Inhibition of Gsk3β in cartilage induces osteoarthritic features through activation of the canonical Wnt signaling pathway


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**SUMMARY**

**Objective:** In the past years, the canonical Wnt/β-catenin signaling pathway has emerged as a critical regulator of cartilage development and homeostasis. In this pathway, glycogen synthase kinase-3β (GSK3β) down-regulates transduction of the canonical Wnt signal by promoting degradation of β-catenin. In this study, we wanted to further investigate the role of Gsk3β in cartilage maintenance.

**Design:** Therefore, we have treated chondrocytes in vitro and in vivo with GIN, a selective GSK3β inhibitor.

**Results:** In E17.5 fetal mouse metatarsals, GIN treatment resulted in loss of expression of cartilage markers and decreased chondrocyte proliferation from day 1 onward. Late (3 days) effects of GIN include cartilage matrix degradation and increased apoptosis. Prolonged (7 days) GIN treatment resulted in resorption of the metaphysis. These changes were confirmed by microarray analysis showing a decrease in expression of typical chondrocyte markers and induction of expression of proteinases involved in cartilage matrix degradation. An intra-articular injection of GIN in rat knee joints induced nuclear accumulation of β-catenin in chondrocytes 72 h later. Three intra-articular GIN injections with a 2 days interval were associated with surface fibrillation, a decrease in glycosaminoglycan expression and chondrocyte hypocellularity 6 weeks later.

**Conclusions:** These results suggest that, by down-regulating β-catenin, Gsk3β preserves the chondrocytic phenotype, and is involved in maintenance of the cartilage extracellular matrix. Short-term β-catenin up-regulation in cartilage secondary to Gsk3β inhibition may be sufficient to induce osteoarthritic-like features in vivo.

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**Introduction**

Differentiated chondrocytes maintain their phenotype via synthesis of cartilage-specific extracellular matrix (ECM) molecules including collagen type II and sulfated proteoglycans, like aggrecan. Chondrocytes easily lose essential characteristics when they are removed from their natural environment and cultured in vitro or expanded for the purpose of cartilage tissue engineering.

Chondrocyte dedifferentiation also occurs in the presence of retinoic acid, nitric oxide, or proinflammatory cytokines like interleukin (IL)-1β and Tumor necrosis factor (TNF)-α, as well as in osteoarthritis (OA).

We and others have shown that both constitutive up- or down-regulation of the canonical Wnt pathway negatively influences cartilage development and maintenance resulting in OA-like features. This suggests that a tight regulation of this signaling cascade is crucial throughout the chondrocyte life cycle. In this pathway, in the absence of a Wnt signal, a destruction complex comprising Axin (Conductin) and Adenomatous polyposis coli (APC) mediates the phosphorylation of β-catenin by glycogen synthase kinase-3β (GSK3β), which induces degradation of cytosolic β-catenin in the proteosome. Binding of Wnt to its transmembrane receptor Frizzled results in activation of Dishevelled. This is followed by reduction of GSK3β activity and accumulation of cytoplasmic β-catenin. Upon its nuclear translocation, β-catenin will function as...
a co-factor of TCF/LEF transcription factors to induce expression of Wnt target genes. GSK3β is constitutively active and, unlike many kinases that are activated following stimulus-dependent phosphorylation, it becomes inactive following phosphorylation. Studies reported so far indicate that GSK3β activity is required for both chondrocyte and osteoblast differentiation and thus for endochondral bone development. However, no data is available regarding the role of GSK3β in maintenance of the chondrocytic phenotype.

To better understand the role of GSK3β in regulation of the chondrocyte life cycle, we inactivated this kinase ex vivo and in vivo by using 3-[9-Fluoro-2-(piperidine-1-carboxyl)-1,2,3,4-tetrahydro-[1,4] diazepino[6,7-hi]indol-7-yl]-4-imidazo[1,2-a]pyridin-3-yl-pyrrole-2,5-dione, a selective and potent GSK3β inhibitor, in this manuscript further referred to as GIN. Our results imply that GSK3β activity is crucial for maintenance of the chondrocytic phenotype and for the integrity of cartilage ECM, mainly by down-regulating the canonical Wnt signaling pathway. The cartilage phenotypic changes induced by GIN bear similarities to some of the clinical features commonly observed in OA.

