FRZB decreased while IL1β, iNOS and AXIN2 are increased in human OA

We first investigated the differences in gene and protein expression of the WNT antagonists DKK1 and FRZB, IL1β, iNOS, AXIN2 and FASL in human OA cartilage as compared to macroscopically healthy looking (preserved) cartilage. In OA cartilage DKK1 and FRZB mRNA expression was significantly decreased accompanied by overexpression of the pro-inflammatory factor IL1β, the inflammatory mediator iNOS, the apoptotic factor FASL and the WNT target gene AXIN2 (Figure 1A). Furthermore, IHC staining for DKK1, FRZB and β-catenin was performed in paired preserved and OA cartilage specimens from ten patients. Preserved cartilage consistently demonstrated high expression of cytosolic DKK1 and FRZB, especially in the superficial layer. In contrast, the matching OA cartilage from the same patient showed significantly decreased DKK1 and FRZB expression and increased nuclear localization of β-catenin. β-catenin was hardly detected in preserved cartilage in which high expression of DKK1 and FRZB was observed (Figure 1B). Interestingly, positive staining of DKK1 was also detected in cell clusters of some OA cartilage samples. The protein expression of DKK1, FRZB and β-catenin was measured in each patient (Supplemental figure 1).


A
DKK1
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Log2 Fold change
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Preserved cartilage
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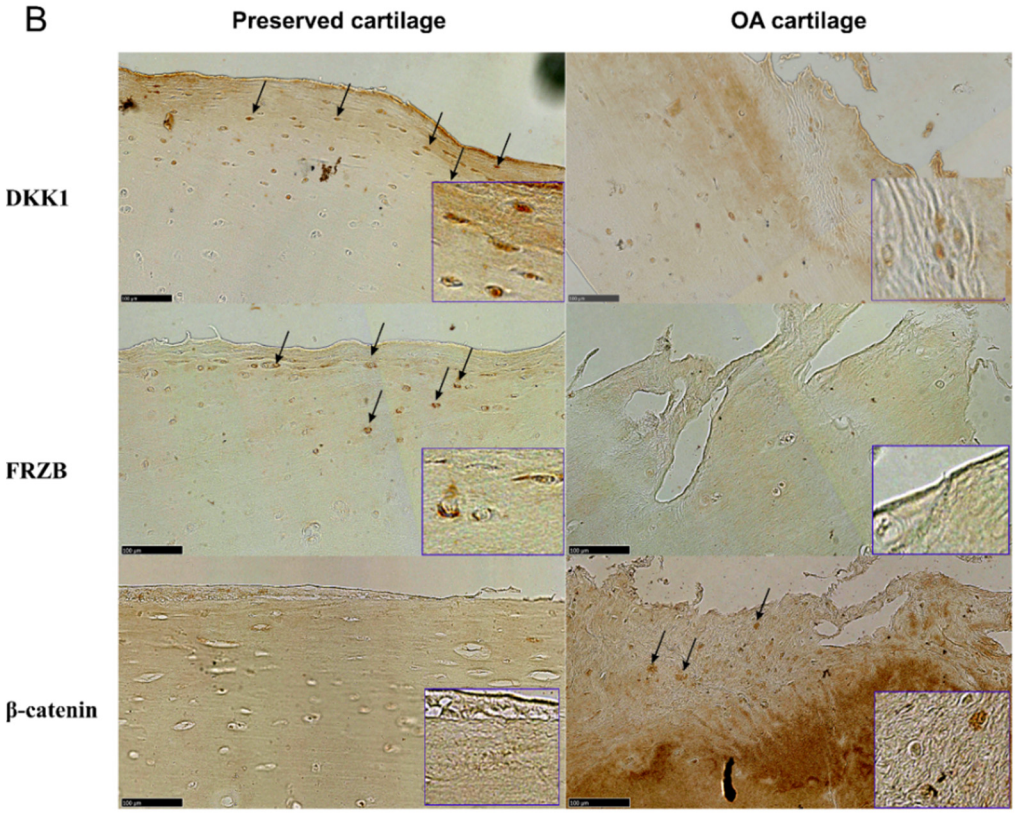
B
Preserved cartilage
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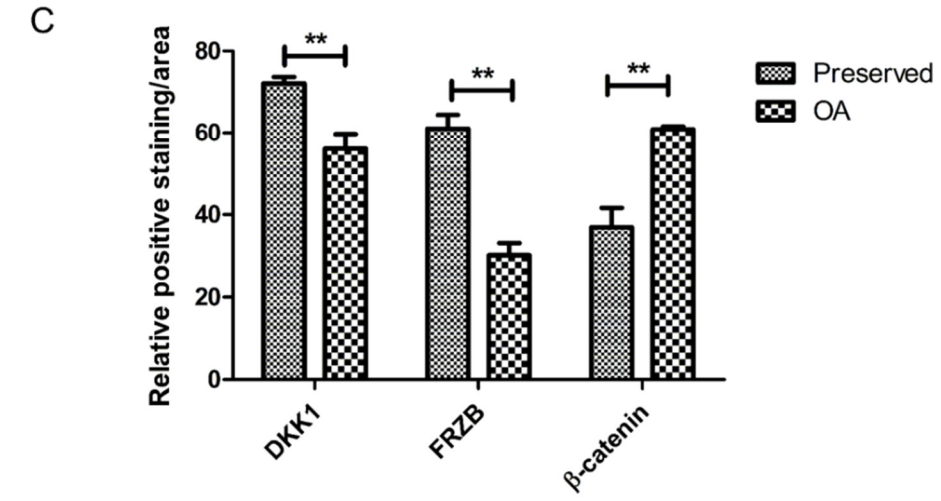
C
Relative positive staining/area
80
60
40
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**
**
**
DKK1
FRZB
β-catenin
Preserved
OA

Figure 1. Gene and protein expression in preserved and OA cartilage. A. RT-qPCR was performed to assess gene expression. B. IHC was used to visualize protein expression. Representative pictures from one donor are shown. Images were taken using the Nanozoomer (scale bar 100μm), magnified pictures were indicated in inserts. C. Quantification of positive staining was performed by ImageJ software.

IL1β decreased DKK1 and FRZB expression in a time, but not dose, dependent manner

To validate that IL1β regulates DKK1 and FRZB expression in healthy hChs, we first measured the effect of IL1β on *DKK1* and *FRZB* mRNA expression by qPCR and the protein levels by ELISA. IL1β significantly decreased expression of DKK1 and FRZB (Figure 2 A, B and C). IF was used to examine the location and expression of DKK1 and FRZB in human chondrocytes. Chondrocytes in the control group demonstrated constitutive expression of DKK1 and FRZB in the cytoplasm and also in the nucleus. IL1β exposure significantly decreased DKK1 and FRZB expression, especially in the cytoplasm (Figure 2D, separate images of the DAPI and DKK1 or FRZB in Supplemental figure 2). In fact, IL1β exposure had a widespread effect on the expression of WNT related genes by increasing *FZD10*, *LEF1* and *TCF4* while downregulating the expression of the WNT4 and the WNT inhibitor WIF2 (Supplemental figure 3A). In addition, the effect of IL1β treatment on expression of cartilage markers, hypertrophic markers, and an apoptotic factor was measured by qPCR. IL1β treatment decreased ACAN and COL2A1 expression while it increased MMP3, BMP2, and FASL expression (Supplemental figure 3B).

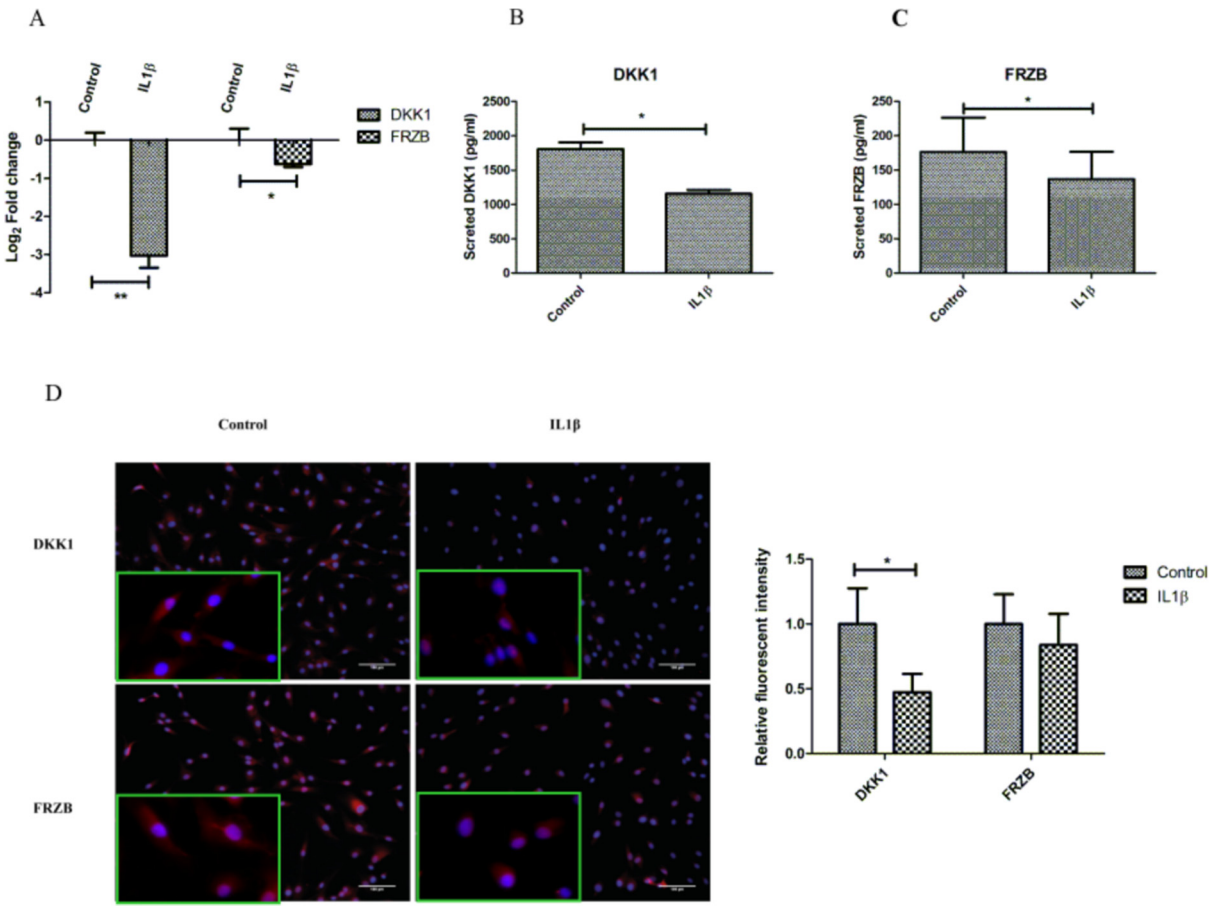

Figure 2. IL1β decreased expression of DKK1 and FRZB at mRNA and protein level. Human primary chondrocytes were treated with IL1β for 24 hours. A, B and C. DKK1 and FRZB gene and protein expression were measured by qPCR and ELISA respectively. D. The expression of DKK1 and FRZB was measured by IF. DKK1 and FRZB are illustrated in red (Alexa 546) and nuclei are in blue (scale bar 100μm), magnified pictures were indicated in inserts. Quantification of immunofluorescence intensity was performed by CellProfiler software.