Materials and methods

**KS483 cell culture, immunofluorescence for β-catenin, transient transfection assays**

Routine culture of KS483 cells, immunofluorescence for β-catenin and transient transfection assays were performed as previously described.

**Ex vivo experiments**

The three middle metatarsals were dissected from E17.5 Swiss Albino mouse embryos. Explants isolated from different animals were randomly distributed and individually cultured in 500 μl α-Minimum Essential Medium (MEM) (Invitrogen) medium containing 10% Fetal Calf Serum (FCS) (Invitrogen), 100 U Pen/Strep (Invitrogen) and 1% GlutaMax (Invitrogen). After an equilibration period of 48 h, metatarsals were challenged with vehicle or GIN as described in the results section. All ex vivo experiments were approved by the ethical committee of the Leiden University Medical Center and complied with national laws relating to the conduct of animal experiments.

**Proliferation and apoptosis assays**

Chondrocyte proliferation was assessed by immunohistochemistry (IHC) for proliferating cell nuclear antigen (PCNA) according to manufacturer's protocol (Santa Cruz Biotechnology). Chondrocyte apoptosis was determined by the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction (Promega), as apoptosis was determined by the Terminal deoxynucleotidyl trans-

**Histology, IHC, in situ hybridization (ISH)**

Histology, IHC, and ISH were performed as previously described.

**Quantification of Glicosaminoglycans (GAGs)**

The GAG content in N = 3 whole metatarsals per condition was quantified related to the amount of DNA using the Blyscan Sulfated GAG Assay kit (Biocolor) according to manufacturer’s protocol. Experiments were repeated at least once.

**Gene expression profiling**

For each condition, RNA was isolated from N = 15 whole metatarsals, checked for quality, amplified and labeled as previously described. Labeled cRNA was further used for the hybridization to Affymetrix GeneChip Mouse Genome 430A 2.0 Array according to the manufacturer’s protocol. The raw and normalized data are deposited on the website of the Department of Tissue Regeneration of the Twente University Institute for Biomedical Technology (http://tr.tnw.utwente.nl).

**Microarray data analysis**

To evaluate the large numbers of genes and to find gene expression trends and noteworthy signaling pathways that are involved in the GIN-mediated effects, we used principal component analysis (PCA). Using a cut-off value of 2 for the expression fold change, a list of 316 differentially expressed genes (225 down- and 91 up-regulated) was generated and used for subsequent analysis (Tables S1 and SII).

Functional annotation of the differentially expressed genes identified by the principal component analysis (PCA) analysis was performed using the DAVID bioinformatics database and the Gene Ontology (GO) terms to describe their (extra)cellular location (GO_CC), molecular functions (GO_MF), and the biological processes (GO_BP) in which they are involved. Enrichment of GO functional groups was determined to be meaningful when the number of probe sets in our list that mapped to a specific GO term was greater than 2 with a P-value ≤ 0.001.

Validation of the microarray analysis was performed by real-time quantitative polymerase chain reaction (PCR) as previously described.

**In vivo experiments**

All in vivo experiments were approved by the ethical committee of the Erasmus University Medical Center and complied with national laws relating to the conduct of animal experiments. Thirteen-week-old male Wistar rats (400–450 g) were housed under standard laboratory conditions (temperature 24°C, 12-h light–dark cycle) with food and water ad libitum. The animals were acclimatized to the laboratory environment for 3 weeks before the start of the experiments.

**GIN treatment**

In a dose-finding study (N = 4), the effect of an intra-articular injection of 100 μl GIN dissolved in phosphate buffered saline (PBS) at concentrations of 3 × 10⁻⁷ M, 3 × 10⁻⁵ M, 3 × 10⁻¹⁵ M, and 10⁻⁵ M in the knee joint was investigated.

In a second experiment, eight rats were injected intra-articularly at day 1, 3 and 5 with 100 μl 10⁻⁷ M GIN. Four rats were injected with GIN in the left knee, the remaining four were injected in the right knee. Contra-lateral joints served as controls and were injected with vehicle. All animals were scanned using contrast-enhanced microCT (CECT) before GIN injection (t = 0) and during follow up as previously described. Rats were sacrificed at the times indicated in the text.

**Microscopical analysis and quantification**

IHC for β-catenin coupled with Alcian Blue (AB) counterstaining for GAGs was carried out as previously described. Quantification of the AB staining was performed using Image-Pro Plus software, version 7.0.

**Statistical analysis**

All values represent median and range for experiments when N ≤ 4 and mean and 95% confidence interval (CI) when N ≥ 5. The paired t-test, the univariate general linear model using simple contrasts and parameter estimates and one-way analysis of variance (ANOVA) were used to assess the data, as appropriate. P values less than 0.05 were considered significant. Statistical analysis was performed using SPSS v16.0 (SPSS).
Results

Inhibition of Gsk3β through GIN results in activation of the canonical Wnt signaling pathway in vitro and ex vivo

We first performed transient transfection experiments in mesenchymal-like K5483 cells using the Wnt-responsive BAT-Luc reporter vector. As expected, GIN induced a dose-dependent increase in luciferase activity, with a maximum response at $10^{-7}$ M [Fig. 1(A)]. At higher concentrations, the luciferase activity decreased, presumably due to toxic effects [Fig. 1(A) and data not shown]. Furthermore, GIN was significantly more potent in inducing the Wnt reporter construct than LiCl, another established inhibitor of GSK3β. Activation of the Wnt reporter construct by $10^{-7}$ M GIN was accompanied by β-catenin accumulation and nuclear translocation as confirmed by immunofluorescence [Fig. 1(B)]. The overall level of β-catenin was notably increased in cells treated with GIN when compared to LiCl (50 mM) or Wnt3a (50 ng/ml) [Fig. 1(B)].

We next investigated the effect of GIN on fetal mouse metatarsals, which represent an established model for studying the chondrocyte life cycle ex vivo. After incubation for 3 days, GIN dose-dependently increased the levels of β-catenin as revealed by IHC [Fig. 1(C)]. Metatarsals treated with either $10^{-7}$ M or $10^{-6}$ M GIN displayed β-catenin staining mainly in the nuclei, indicating an efficient activation of the canonical Wnt signal. The latter concentration resulted in immunohistochemically detectable nuclear β-catenin expression in almost all chondrocytes. Strikingly, metatarsals treated with $10^{-6}$ M GIN displayed a much fainter AB counterstaining in comparison to controls, indicative for loss of GAGs. Additional morphological analysis revealed no evidence of cell death, cell shrinkage, picnotic nuclei, blebbing of the cytoplasm or necrosis. Subsequent experiments using the fetal mouse metatarsal ex vivo culture model were performed with $10^{-6}$ M GIN.

GIN inhibits chondrocyte proliferation and increases cartilage apoptosis

We assessed the effect of GIN on chondrocyte proliferation using IHC for PCNA. The percentage of proliferating cells was smaller in the GIN-treated group compared to controls at all time points examined [Fig. 2(A–E)]. Chondrocyte proliferation was significantly inhibited both at d1 (11.3% vs 25.9%, $P = 0.048$) and at d3 (7.6% vs 27.8%, $P = 0.014$). TUNEL staining in combination with histological evaluation was used to assess chondrocyte apoptosis. GIN did not have an effect on TUNEL positivity at 6h, d1 and d3 [Fig. 2(F–J)]. Only after prolonged GIN treatment (d7), TUNEL staining was significantly increased (22.9% vs 6.0%, $P = 0.043$). At d7, TUNEL-positive cells were predominantly identified among the hypertrophic chondrocytes in controls. In contrast, TUNEL-positive cells were also observed among the resting and proliferative chondrocytes in the GIN-treated group. Based on histological evaluation, TUNEL-positive cells underwent apoptosis.