Next, we explored if this IL1β regulation of DKK1 and FRZB was time and dose-dependent. We therefore performed a time-course experiment to examine the effects of IL1β treatment for up to 72 hours after stimulation. To ensure the efficacy the stimulation, we first measured IL16, IL1β and MMP3 expression, which are well-established target genes of IL1β. As expected, IL1β strongly induced the mRNA levels of all these target genes, which progressively increased until at least 72 hours after treatment (Supplemental figure 4A). The expression of DKK1 and FRZB in response to IL1β was time dependent, but not concentration dependent. DKK1 and FRZB mRNA expression started to decrease from 12 hours after stimulation and reached the lowest expression levels at 72 and 48 hours respectively (Figure 3A). The decrease in FRZB

mRNA level occurred more slowly. In line with the qPCR results, the secreted protein levels of DKK1 and FRZB were downregulated after IL1β stimulation (Figure 3B and C). When we measured the dose-dependent effects of IL1β on DKK1 and FRZB gene expression level after 12 hours using a range of 0.4 ng/ml to 50ng/ml, we revealed that IL1β treatment was already effective at the lowest concentration of 0.4 ng/ml. There was an increase of IL1β expression with increased concentration (Supplemental figure 4B). Exposure to IL1β at any concentration significantly downregulated DKK1 and FRZB expression as compared to the control. However, no significant difference was observed between any of the different IL1β concentrations (Figure 3D).

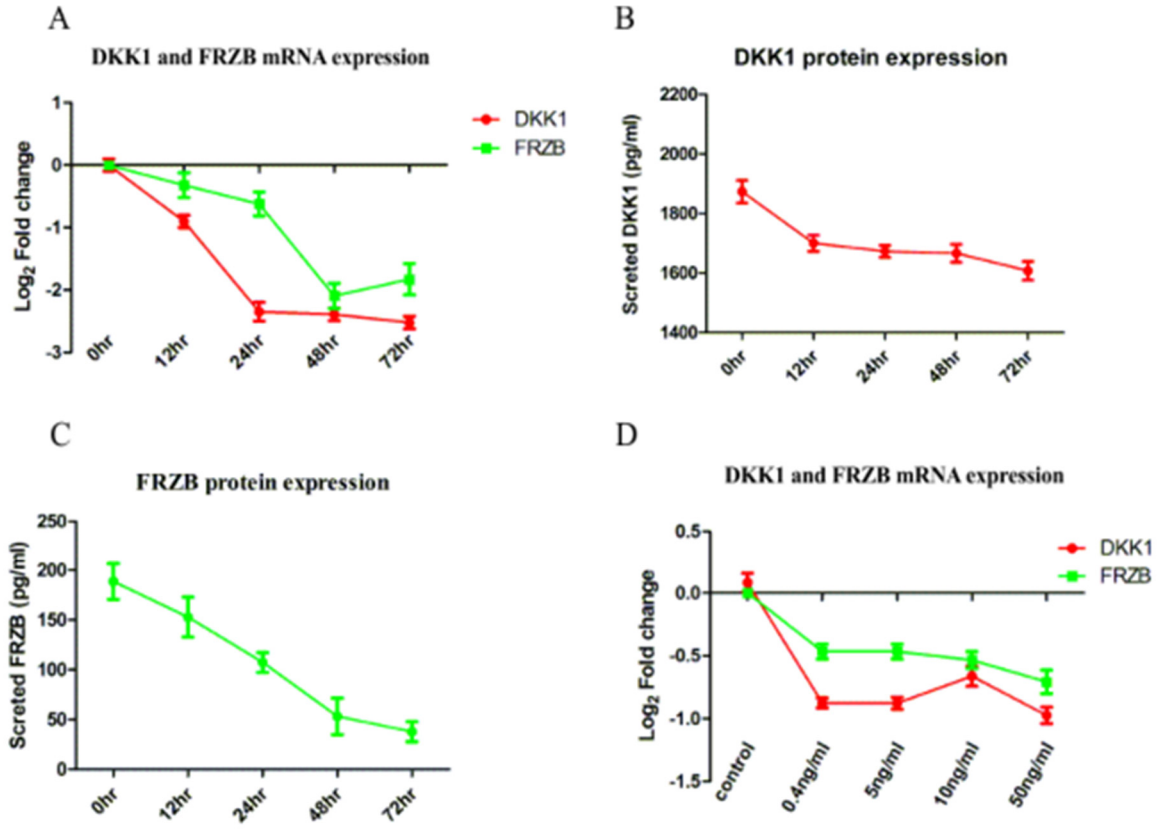

Figure 3. IL1β decreased DKK1 and FRZB expression is time dependent. A, B and C. Time course evaluation of DKK1 and FRZB expression after IL1β stimulation. Cells and cell medium were collected in indicated time point. qPCR and ELISA were used to measure mRNA expression and the concentration of secreted protein of DKK1 and FRZB in medium. D. Human chondrocytes were exposed to concentrations of 0.4ng/ml to 50ng/ml IL1β for 12 hours. The mRNA expression of DKK1 and FRZB was measured by qPCR.

IL1β decreased DKK1 and FRZB expression through upregulation of iNOS

To determine if IL1β decreased DKK1 and FRZB by upregulating iNOS, we measured iNOS expression and NO production 24 hours after IL1β treatment. iNOS expression at mRNA level was significantly induced by IL1β (Figure 4A). The concentration of the end product of iNOS, nitrite, was increased in cell medium as determined by a Griess assay (Figure 4B). Moreover, iNOS protein can hardly be detected in normal human chondrocytes while iNOS protein was strongly increased after IL1β stimulation as determined by IF and western blot, respectively (Figure 4C and D). In addition, IL1β almost linearly (R^2=0.9875) increased iNOS expression over 48 hours stimulation (Figure 4E).

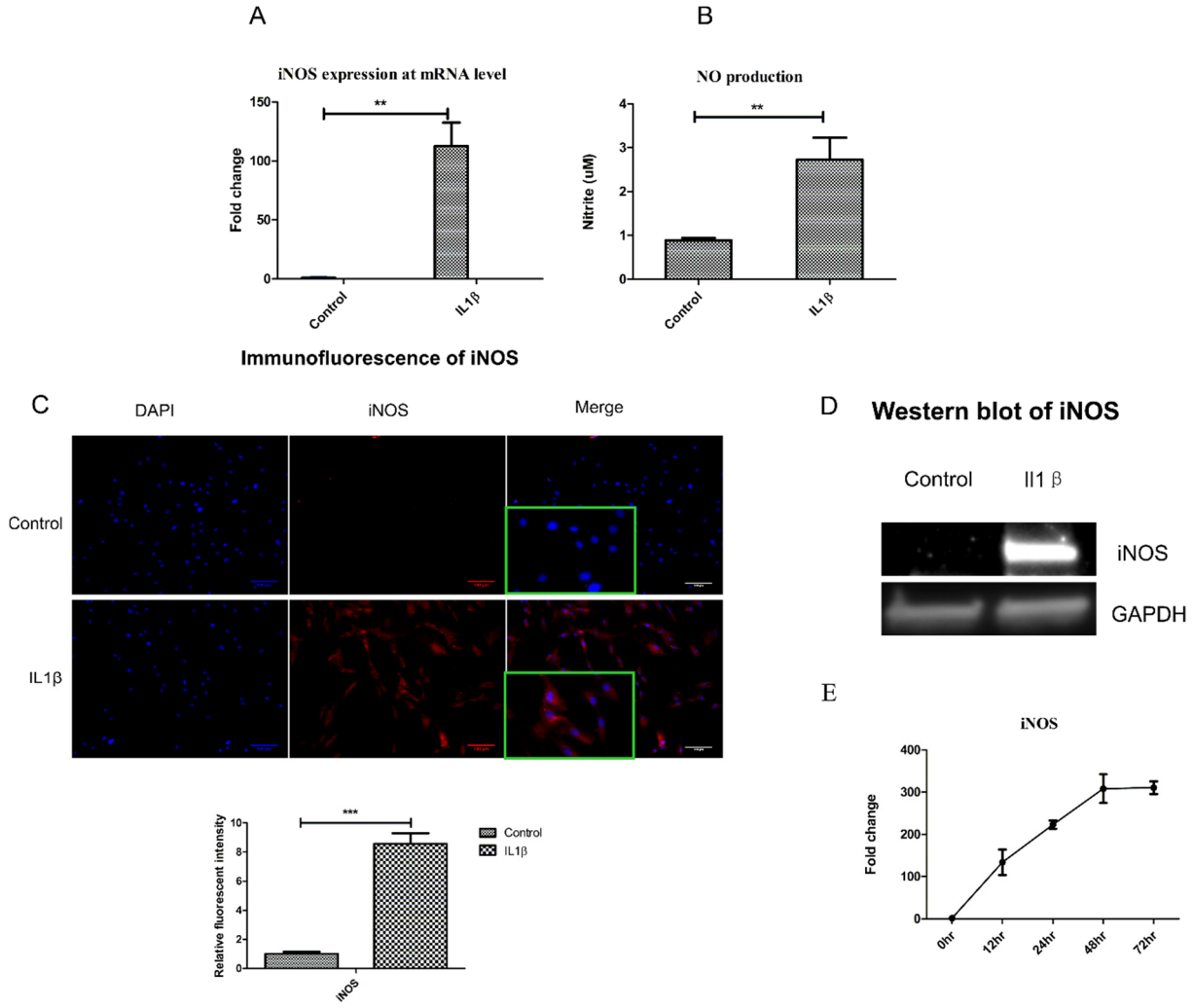

Figure 4. IL1β significantly induced iNOS expression at mRNA and protein level. A, B. human chondrocytes were treated with IL1β for 24 hours. Cells and cell medium were collected. iNOS mRNA expression was detected by qPCR and NO production was measured by Griess assay. C, D. IF was used

to measure iNOS expression, magnified pictures were indicated in inserts. Quantification of immunofluorescence intensity was performed by CellProfiler software. D. Western blot were used detect iNOS protein expression. E. Time course evaluation of iNOS mRNA expression after IL1β treatment.

Next, we test our hypothesis in human primary chondrocytes that blocking iNOS using iNOS inhibitor 1400W will be sufficient to block WNT signaling by recovery of DKK1 and FRZB expression. The iNOS-selective inhibitor 1400W was used to block iNOS-generated nitric oxide, as validated via Griess assay (Figure 5A). Blocking iNOS simultaneously rescued DKK1 and FRZB expression at mRNA and protein level as determined by qPCR (Figure 5B) and ELISA (Figure 5C), and corroborated by IF (Figure 5D, Supplemental figure 5).

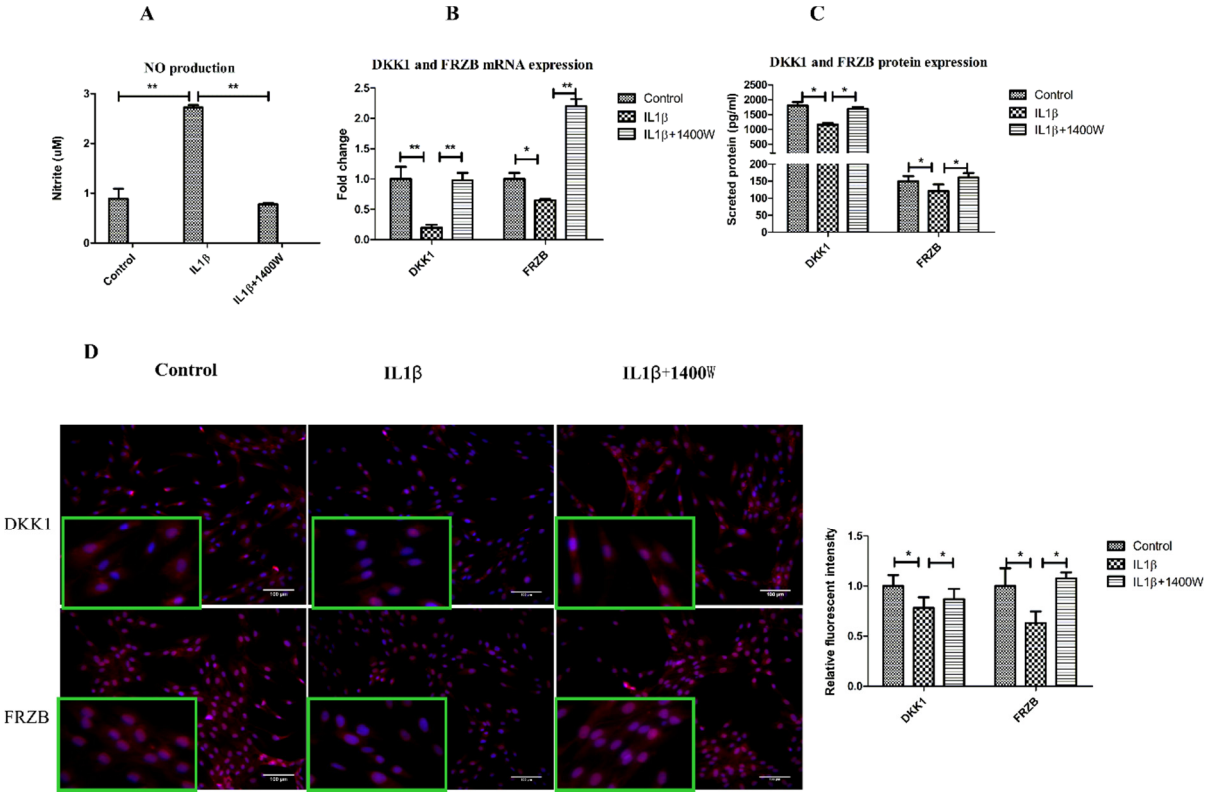

Figure 5. iNOS inhibitor 1400W blocked NO production and rescued the expression of DKK1 and FRZB. A. 1400W inhibited IL1β induced NO production, measured by Griess assay. B and C. The mRNA and protein expression of DKK1 and FRZB was rescued after adding of iNOS inhibitor,

measured by qPCR and ELISA D. The protein expression of DKK1 and FRZB was also measured by IF, magnified pictures were indicated in inserts. Quantification of immunofluorescence intensity was performed by CellProfiler software.

Blocking IL1β-induced iNOS decreased β-catenin expression

To determine the relationship between IL1β, iNOS, and WNT/β-catenin signaling, we measured β-catenin expression by Western blot and IF following exposure IL1β in the presence and absence of 1400W (Figure 6A). The control group showed low level expression of membrane bound cytosolic β-catenin. IL1β highly increased cytosolic expression and nuclear localization of β-catenin and blocking IL1β-induced iNOS by 1400W decreased β-catenin expression (Figure 6B, Supplemental figure 6). In addition, we found that 1400W inhibited IL1β-induced MMP-1, -3, and -13 expression and chondrocyte apoptosis (Figure 6C and D).

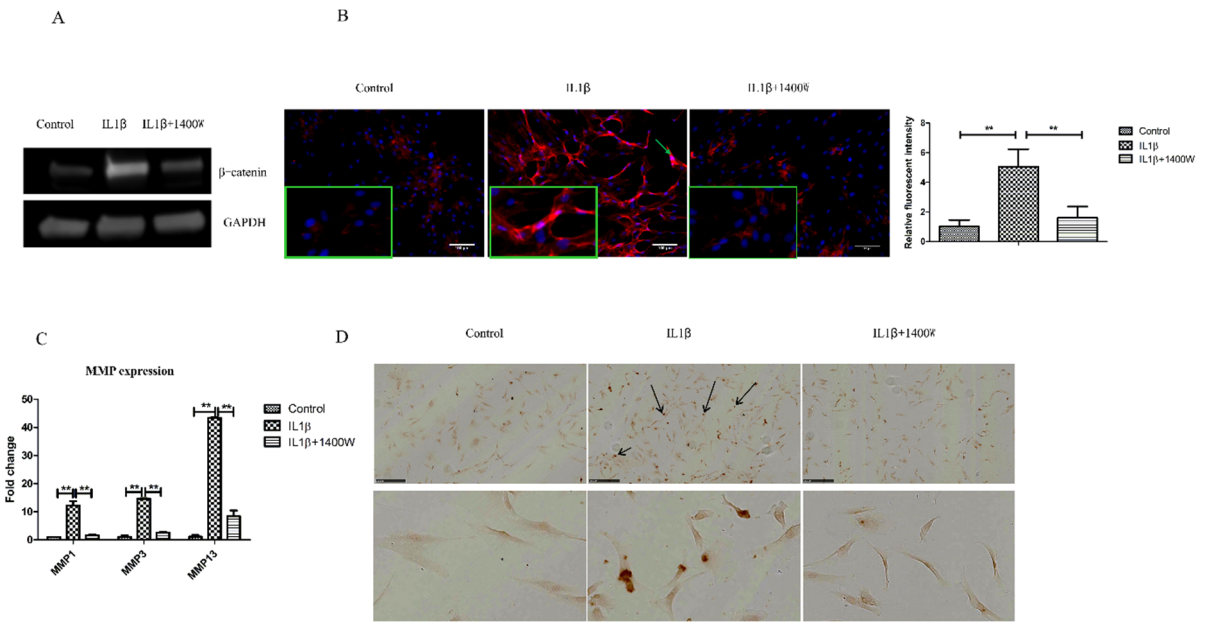

Figure 6. Blocking of iNOS decreased β-catenin, MMPs expression and inhibited apoptosis. A, B. Chondrocytes were treated by 10ng/ml recombinant human IL-1β or 100uM iNOS inhibitor 1400W for 24 hours. The protein expression of β-catenin was detected by Western blot and IF, green arrow indicated nuclear positive staining of β-catenin, magnified pictures were indicated in inserts. Quantification of immunofluorescence intensity was performed by CellProfiler software . C. MMPs mRNA expression was measured by qPCR. D. Apoptosis of human chondrocytes was detected using the DeadEnd colorimetric TUNEL assay. Apoptotic nuclei were stained dark brown (dark arrow).

Images were taken using Hamamatsu Nanozoomer. Scale bar= 250μm, top panel indicate overview of cell apoptosis, below panel indicates enlarged picture.

Whole genome gene expression analysis of IL1β treated chondrocytes

Our results showed that IL1β treatment of human chondrocytes mediated its effects not purely via the IL1β pathway, but also for an important part via crosstalk with the canonical WNT signaling pathway by influencing β-catenin expression. To identify whether exposure to IL1β changes other pathways as well, we performed global gene expression microarray analysis. For this, chondrocytes of preserved human cartilage were cultured in the presence or absence of 10 ng/ml of IL1β for 48 hours and subsequently analyzed using whole genome gene expression analysis. All genes with at least a two-fold change in expression are detailed in supplemental table 1. One hundred fifty genes showed over two-fold change after IL1β treatment. Functional annotation using ingenuity pathway analysis (IPA) showed that significantly changed genes were involved in multiple biological processes (Figure 7A). Notably, cellular growth and proliferation, cell death, cell morphology were significantly changed, which correlates to IL1β induced chondrocyte apoptosis [39]. Other genes related to cartilage biology, such as skeletal and muscular system development, connective tissue development and function and tissue development also showed a significant change. In addition, clustering of differentially expressed genes is listed in supplemental table 1.

Network analysis was performed to gain a system-level understanding of the changes in gene expression between the control and IL1β treated group. This analysis indicated the involvement of multiple interaction hubs (Figure 7B). Specifically, key upregulated pro-inflammatory factors or mediators or catabolic factors, such as IL8, IL16, NF-κB and MMPs, formed the hubs of the networks upon response to IL1β stimulation. This indicated that these factors cooperated with IL1β to play a role in chondrocytes. Importantly, signaling factors involved in cartilage matrix formation formed another cluster, represented by COL1A1, COL1A2, VCAN, BMP2 and COL5A1. This suggested that IL1β treatment has effects on cartilage matrix formation and production. Other factors, such as factors related to apoptosis and inflammation, were expressed, exemplified by members of the Interferon alpha-inducible protein (IFI) family and CXC chemokine family. No significant difference in expression of DKK1 and FRZB between the control and the IL1β treated group was found, probably due the low BeadChip sensitivity for both DKK1 and FRZB. These genes, therefore, did not meet the quality control cut-off.

Regardless, this whole genome gene expression provides global insight into the effects of IL1β on the chondrocyte gene expression landscape, which can be used as a blue print for future studies.

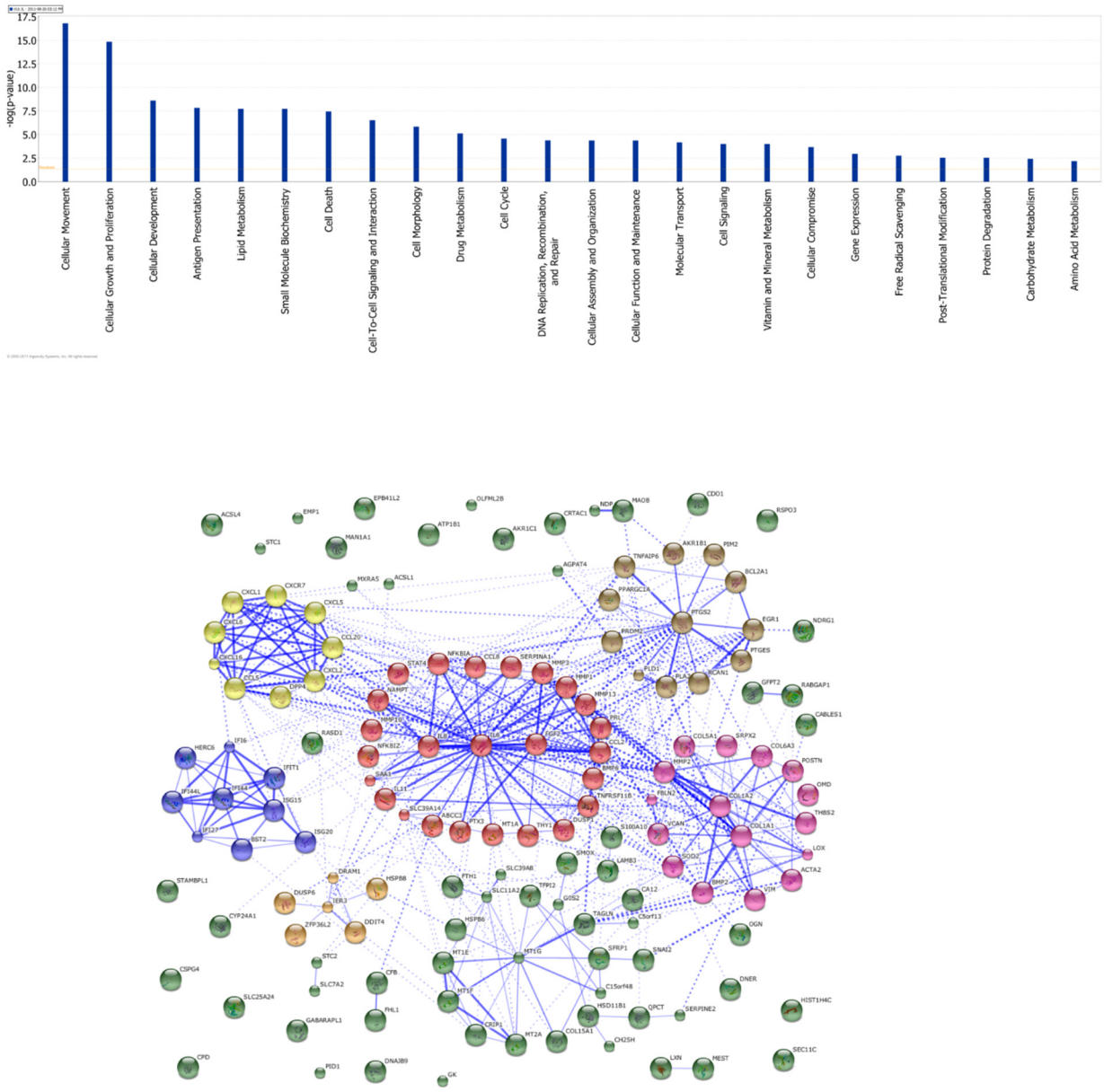

Figure 7. Whole genome gene expression analysis of IL1β treated human chondrocytes. A. Significantly changed biological processes upon IL1β stimulation according to ingenuity pathway analysis (IPA). B. Pathway networks were analyzed using significantly changed genes comparing IL1β treated group with control group.