Inhibition of Gsk3β induces degradation of cartilage matrix and loss of the chondrocytic phenotype ex vivo

We investigated the effect of GIN at the cellular level by IHC analysis for β-catenin, collagen type II and X, and ISH analysis for Col2a1 and Col10a1. No microscopical differences between vehicle- and GIN-treated metatarsals were observed at 6 h (data not

![Fig. 1](image-url)
shown). β-catenin expression at the start of the experiment and in the control metatarsals at d1, d3 and d7 was restricted to the cytoplasm of a minority of perichondrial and periostial cells [Fig. 3(A, Aii, Aiii, Aiv)]. We first noticed a clear increase in the level of nuclear β-catenin after 1 day of GIN treatment [Fig. 3(Aii)]. β-catenin accumulation was observed at d3 and d7 as well [Fig. 3(Aiv, Eiv)]. The level of nuclear β-catenin was inversely correlated with the intensity of the AB counterstaining [Fig. 3(A–Aiv)]. GIN treatment progressively decreased the GAG content in the ECM, with a near complete loss of GAGs at d7 [Fig. 3(F)]. GIN did not have an effect on mineral deposition and ossification of the metatarsals (data not shown).

Additional microscopical analysis revealed Col2a1 mRNA and collagen type II protein expression in the resting, proliferative and prehypertrophic chondrocytes, and their matrix, respectively, at all time points in the controls [Fig. 3(B, Bii, Biii, C, Cii, Ciii, Civ)]. GIN treatment for 1 day resulted in a considerable inhibition of the mRNA expression of this chondrocyte marker, whereas its protein expression was not changed [Fig. 3(Bii, Cii)]. At d3, most of the chondrocytes in the GIN-treated metatarsals, although surrounded by a matrix rich in collagen type II, failed to express Col2a1 [Fig. 3(Biii, Ciii)]. At d7, neither Col2a1 nor collagen type II expression was found in the GIN-treated metatarsals [Fig. 3(Biv, Civ)].

Furthermore, control metatarsals displayed Col10a1 mRNA and collagen type X protein expression in the hypertrophic zone [Fig. 3(D, Dii, Diii, Dv, E, Ev, Evii, Eviii)]. At d1, there were no differences in the expression of this mature chondrocyte marker between the control and the GIN-treated group, neither at the mRNA nor at the protein level [Fig. 3(Dii, Dv, Ev)]. At d3, GIN-treated explants displayed no Col10a1 expression, whereas collagen type X was still present in the ECM [Fig. 3(Diii, Evii)]. Ultimately, at d7, GIN induced a complete absence of both Col10a1 and collagen type X [Fig. 3(Dv, Eviii)].

### Microarray analysis confirms GIN’s proteolytic effects on cartilage

To further examine the effects of GIN on gene expression patterns in the femur mouse metatarsal model, we performed cDNA microarray analysis on mRNA isolated from GIN-treated and control explants at T0, 6h, d1 and d3. We particularly designed our microarray analysis as such since GIN-treated metatarsals at d7 showed only aggravated features of the ones observed at d3. Furthermore, the increased apoptosis at d7 would have jeopardized the specificity of the results and mainly revealed differentially expressed genes related to cell death, an indirect effect of GIN treatment.

According to GO_CC terms, the vast majority of the 316 differentially expressed genes (225 down- and 91 up-regulated) encoded proteins that are active in the ECM [Fig. 4(A)]. Classification according to GO_MF and GO_BP terms is represented in Fig. 4(B and C), respectively. The large number of up-regulated genes and the fact that they did not categorize under any GO terms related to cell death suggested that our microarray data efficiently revealed biological effects caused by GIN treatment and not by toxicity.

In consistence with the microscopical findings indicating significant cartilage matrix degradation, we found among the 91 up-regulated genes numerous transcripts encoding established proteases: Matrix metalloproteinase 9 (Mmp9), Mmp10, Mmp11, and HtrA serine peptidase 1 (HtrA1). Given the role of GSK3β in canonical Wnt signaling, the microarray data showed evidence for a Wnt/β-catenin signature as evidenced by the up-regulation of established direct targets of the β-catenin/Tcf4 complex, like Axin2 and adenomatosis polyposis coli down-regulated 1 (Apdcd1). Microarray and pathway analysis did not reveal clear signatures of changes in other signaling pathways, such as Hedgehog (Hh) and Fibroblast growth factor (FGF).