Discussion

The major and novel findings in this study are: i) In paired samples of the same donor we show DKK1 and FRZB protein is highly expressed in the superficial layer of preserved cartilage while it is lost in OA cartilage. ii) IL1β induced iNOS expression in chondrocytes, which, as a mediator, simultaneously decreased endogenous DKK1 and FRZB expression, thereby activating WNT signaling in primary human chondrocytes. iii) Multiple signaling mediators were changed upon IL1β stimulation in human chondrocytes, especially signaling factors related to cartilage matrix production, cellular survival and migration, and lipid metabolism.

We first measured the expression of WNT antagonists, inflammatory factors and apoptosis in preserved and OA cartilage as paired samples from the same donor, for 11 donors. We found that the expression of DKK1 and FRZB was decreased while IL1β, iNOS and AXIN2 was increased in OA cartilage. This is in line with our and other previous work [11, 40, 18]. Furthermore, we observed that the protein expression of DKK1 and FRZB is highly present in relative healthy cartilage while the expression is lost in the paired OA cartilage. In contrast, β-catenin showed an opposite expression pattern with high expression in OA and low expression in the healthy sample. This indicates that diminished DKK1 and FRZB expression favors activation of canonical WNT signaling and consequently contributes to OA. Interestingly, in some patients, DKK1 positive staining was also observed in some cell clusters in the middle layer of OA cartilage. It has been reported that overexpression of cartilage markers such as SOX9, ACAN and COLII is observed in cell clusters in OA [41]. Given the anabolic role of DKK1 in cartilage homeostasis, it is not surprising that some repopulated cells produce DKK1 to antagonize WNT signaling in OA cartilage. However, this self-repair ability is overridden in OA progression.

IL1β is known as a non-specific activator of WNT signaling [42, 43]. A previous study from our group has shown that IL1β induces β-catenin accumulation and that this might be through inhibition of WNT inhibitors in human chondrocytes [13]. We have previously shown that TCF4, the downstream mediator of WNT/β-catenin signaling induces MMP expression by binding to p65-NFkB thereby activating NF-κB signaling in human chondrocytes [33]. We showed here that in human chondrocytes, IL1β significantly downregulated DKK1 and FRZB, and that this regulation is time dependent, but not dose dependent. In addition, IL1β induced expression of several WNT related gene such as FRZD10, LEF1 and TCF4 while expression of WNT inhibitor WIF2 was reduced. This matches with findings in a cancer cell line [36]. It has

been shown that inflammatory factors reduce cartilage proteoglycan synthesis [27] and induce chondrocyte hypertrophy [44-47]. We found that IL1β inhibited expression of the cartilage markers ACAN and COL2A1, while it induced expression of the hypertrophic markers COL10A1, MMP13 and BMP2. Given the role of DKK1 and FRZB in preventing hypertrophic differentiation in chondrocytes, it might be that IL1β induces chondrocytes to switch their stable articular phenotype to a hypertrophic state by decreasing DKK1 and FRZB expression.

It was shown in cancer cells that IL1β induced nitric oxide production and that this upregulated WNT/β-catenin signaling by inhibiting DKK1 [36]. We therefore studied if IL1β decreased DKK1 and FRZB expression through upregulating iNOS in human chondrocytes. iNOS is induced by both mechanical and biochemical factors, including inflammatory factors such as IL1β and TNFα [48]. We found that while iNOS can hardly be detected in normal cells, IL1β significantly upregulated iNOS while simultaneously decreasing the expression of DKK1 and FRZB. Blocking iNOS rescued the expression of both of DKK1 and FRZB and also inhibited IL1β-induced MMP expression and chondrocyte apoptosis. This suggests that the IL1β induced decrease in WNT antagonist expression directly results in apoptosis and in a β-catenin mediated induction of MMP expression mediated via iNOS expression. This matches with work by Pelletier et al in which another iNOS inhibitor, L-NIL, was shown to reduce MMP1 and MMP3 expression [29] and inhibits chondrocyte apoptosis in OA dogs [49].

Although both DKK1 and FRZB are antagonists of WNT signaling, they antagonize WNT signaling through different mechanisms. DKK1 binds to the Frizzled co-receptors low-density lipoprotein receptor–related protein 5 (LRP-5) and LRP-6, and thereby inhibits the canonical pathway [50, 51]. In contrast, FRZB antagonizes WNT signaling through interaction with WNTs and/or through the formation of non-functional complexes with the Frizzled receptors [52-54], which results in the inhibition of both canonical and noncanonical pathways [55]. It has been show that WNT signaling in chondrocytes occurs through both the canonical and noncanonical pathways, in which the canonical pathway regulates proliferation and the noncanonical pathway regulates differentiation [56]. In OA cartilage, chondrocytes not only show abnormal proliferation but also hypertrophic differentiation and dedifferentiation, which suggests that both canonical and noncanonical WNT pathways were activated due to the inhibition of DKK1 and FRZB expression. In this manuscript we focused on the crosstalk of the canonical WNT pathway and IL1β signaling. In the canonical pathway FRZB is able to compensate for the loss of DKK1 and vice versa. The functional redundancy of DKK1 and FRZB is also observed in knockout mouse models. For example, a FRZB knock out mouse does not show any cartilage phenotypic changes, but are more susceptible to OA after

mechanical injury [6]. DKK1 knockout mice shows no change in cartilage degeneration, only osteophyte formation is increased upon mechanical loading [6]. This explain our data that both DKK1 and FRZB were inhibited by IL1β to regulate β-catenin expression, through re-activation of the WNT signaling pathway.

A schematic representation of the relationship between IL1β, WNT antagonists, iNOS and WNT signaling is pictured in Figure 8. We propose that IL1β-induced iNOS contributes to OA by upregulating WNT signaling and other catabolic mechanisms and by simultaneously downregulating expression of WNT inhibitors and other anabolic factors. It is of note that other possible mechanisms might also contribute to the inhibition of DKK1 and FRZB. For example, our group has previously shown that abnormal mechanical loading and tonicity decreased DKK1 and FRZB expression in human chondrocytes [11]. It is also possible that NO directly influences other factors controlling DKK1 and FRZB expression [36].

In addition to regulating the WNT signaling pathway, global gene expression analysis has revealed the effects of IL1β on the chondrocyte gene expression landscape. Such blue prints provide a road map for future studies that can help navigate potential crosstalk effects via IL1β and a secondary pathway. In fact, it is expected that IL1β signaling crosstalks with additional signaling pathways. Developing a comprehensive yet exhaustive understanding of the crosstalks between the various signaling mechanisms will provide unique insight in cartilage homeostasis and disease development, and is likely to prove valuable for drug development. Regardless, much additional research is needed to gain such a knowledge framework.

Although we have revealed that IL1β interacts with WNT/β-catenin signaling via iNOS mediated downregulation of DKK1 and FRZB, the exact molecular mechanism by which iNOS decreases the expression of these WNT inhibitors is still not known. However, the DKK1 promoter contains NF-κB response elements, and NO has been shown to inhibit NF-κB DNA-binding activity [57, 58]. In addition, it has been shown that iNOS is a target of the WNT signaling pathway [37], suggesting there might be a positive feedback by which the activation of WNT signaling due to loss of its antagonists increases iNOS expression, which further decreases DKK1 and FRZB expression.

In conclusion, our data identify a mechanism by which IL1β-induced iNOS mediated catabolic activities including: upregulation of WNT signaling by decreasing its antagonists, induction of MMP expression, inhibition of the synthesis of collagen II and proteoglycans, mediation of chondrocyte apoptosis. This suggests a pivotal role of iNOS in the inflammatory response of human OA. These findings provide evidence of how IL1β contributes to OA

through regulating iNOS expression. Blocking NO production using pharmacological methods may inhibit the loss of the articular phenotype in OA by preventing downregulation of the expression of DKK1 and FRZB.

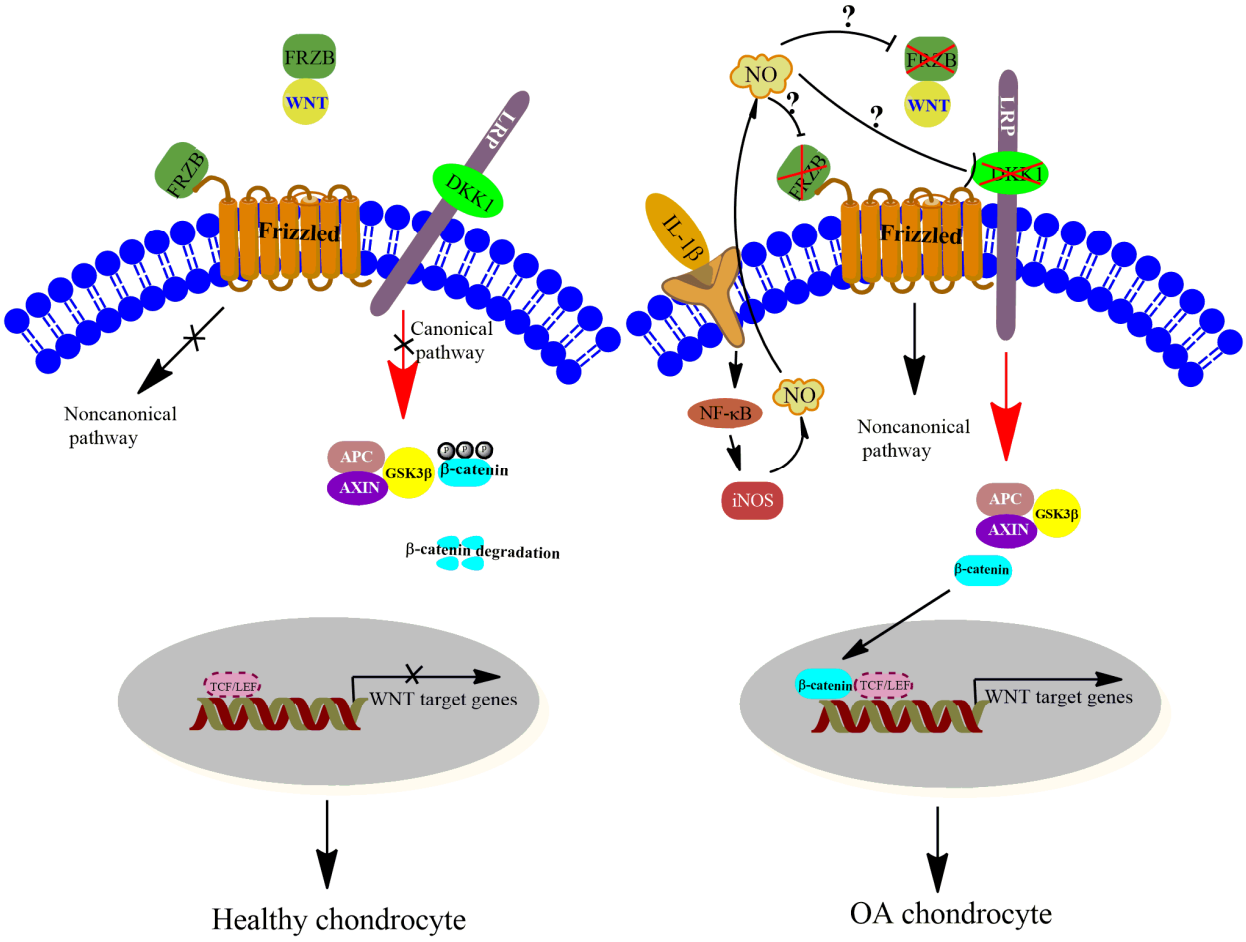

Figure 8. iNOS contribute to the pathogenesis of OA by decreasing DKK1 and FRZB. A. In normal condition, the presence of WNT antagonists DKK1 and FRZB keep β-catenin at proper level in chondrocytes, thus maintain joint homeostasis. B. In abnormal circumstances, chondrocytes were exposed in inflammatory environment, such as IL1β, which induces NO production. The presence of NO induces chondrocyte apoptosis and blocks expression of DKK1 and FRZB, results in unleash of β-catenin. It remains unclear how dose NO inhibits expression of DKK1 and FRZB. Overexpression of β-catenin induces chondrocyte dedifferentiation and hypertrophic differentiation consequently contributing to OA.

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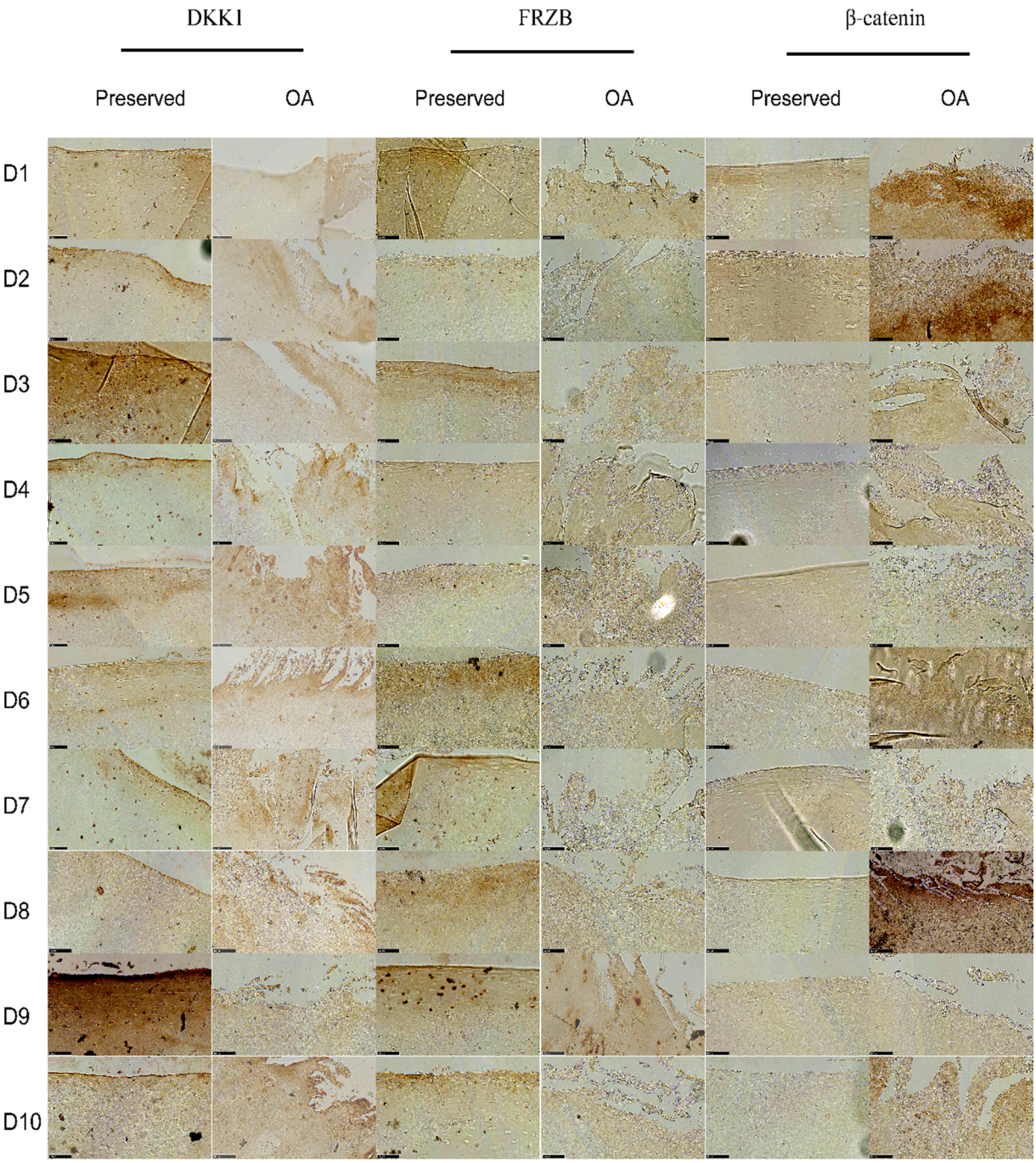
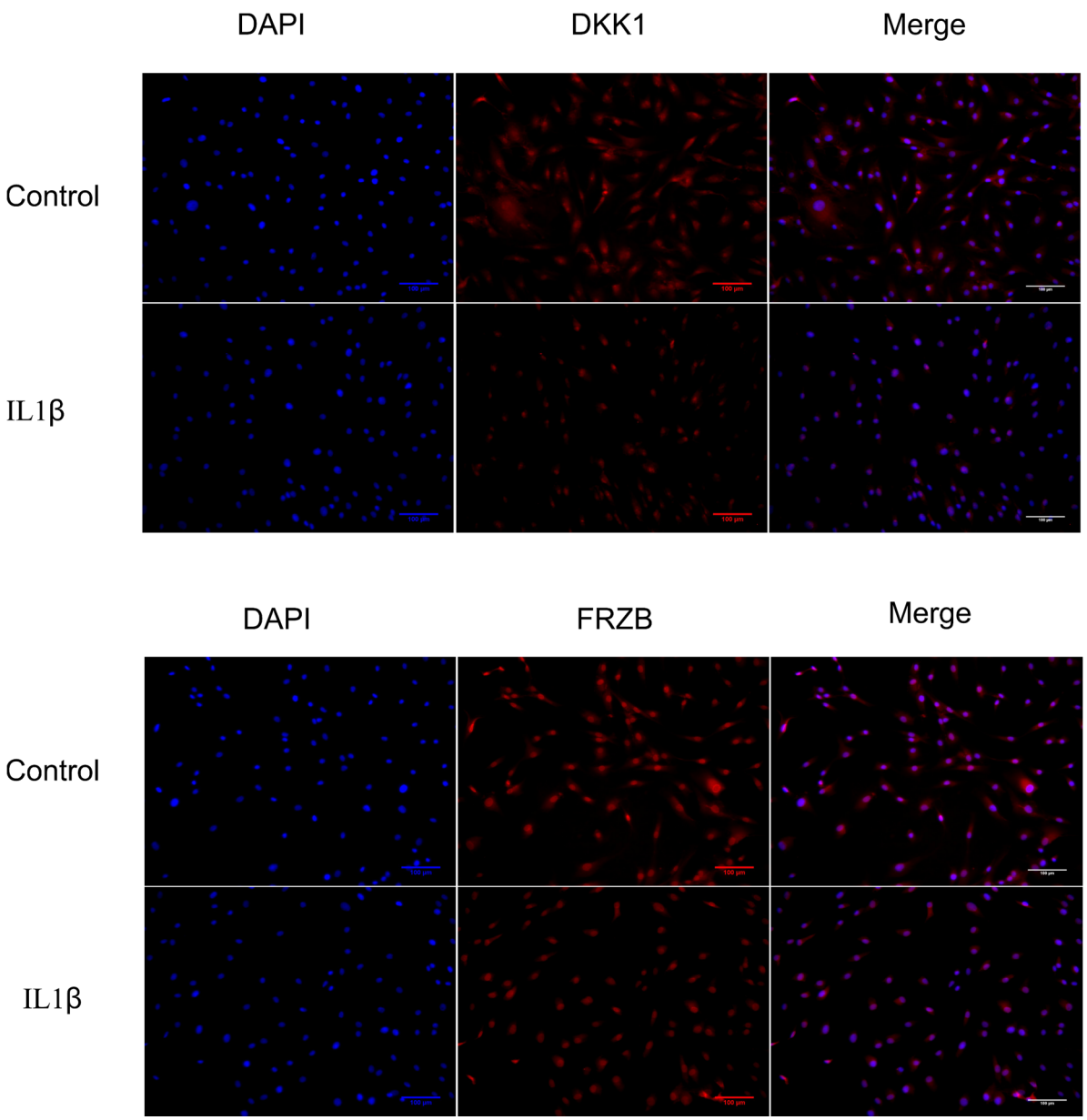
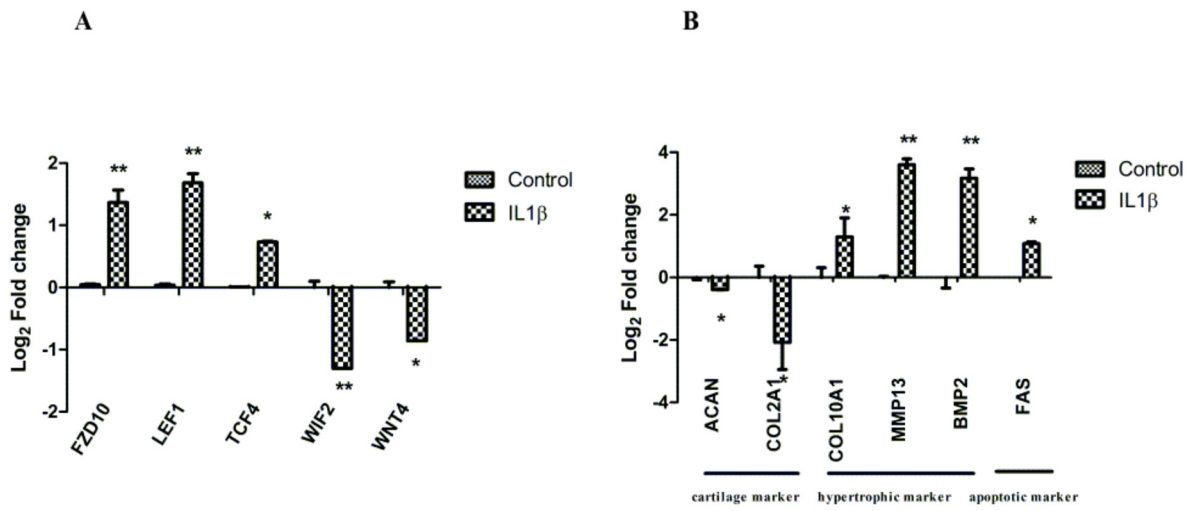
Supplemental data