Furthermore, several cartilage ECM proteins were identified among the 225 down-regulated genes: unique cartilage
matrix-associated protein (Ucma), matrilin 1 (Matn1), Matn3, Matn4, hyaluronan and proteoglycan link protein 1 (Hapln1), collagen, type XI, alpha 1 (Col11a1), epiphycan (Epyc), fibromodulin (Fmod), matrix Gla protein (Mgp), Col14a1, and (Col9a3). In the list of repressed genes, we also found transcripts known to encode non-cartilaginous matrix proteins like osteomodulin (Omd), osteoglycin (Ogn), microfibrillar-associated protein 4 (Mfap4), tenomodulin (Tnmd), asporin (Aspn), and fibulin7 (Fbnl7), suggesting a more complex effect of the GIN treatment on the ECM.

To independently validate the results of the microarray analysis, 16 genes were selected for confirmation by quantitative real-time RT-PCR analysis. For the transcriptional analysis we therefore
isolated RNA from a separate experiment that mirrored the one used to generate the microarray data. Four of these 16 genes are known to be involved in chondrocyte differentiation and cartilage maintenance (Sox9, Col2a1, Acan and Col10a1), four are members of the canonical Wnt signaling pathway (Axin1, Axin2, Gsk3b and Ctnnb1), and eight encode proteinases known to regulate maintenance and degradation of the ECM (Mmp2, Mmp3, Mmp9, Mmp13, Adamts4, Adamts5, Hyal1 and CtsK). We found a similar expression pattern of the analyzed genes, indicating that our microarray data specifically corresponded to actual gene expression patterns (Fig. 5).

**GIN induces OA-like effects in vivo**

We next investigated whether GIN can induce the same biological effects in an *in vivo* experimental model. In an initial experiment, we observed 72 h after GIN injection nuclear translocation of β-catenin in a dose-dependent fashion in rat knee articular chondrocytes, whereas vehicle treatment did not induce a change in β-catenin expression in the control joints [Fig. 6(A, Ai,B, Bi and data not shown)]. Virtually all articular chondrocytes treated with the highest GIN concentration (10^{-5} M) showed nuclear β-catenin expression, yet they did not display any morphological changes or alterations of their ECM. We did not detect β-catenin up-regulation in other tissues such as synovium, tendons, or bone at the examined time point, nor evidence of inflammation.

In a second experiment, we injected 10^{-5} M GIN on day 1, 3 and 5. Four rats ("early" group) displayed signs of severe acute inflammation of the GIN-treated knee beginning at day 7 and these animals were therefore sacrificed already at day 10. No difference in β-catenin expression was observed between the GIN-treated and control knees of these animals (data not shown). In the vehicle-injected knee, the surface of the articular cartilage (AC) was smooth, the matrix was densely stained with AB and showed no signs of degeneration [Fig. 6(C, Ci)]. Besides displaying histological signs of inflammation (intra-articular infiltration of neutrophils and macrophages, synoviocyte hyperplasia, fibrin exudation etc.), the GIN-treated knees in the "early" group displayed intensely degraded AC, containing almost no GAGs, as indicated by absence of AB staining [Fig. 6(D, D, and data not shown)] in each of the four animals.

The other four rats ("late" group) from this experiment were sacrificed after 6 weeks and again showed no difference in the β-catenin expression pattern between the GIN-treated and control knees [Fig. 6(E, Ei,F, Fi)]. Whereas no morphological changes were observed in the control knees of the "late" group, GIN-treated samples from all four rats displayed superficial fibrillation of AC, focal hypocellularity of chondrocytes, and reduced AB staining. Quantification of the intensity of the AB staining revealed significantly less staining in the cartilage of GIN-treated knees in comparison to contralateral control knees [Fig. 6(G), *P = 0.05*]. Although not statistically significant, CECT analysis of condylar cartilage revealed a trend in reduction of cartilage volume as well as GAG-depletion (expressed by increased attenuation) in the GIN-treated knee joints in comparison to control knees [Fig. 6(H, I)].

**Discussion**

Here we show that Gsk3β, by controlling the canonical Wnt signaling pathway, is critical for maintenance of the chondrocytic phenotype. Inhibition of Gsk3β in chondrocytes *ex vivo* leads to loss of cartilage markers expression, induces matrix degradation by stimulating the expression of Mmps, inhibits chondrocyte proliferation and, most likely as a consequence of these effects, induces...
chondrocyte apoptosis. In addition, we demonstrate that transient inhibition of Gsk3β, following three intra-articular injections of GIN in rat knees during 1 week is associated with the appearance of OA-like features 6 weeks later. In agreement with our results, recent findings suggest that up-regulation of β-catenin through induction of proteasomal degradation of Gsk3β in chondrocytes initiates early events of OA, while inhibition of Gsk3β may block chondrogenesis.\(^{20,21}\)

Besides the canonical Wnt pathway, GSK3β also regulates signal transduction of the Hh and Fibroblast growth factor (Fgf) family of secreted proteins.\(^{22,23}\) Given that both Hh and Fgf growth factors play important roles in the chondrocyte life cycle, we searched in our microarray results for possible target genes of these proteins among the list of transcripts differentially regulated by GIN.\(^{24,25}\) Only PR domain containing 1, with zinc finger (ZNF) domain (Prdm1) matched this criterion, acting downstream of a sequential Wnt and Fgf signaling cascade.\(^{26}\) Our microarray expression data indicated that GIN treatment up-regulated the canonical Wnt target genes Axin2 and Apocd1, transcripts that have previously been shown to be induced only by Wnt and not by Fgf signaling.\(^{27–29}\) The protein products of Axin2 and Naked cuticle homolog 2, both of which are up-regulated in the microarray, are both renowned antagonists of the canonical Wnt signal. They have been shown to participate in negative feedback regulation of β-catenin activity.\(^{30–32}\) Taken together, these findings suggest that the intense cartilage matrix degeneration as well as the loss of the chondrocytic phenotype following GIN treatment occurred, at least in our experimental set-up, mainly due and can be explained by the activation of the Wnt/β-catenin pathway, although we cannot exclude minor roles of other signaling pathways in which GSK3β is known to be implicated nor of minor off-target effects of GIN.

Previously, we and others have reported that continuous exposure of chondrocytes to extensive levels of β-catenin in vivo induces loss of the chondrocytic phenotype as evidenced by the loss in expression of typical chondrocyte markers.\(^{5,6}\) Microarray analysis of GIN-treated metatarsals confirmed and extended this observation. Furthermore, our microscopical analysis suggests that GIN not only induced an enhanced degradation of the ECM, but also inhibited the expression of several ECM constituents in a time-dependent manner. Note-worthy, in the metatarsal experiments the loss of expression of typical cartilage markers at the mRNA level was observed before protein degradation was noticeable. GIN treatment inhibited the expression of genes encoding collagenous (Col9a3, Col11a1, and Col14a1), and non-collagenous ECM proteins (Ucma, Matn1, Matn3, Matn4, Hapln1, Finod, Mgp), as well as proteoglycans (Epyc). In addition, GIN stimulated the expression of proteinases Mmp9, Mmp10, Mmp11, and Htra1, which promote ECM degradation, suggesting that the loss of tissue integrity observed in the treated...
metatarsals is due not only to a loss of the links between the collagenous and the non-collagenous proteins in the matrix, but also to active matrix degradation.