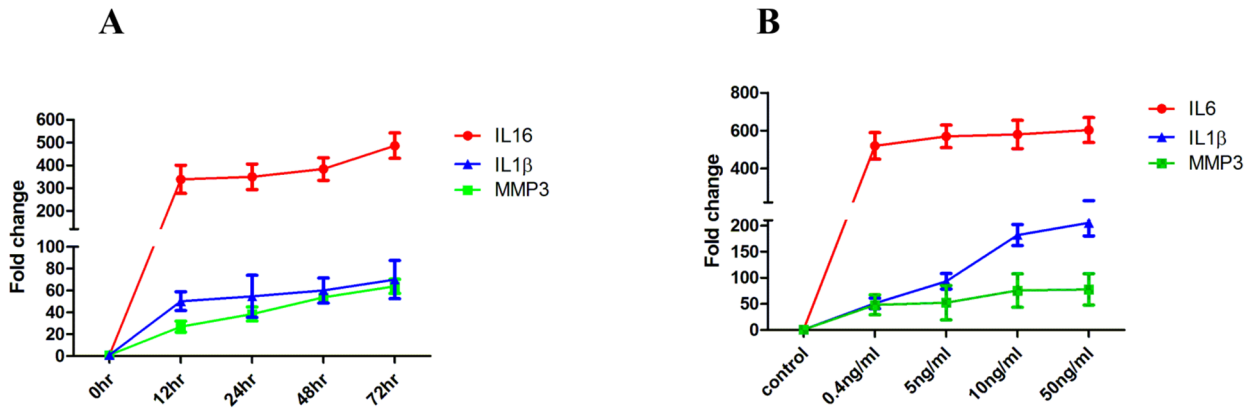
Supplemental figure 1. The protein expression of DKK1, FRZB and β-catenin was detected by IHC in each donor. Images were taken using the Nanozoomer (scale bar 100µm). D1-D10=Donor 1-10.



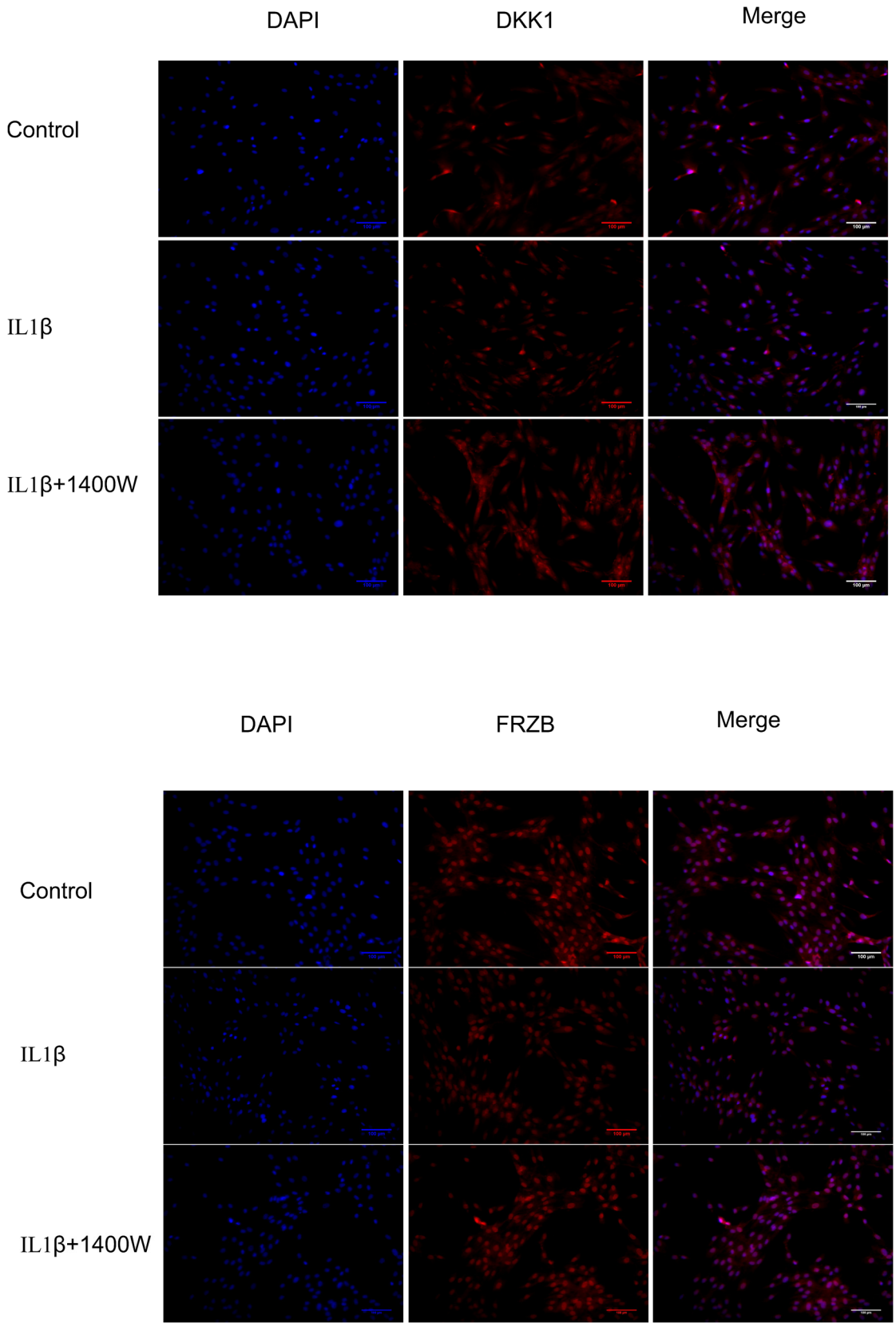
Supplemental figure 2. **The effects of IL1β on DKK1 and FRZB were measured by IF**. DKK1 and FRZB are illustrated in red (Alexa 546) and nuclei are in blue (DAPI).



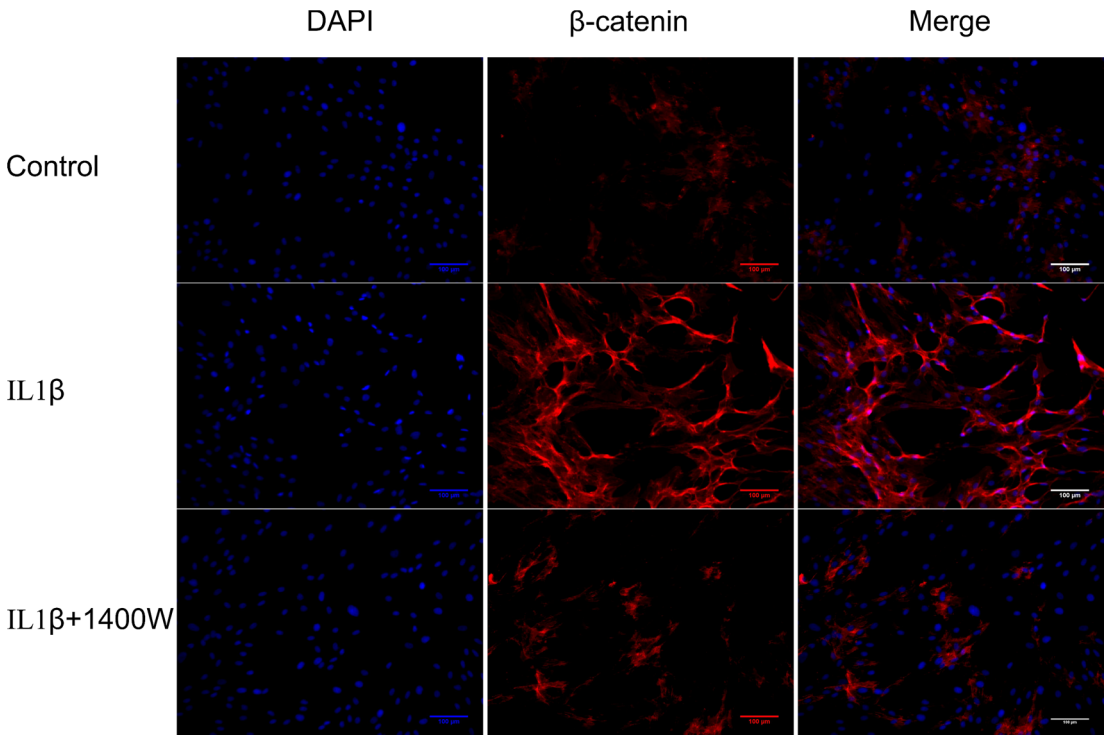
Supplemental figure 3. The expression of Cartilage and WNT related genes was measured by qPCR after IL1β treatment. A. IL1β decreased expression of cartilage markers ACAN and COL2A1 while increased hypertrophic and apoptotic markers. B. WNT receptor FRZD10 and transcription factors TCF4 and LEF1 were induced by IL1β. WNT inhibitor WIF2, WNT4 expression was decreased upon IL1β stimulation.



Supplemental figure 4. The expression of IL1β target genes was measured at indicated time and dose points. Human chondrocytes was collected for RNA isolation in indicated time and dose point after IL1β treatment. qPCR was used to measure the expression of IL1β target gene IL16, IL1β and MMP3.



Supplemental figure 5. **The effects of IL1β and iNOS inhibitor on DKK1 and FRZB** were measured by IF. DKK1 and FRZB are illustrated in red (Alexa 546) and nuclei are in blue (DAPI).



Supplemental figure 6. **The effects of IL1β and iNOS inhibitor on β-catenin were measured by IF**. β-catenin are illustrated in red (Alexa 546) and nuclei are in blue (DAPI).