Decreased chondrocyte proliferation, augmented apoptosis, loss of the chondrocytic phenotype and degradation of ECM together characterize the “degradative phase” of OA, the most common form of arthritis. These pathological phenomena were observed after up-regulated canonical Wnt signaling by GIN treatment in our experimental set-ups ex vivo and in vivo, in agreement with recent data suggesting a link between excess signaling through the Wnt/β-catenin pathway and OA. Moreover, many genes reportedly induced in OA cartilage were up-regulated by GIN treatment: *Mmp9, Mmp10, Mmp11, Axin2, Htrat1, angiopoietin-like 2 (Angpt2)*, and met proto-oncogene (*Met*). *Htrat1*, which is increased several-fold in joint cartilage of OA patients, promotes degeneration of cartilage, while *Met*, besides contributing to the altered metabolism during OA, also stimulates osteophyte development. *Serping1*, previously reported to be repressed in OA, and *Matt3*, whose inactivation leads to higher incidence of OA, were both down-regulated by GIN. In addition, inactivation of *Frzb*, another transcript repressed by GIN treatment, renders joints more susceptible for osteoarthritic changes.

Our results suggest that treatment with GIN can induce cartilage degeneration of rat AC after three intra-articular injections of GIN with 2 days interval. We observed two distinct phenotypes, most likely explained by a difference in retention time of GIN in the knee: a severe form with acute inflammation associated with resorption already 10 days after the first injection a milder phenotype. The potent catabolic effects of GIN on cartilage may have caused rapid and excessive cartilage degradation. These degradation products may have triggered an acute form of inflammation through the release of for example collagen type II fragments. In animals with the milder phenotype, microscopical analysis demonstrated the presence of the first signs of OA-like changes such as surface fibrillation, focal chondrocyte hypocellularity and a decrease in GAG staining in GIN-treated knees but not in the contralateral control knees 6 weeks after the last GIN injection. CECT analysis revealed a trend of less cartilage volume and more attenuation, indicative for GAG loss in the
GIN-treated animals; however, this observation did not reach significance. In contrast to the increased β-catenin expression in AC present 3 days after GIN injection, we did not detect increased β-catenin staining 6 weeks after GIN injection nor did we found evidence for nuclear β-catenin accumulation in the synovium, tendons or bone at each of the analyzed time points. This suggests that a transient rise in β-catenin in AC may be sufficient to trigger development of OA-like features, an observation that extends findings in conditional constitutive mice carrying a stabilized, oncogenic variant of β-catenin, which also develop OA. Although we did not find evidence for increased β-catenin accumulation in other joint tissues besides cartilage at the examined time points, we cannot exclude that GIN injection has resulted in a more rapid and transient rise of β-catenin in these tissues which was normalized 72 h after the injection. This may have also contributed to the observed pathology. Furthermore, we cannot exclude that the mild cartilage phenotype was due to a milder form of inflammation in the first weeks after injection. However, given the clear evidence of increased nuclear β-catenin accumulation in AC 72 h after GIN injection and the consistency of the in vivo findings with the phenotypic changes and effects on gene expression of GIN in our ex vivo cartilage explant model, we favor the hypothesis that these first indications of cartilage degeneration were due to a transient rise in β-catenin in AC triggering cartilage catabolism and changes in the chondrocyte phenotype.

Abnormally regulated GSK3β has been associated with many pathological conditions like Alzheimer’s disease, mood disorders, diabetes and cancer. However, a direct link between GSK3 and the pathophysiology of OA has not yet been reported. Since in our experimental set-ups the GIN-induced effects resemble results normally seen in osteoarthritic chondrocytes, we speculate that Gsk3β plays a role in the pathophysiology of this degenerative cartilage disease as well, most likely by regulating the levels of β-catenin. Whether pharmacological modulation of Gsk3β might represent a potential novel therapeutic approach for the management of OA remains to be elucidated.

Contributions

Conception and design: RLM, MK; Collection of data: RLM, MS; Analysis and interpretation of data: RLM, MS, LF, JJJ, HW, JMW, ECR-M, MK; Drafting of the article: RLM; Critical revision of the article for important intellectual content: RLM, MS, LF, JJJ, CWGML, WO, HW, JMW, ECR-M, MK; Final approval of the article: RLM, MS, LF, JJJ, CWGML, WO, HW, JMW, ECR-M, MK; Statistical expertise: LF, JJJ.

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Not applicable.

Conflict of interest

All authors declare that they have no conflict of interest.

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Supplementary material

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