Gene	Fold change
IL8	-30,1827
CXCL6	-23,5713
IL6	-21,8063
MMP3	-19,6971
PTGS2	-13,2632
CXCL1	-12,8736
C15orf48	-6,6582
MMP1	-6,45924

TNFAIP6	-6,3038
CXCL5	-6,28527
NDP	-5,9162
DNER	-5,72511
CFB	-5,50234
TNFRSF11B	-5,47381
STC1	-5,42969
PID1	-5,38972
IFI27	-5,05603
PTGES	-5,01198
TFPI2	-4,94264
NAMPT/PBEF1	-4,77533
TMEM158	-4,77358
G0S2	-4,52263
MT1F	-4,43741
IER3	-4,41128
SLC39A8	-4,34507
MT1A	-4,13811
MT2A	-4,09345
BCL2A1	-4,09216
SLC7A2	-4,0149
SOD2	-3,99437
IL11	-3,94944
CCL20	-3,86043
HSD11B1	-3,83716

MT1G	-3,81688
ACSL4	-3,79873
PLD1	-3,58006
LOC100129681	-3,57193
CXCL2	-3,54468
STAMBPL1	-3,52679
LAMB3	-3,47943
PPARGC1A	-3,47907
FTH1	-3,41364
ISG15	-3,37738
MTE	-3,34765
CA12	-3,32187
IFIT1	-3,31601
FGF2	-3,25836
DPP4	-3,25504
DUSP1	-3,16691
IFI6	-3,06267
CRTAC1	-3,05287
SLC39A14	-3,03126
GFPT2	-2,98759
DDIT4	-2,96941
CDO1	-2,92936
CYP24A1	-2,92598
CH25H	-2,89888

RCAN1	-2,78107
CXCL16	-2,77778
HERC6	-2,72143
MAOB	-2,69816
PLA2G4A	-2,66564
SLC11A2	-2,63407
ABCC3	-2,61275
NFKBIZ	-2,60965
AKR1C1	-2,60606
MT1E	-2,60594
AKR1B1	-2,60117
LOC283050	-2,56608
BMP2	-2,56008
STAT4	-2,55203
BST2	-2,54564
SLC25A24	-2,51723
LOC441019	-2,51257
CCL2	-2,49939
PRL	-2,47894
SERPINE2	-2,45862
ATP1B1	-2,45123
SAA1	-2,43452
RASD1	-2,4257
CCL5	-2,40784
GK	-2,40533

SEC11C	-2,40515
DNAJB9	-2,36376
DRAM1	-2,36198
IFI44L	-2,33768
LOC654103	-2,33707
MMP13	-2,32431
ACSL1	-2,31724
IFI44	-2,30451
MMP10	-2,29648
SERPINA1	-2,28294
BMP6	-2,27756
HSPB8	-2,25512
MMP2	-2,24835
QPCT	-2,2368
GABARAPL1	-2,21755
PIM2	-2,21302
LOC653778	-2,18883
STC2	-2,18008
DUSP6	-2,12969
SMOX	-2,11453
MAN1A1	-2,104
NFKBIA	-2,10167
CABLES1	-2,09236
CCL8	-2,08395

RSPO3	-2,07804
AGPAT4	-2,07781
ISG20	-2,07358
CPD	-2,04299
SFRP1	-2,03265
NDRG1	-2,02785
CXCR7	-2,02669
SRPX2	-2,02351
LOC645638	-2,00014
TAGLN	2,01235
PTX3	2,022333
FBLN2	2,023117
LXN	2,039604
EGR1	2,040302
OMD	2,042948
CSPG4	2,050275
CRIP1	2,074152
EMP1	2,086073
RABGAP1	2,133587
HIST1H4C	2,136093
SNAI2	2,250008
EPB41L2	2,264162
COL1A1	2,288933
OGN	2,290665
ACTA2	2,29596

COL15A1	2,297734
COL1A2	2,314732
COL6A3	2,326305
FHL1	2,327872
OLFML2B	2,575337
THBS2	2,617987
COL5A1	2,630744
VCAN	2,646043
HSPB6	2,665845
VIM	2,721598
MXRA5	2,731083
LOX	2,764629
ZFP36L2	2,821675
LOC100129667	3,094821
S100A10	3,11978
THY1	3,227556
MEST	3,390932
C5orf13	3,601008
POSTN	4,393478

Supplemental table 1. Significantly changed genes were listed in table. Fold change indicated control compared with IL1β group, thus a negative value was more expressed in the presence of IL1β.

Chapter 8

General Discussion and outlook

A good beginning makes a good ending (善始者善终)

WNT antagonists DKK1 and FRZB maintain healthy cartilage

Previously, we have identified DKK1, FRZB, and GREM1, naturally occurring antagonists of the WNT or BMP-signaling pathway, as key factors in controlling the articular cartilage phenotype by preventing hypertrophic differentiation [1]. We hypothesize that DKK1 and FRZB are the gatekeepers for the maintenance of homeostasis in articular cartilage and that when their expression changes in joint disorders the consequences and mechanism behind this change is of the utmost importance. Therefore, in this thesis, we investigated the role of these factors in healthy and OA cartilage. We investigated how these factors change during joint disorders and the cause and effects of these changes in human articular cartilage. We tested our hypothesis in different human cell and tissue systems.

Inflammation is the initiator leading to loss of WNT antagonists, thereby indirectly activating WNT signaling

In this thesis we show that our hypothesis is proven. The chronology of our evidence can be best summarized as shown in Figure 1. OA can be the result of trauma in at least a subset of OA patients, After joint trauma inflammation is induced (chapter 4), at least temporarily. Because of expression of inflammatory factor, such as IL1β, the expression of WNT antagonists DKK1 and FRZB is inhibited (chapter 7), resulting in activation of WNT signaling (chapter 3, 5, 6 and 7), the high level of WNT signaling induces hypertrophic differentiation (chapter 3 and 6), consequently lead to OA (chapter 3).

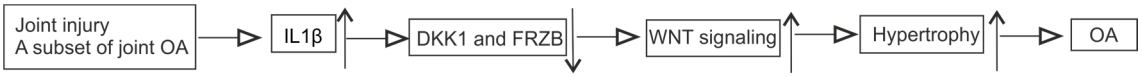


Figure 1. Schematics of the role of WNT signaling mediated by IL1β in human OA.

OA has long been considered as a non-inflammatory disease. Now accumulating evidence shows a strong implication of inflammation in the pathogenesis of OA [2]. Synovitis is inflammation of the synovial membrane and the presence of synovitis is observed in at least a subset of patients with primary OA [3]. In addition, inflammation also can be observed in joint fluids and tissues from patients with joint injury [4].

We observed that IL1β and iNOS are highly expressed in OA cartilage while expression of the WNT antagonists was lost as compared with paired preserved cartilage (Chapter 7). Based

on this observation, we studied the indirect interplay of IL1β/NO with WNT signaling pathway in human cartilage. It has been reported that iNOS decreases DKK1 expression in cancer cells [5]. We found that in human chondrocytes, IL1β downregulates expression of the WNT antagonists by upregulating iNOS, resulting in the increased activation of WNT/β-catenin signaling. In addition, a negative correlation between the expression level of IL1β and WNT antagonists was observed in human OA synovial fluid (manuscript in preparation, not included in this thesis). Moreover, we found that IL1β not only decreased the expression of WNT antagonists, it also downregulated expression of cartilage markers and simultaneously upregulated catabolic factors. All these events disrupt the cartilage homeostasis, consequently leading to cartilage degeneration.

NF-κB signaling has long been considered a classical proinflammatory signaling pathway [6]. It is activated by proinflammatory cytokines such as IL1β and TNFα and mediates inducing proinflammatory genes. Our study on the role of TCF4 transcription factors, the downstream targets of WNT/β-catenin signaling in human chondrocytes, showed that TCF4 is a pro-catabolic and apoptotic factor by potentiating NF-κB signaling in human chondrocytes, and that this effect is likely independent of β-catenin (Chapter 5). Moreover, high expression level of TCF4 mRNA was observed in human OA cartilage suggesting that TCF4 might be a pathogenic factor in human cartilage degeneration by potentiating NF-κB signaling. The interaction between TCF4 and NF-κB further proves the interplay between WNT and IL1β signaling.

We found that the expression of WNT antagonists DKK1 and FRZB was lost during OA and joint injury progression (Chapter 3 and 4). As describe above, chronic inflammation can be initiated in joint trauma and OA, at least in a subset of OA patients. It is therefore not a surprise that the loss of DKK1 and FRZB was caused by the presence of IL1β in joint injury. Given the inhibitory role of DKK1 and FRZB on WNT signaling, loss of their expression will unleash WNT/β-catenin signaling. In human OA and in injured cartilage, increased levels of WNT signaling results in loss of the chondrocyte phenotype and premature hypertrophic differentiation [7-9].

In chapter 6, we investigated the combined role of endogenously expressed WNT antagonists DKK1 and FRZB on the chondrogenic potential and on preventing cell hypertrophy by using neutralizing antibodies. We found that endogenously produced WNT antagonists DKK1 and FRZB is sufficient in repressing WNT signaling and to thereby inhibit dedifferentiation, initiate chondrogenesis and inhibit hypertrophic differentiation. We further explored this study in cartilage explants at tissue level (data not included in this thesis). Blocking of DKK1 and FRZB induced cell clusters and increased OA severity. The formation of cell clusters is a histological

hallmark of OA cartilage [10]. Our data is preliminary due to the limited donors used, however, others have demonstrated that WNT signaling induces cell proliferation in cartilage / chondrocytes. For example, WNT3a induces human chondrocyte proliferation though the β-catenin dependent canonical pathway [11]. In addition, BMP2 promotes rat chondrocyte proliferation by WNT/β-catenin signaling [12]. Hence, blocking DKK1 and FRZB released the break on WNT/β-catenin signaling, thereby inducing chondrocyte proliferation. These cells appear to be involved in an attempt at repair, but probably contribute to further cartilage damage by producing MMPs [13].

In this thesis, we proved the protective role of DKK1 and FRZB on cartilage since blocking with VHHs lead to chondrocyte dedifferentiation, inhibition of chondrogenesis and induction of hypertrophy of MSCs. In addition, blocking of DKK1 and FRZB also increased OA severity in OA cartilage explants. This is in line with several animal studies. For example, overexpression of WNT inhibitor DKK1 in mouse inhibits destabilization of the medical meniscus (DMM)-induced OA cartilage destruction and MMP13 expression [14]. Furthermore, in transgenic mice overexpressing DKK1, DKK1 ameliorates OA by inhibiting chondrocyte hypertrophy and VEGF secretion [15]. Similar patterns are seen for FRZB. Although FRZB knockout mice show no phenotypical changes during normal development [16], increased WNT signaling and increased expression and activity of MMPs are observed in response to instability, enzymatic injury, or inflammation [16]. Our group has previously shown that abnormal mechanical loading and tonicity decreases DKK1 and FRZB expression in human chondrocytes [17]. In this thesis, we also shown that inflammatory factor IL1β inhibited the expression of DKK1 and FRZB (chapter 7). All this evidence indicates that mechanical destabilization and inflammation might predispose cartilage to destruction by decreasing DKK1 and FRZB.

Although we have measured expression of GREM1 in human cartilage and synovial fluid, we did not perform GREM1 functional studies since no neutralizing antibodies were available at the time of these studies. We did however see that GREM1 expression increased with OA progression in human cartilage (chapter 3). The concentration GREM1 in SF was increased in recent injury compared to the old injury and reference group (chapter 4). In future studies, we plan to focus on functional mechanisms of regulation of GREM1 expression and activity.

In this thesis we use *in vitro* experiments to show the role of WNT antagonists DKK1 and FRZB in loss of cartilage tissue homeostasis. However, to provide strong functional evidence about the role of main players in joint, loss/gain of function experiment *in vivo* studies are

extremely important. A start in the right direction is the use of human cartilage explants, which we are currently testing for the importance of DKK1 and FRZB expression in homeostasis.

Future perspectives

Osteoarthritis (OA) is a degenerative joint disease with gradual loss of cartilage and joint function and also is most often not diagnosed in earlier stages due to the absence of obvious clinical symptoms and proper biomarkers. Cartilage has no blood vessels and nerves. Nutrients for chondrocytes are transported from the synovial fluid. Most recently, biomarker measurements in body fluids like urine, serum and synovial fluid show promising for diagnosis of OA. Biomarker measurements in synovial fluid may be better predictive for the health status of a joint than measurements in serum or urine due to the dilution effect of biomarkers in these bodily fluids. Here we propose that DKK1, FRZB and GREM1 play and important role in maintaining cartilage homeostasis and that they could be used as biomarkers for diagnosing posttraumatic joint diseases. The effectiveness and accuracy of these three factors as biomarkers need to be further verified in the future.

Although a big leap has been made for understanding of pathophysiology of OA, there are still limited treatment options. Most of OA studies are performed in animal models. However, how accurately the animal OA model represents human OA is still largely unknown. In addition, there are some restrictions of using animal models, including the variability of development and symptoms of OA in different species. This makes the translation of the results from animal to human more difficult and risky. Research should therefore be focused on human chondrocytes, human cartilage and/or human synovial fluid instead of animal models for better understanding of the role of the WNT signaling pathway in human cartilage disease.

This thesis provides more fundamental evidence on how pathogenic factors change during OA progression and the role of the main factors such as WNT, WNT antagonists and IL1β in human samples. Combined with the knowledge presented in this thesis, the IL1β and WNT pathways seem like attractive therapeutic targets. However, direct targeting of WNT signaling is assumed to be too risky, since its importance for the maintenance of stability of articular cartilage and its proven role in carcinogenesis [18]. A more feasible and safer strategy might be identifying the mechanisms that lead to dysregulation of WNT signaling in OA. In this thesis we show that increased expression of IL1β may be an initiator of at least the subset of OA patients that suffered from earlier joint trauma. Thus, anti-inflammatory interventions or

disturbing the interaction between IL1β and iNOS to restore expression of WNT antagonists, such as DKK1 and FRZB, might be a more effective strategy than only targeting WNT signaling for the treatment of degenerative cartilage diseases.

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Summary

Signaling of the secreted wingless-type MMTV integration site (WNT) has been implicated as a driving factor in osteoarthritis (OA). The induction of chondrocyte hypertrophy is a role attributed to WNT in OA, which may explain why chondrocytes in articular cartilage express high levels of WNT antagonists to modulate the activity of WNT signaling. Dickkopf 1 homolog (DKK1) and frizzled-related protein (FRZB) are naturally occurring antagonists of the WNT signaling pathway. Previously, we identified DKK1 and FRZB as key factors in controlling the articular chondrocyte phenotype by preventing endochondral ossification.

The aim of this thesis is to study the role of WNT signaling and its antagonists DKK1 and FRZB in human articular cartilage. In this thesis, we have provided answers for the following questions: i) how does expression of joint-related factors change in cartilage and synovial fluid during OA progression, with a focus on the changes in expression of WNT and WNT antagonists; ii) what is the fundamental role of WNT/β-catenin signaling and its antagonists (DKK1 and FRZB) in human chondrocytes and mesenchymal stem cells; iii) how are WNT antagonists regulated by the inflammatory factor IL1β; and iv) how can we use our knowledge to improve the understanding OA pathology and its treatment.

Chapter 2. We described the most recent literature, which focuses on the regulatory role of main signaling crosstalk in hypertrophy of MSCs and human articular chondrocytes.

Chapter 3. We showed that the cellular and molecular behavior of chondrocytes was changed during OA. There is a strong inverse correlation between the expression of DKK1, FRZB and the severity of OA on one hand and a positive correlation between RUNX2, IHH and OA severity on the other hand.

Chapter 4. We investigated the expression level of the natural antagonists (DKK1 and FRZB for WNT, GREM1 for BMP) and their correlation with other cartilage and bone turnover markers and pro-inflammatory cytokines in human synovial fluid (SF) collected from injured knees (acute and chronic), osteoarthritis (OA) and healthy subjects.

Chapter 5. We provided a detailed study of transcription factors of WNT/β-catenin signaling in human articular chondrocytes. In human chondrocytes TCF4 was found to increase MMP expression and chondrocyte apoptosis by directly interacting with NF-κB activating established NF-κB target genes such as MMPs and IL6. This effect of TCF4 is likely independent of β-catenin.

Chapter 6. We described that endogenous expression of the WNT antagonists DKK1 and FRZB is necessary for multiple steps during chondrogenesis: firstly DKK1 and FRZB are indispensable for the initial steps of chondrogenic differentiation of hMSCs, secondly they are

necessary for chondrocyte redifferentiation, and finally in preventing hypertrophic differentiation of articular chondrocytes.

Chapter 7. We revealed an unknown mechanism that IL1β decreased WNT antagonists expression level via upregulating iNOS and Nitric Oxide signaling in human chondrocytes. This indicates the loss of DKK1 and FRZB in OA cartilage is at least partially because of inflammatory factor IL1β.

Chapter 8. The main conclusions of this thesis based on overall results are described and an outlook for using our current knowledge to improve the understanding OA pathology and its treatment is provided.

Samenvatting

De signaaltransductie van het uitgescheiden wingless-type MMTC integration site (WNT) is geïmpliceerd als een drijvende factor in osteoartritis (OA). The inductie van hypertrofie in chondrocyten in OA wordt in verband gebracht met WNT, wat kan verklaren waarom chondrocyten in articulair kraakbeen hoge niveaus van WNT antagonisten uitscheiden om de activiteit van WNT signaaltransductie te moduleren. Dickkopf 1 homolog (DKK1) en frizzled-related protein (FRZB) zijn natuurlijk voorkomende antagonisten van de WNT signaaltransductie cascade. Voorheen hebben we DKK1 en FRZB geïdentificeerd als bepalende factoren in het in stand houden van het articulaire fenotype van chondrocyten door het voorkomen van endochondrale ossificatie.

Het doel van dit werk is om de rol van WNT signaaltransductie en zijn antagonisten DKK1 en FRZB te onderzoeken in menselijk articulair kraakbeen. In dit proefschrift hebben we de volgende vragen beantwoord: i) hoe veranderd de expressie van gewrichts-gerelateerde factoren in kraakbeen en synoviaal vocht gedurende de progressie van OA, met focus op de veranderingen in de expressie van WNT en WNT antagonisten; ii) wat is de fundamentele rol van WNT/β-catenine signaaltransductie en zijn antagonisten (DKK1 en FRZB) in menselijke chondrocyten en mesenchymale stamcellen (MSCs); iii) hoe zijn WNT antagonisten gereguleerd door de ontstekingsfactor IL-1β; en iv) hoe kunnen we onze kennis gebruiken om ons begrip van OA pathologie en de behandeling te verbeteren.

Hoofdstuk 2. We beschrijven de meest recente literatuur gefocust op de regulerende rol van de belangrijkste signaaltransductie crosstalk bij hypertrofie van MSCs en menselijke articulaire chondrocyten.

Hoofdstuk 3. We laten zien dat het cellulaire en moleculaire gedrag van chondrocyten veranderd gedurende OA. Er is een sterke inverse correlatie tussen de expressie van DKK1, FRZB en de intensiteit van OA enerzijds en een positieve correlatie tussen RUNX2, IHH en OA intensiteit anderzijds.

Hoofdstuk 4. We hebben onderzoek gedaan naar het expressie niveau van natuurlijke antagonisten (DKK1 en FRZB voor WNT, GREM1 voor BMP) in menselijk synoviaal vocht verkregen uit beschadigde knieën (acute en chronisch), OA patiënten en gezonde personen. Daarbij hebben we gekeken naar de correlatie tussen deze antagonisten met andere kraakbeen- en botontwikkeling markers, en pro-ontsteking cytokines.

Hoofdstuk 5. We beschrijven een gedetailleerd onderzoek naar transcriptie factoren van WNT/β-catenin signaaltransductie in menselijke articulaire chondrocyten. We hebben ontdekt dat in menselijke chondrocyten TCF4 leidt tot een verhoogde MMP expressie en apoptose van

chondrocyten door directe interactie met NF-κB activerende target genen zoals MMPs en IL-6. Dit effect van TCF4 is waarschijnlijk onafhankelijk van β-catenin.

Hoofdstuk 6. We beschrijven dat endogene expressie van de WNT antagonisten DKK1 en FRZB noodzakelijk is voor verscheidene stappen gedurende chondrogenese: ten eerste zijn DKK1 en FRZB essentieel voor de initiële stappen van chondrogene differentiatie van hMSCs, ten tweede zijn ze noodzakelijk voor re-differentiatie van chondrocyten, en als laatste zijn ze belangrijk in het voorkomen van hypertrofe differentiatie van articulaire chondrocyten.

Hoofdstuk 7. We onthullen een onbekend mechanisme waaruit blijk dat IL-1β de WNT-antagonist expressie verminderd door middel van opregulatie van iNOS en stikstofmonoxide (NO) signaaltransductie in menselijke chondrocyten. Dit geeft een indicatie dat het verlies van DKK1 en FRZB in OA kraakbeen op zijn minst voor een deel wordt veroorzaakt door de ontstekingsfactor IL-1β.

Hoofstuk 8. De belangrijkste conclusies van dit proefschrift, gebaseerd op een geheel van de resultaten, worden beschreven en een toekomstperspectief wordt geleverd voor het toepassen van de verkregen kennis voor het verbeteren van ons begrip van OA pathologie en zijn behandeling.

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Curriculum Vitae

Leilei Zhong was born on 20th of October 1987 in Shandong Province, China. In 2009, she received her Bachelor's degree in life sciences from Linyi University, Linyi, China. In September 2009 she started her Master program in cellular and molecular biology at Peking Union Medical College, Beijing, China. In 2012 she received her Master's degree and start her PhD project in Developmental BioEngineering group in University of Twente under supervision of Dr. Janine N. Post and Prof. Marcel Karperien. Her PhD research focused on the role of WNT antagonists in cartilage development and degeneration. In 2016, she completed her PhD thesis entitled " Joint players: endogenous WNT antagonists determine joint health".

List of publications

Publications related to this thesis:

Leilei Zhong, Xiaobin Huang, Marcel Karperien and Janine N. Post. The Regulatory Role of Signaling Crosstalk in Hypertrophy of MSCs and Human Articular Chondrocytes. Int. J. Mol. Sci. 2015;16(8):19225-47. doi:10.3390/ijms160819225.

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Ma B, **Zhong L**, van Blitterswijk CA, Post JN, Karperien M. T cell factor 4 is a pro-catabolic and apoptotic factor in human articular chondrocytes by potentiating nuclear factor κB signaling. J Biol Chem. 2013 Jun 14;288(24):17552-8

Leilei Zhong, Xiaobin Huang, Emilie Dooms Rodrigues, Jeroen C.H. Leijten, Theo Verrips, Mohamed El Khattabi, Marcel Karperien, and Janine N. Post. Endogenous DKK1 and FRZB regulate chondrogenesis and hypertrophy in 3D cultures of human chondrocytes and human mesenchymal stem cells. *Submitted.*

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Abstracts selected for poster presentations

Leilei Zhong, Xiaobin Huang, Emilie Dooms Rodrigues, Jeroen C.H. Leijten, Theo Verrips, Mohamed El Khattabi, Marcel Karperien, and Janine N. Post. Endogenous DKK1 and FRZB regulate chondrogenesis and hypertrophy in 3D cultures of human chondrocytes and human mesenchymal stem cells. World congress on osteoarthritis (OARSI), Amsterdam, the Netherlands, April 2016.

Leilei Zhong, Marcel Karperien and Janine N. Post. DKK1, FRZB and GREM1 prevent IL1β induced articular cartilage degradation. World congress on osteoarthritis (OARSI), Paris, France, April 2014.

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Bin Ma, **Leilei Zhong**, Janine N. Post, and Marcel Karperien. T cell factor 4 is a pro-catabolic and apoptotic factor in human articular chondrocytes by potentiating nuclear factor κB signaling. World congress on osteoarthritis (OARSI), Philadelphia, USA, April 2013.

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