Osteoarthritis and Cartilage 27 (2019) 1702–1710

Osteoarthritis and Cartilage

Exostosin-1 enhances canonical Wnt signaling activity during chondrogenic differentiation

X. Wang †, F.M.F. Cornelis †, R.J. Lories † ‡, S. Monteagudo † *

† Laboratory of Tissue Homeostasis and Disease, Skeletal Biology and Engineering Research Center, KU Leuven, Leuven, Belgium
‡ Division of Rheumatology, University Hospitals Leuven, Leuven, Belgium

ARTICLE INFO

Article history:
Received 11 February 2019
Accepted 9 July 2019

Keywords:
EXT1
Heparan sulfate proteoglycans
Wnt/β-catenin signaling
Chondrogenesis
Cartilage

SUMMARY

Objective: Exostosin-1 (*Ext1*) encodes a glycosyltransferase required for heparan sulfate (HS) chain elongation in HS-proteoglycan biosynthesis. HS chains serve as binding partners for signaling proteins, affecting their distribution and activity. The Wnt/β-catenin pathway emerged as critical regulator of chondrogenesis. Yet, how EXT1 and HS affect Wnt/β-catenin signaling during chondrogenesis remains unexplored.

Method: Ext1 was stably knocked-down or overexpressed in ATDC5 chondrogenic cells cultured as micromasses. HS content was determined using ELISA. Chondrogenic markers *Sox9, Col2a1, Aggrecan*, and Wnt direct target gene *Axin2* were measured by RT-qPCR. Proteoglycan content was evaluated by Alcian blue and DMMB assay, canonical Wnt signaling activation by β-catenin Western blot and TOP/FOP assay. ATDC5 cells and human articular chondrocytes were treated with Wnt activators CHIR99021 and recombinant WNT3A.

Results: Ext1 knock-down reduced HS, and increased chondrogenic markers and proteoglycan accumulation. *Ext1* knock-down reduced active Wnt/β-catenin signaling. Conversely, *Ext1* overexpressing cells, with higher HS content, showed decreased chondrogenic differentiation and enhanced Wnt/β-catenin signaling. Wnt/β-catenin signaling activation led to a down-regulation of *Ext1* expression in ATDC5 cells and in human articular chondrocytes.

Conclusions: EXT1 affects chondrogenic differentiation of precursor cells, in part via changes in the activity of Wnt/β-catenin signaling. Wnt/β-catenin signaling controls *Ext1* expression, suggesting a regulatory loop between EXT1 and Wnt/β-catenin signaling during chondrogenesis.

Introduction

The majority of bones in our body are formed by endochondral ossification. This process is initiated by condensation of mesenchymal progenitor cells, which then differentiate into cartilage-forming chondrocytes to generate a template of the future bone. Chondrocytes in these templates undergo a differentiation program from proliferating into mature chondrocytes, which eventually become hypertrophic and are ultimately replaced by bone[1]. Chondrogenic differentiation is tightly controlled by a complex network of signaling cascades. The Wnt/β-catenin pathway (also known as canonical Wnt signaling), composed of Wnt ligands, receptors, antagonists, and intracellular signaling molecules, has emerged as a critical regulator of skeletal cell differentiation processes[2,3].

The expression patterns and the modulation of Wnt pathway components in chick and mouse models of the developing limb provide strong evidence for the involvement of this cascade in chondrogenesis and endochondral bone formation[4–8]. In the early phase of skeletogenesis, expression of Wnts, such as WNT3A, in distal cell populations at the apical ectodermal ridge keeps the mesenchymal progenitor cells in a proliferative and undifferentiated state that is essential for limb outgrowth, preventing differentiation into chondrocytes. However, low Wnt signaling in cells distant from the Wnt source allows them to express the chondrogenic master transcription factor SOX9 and differentiate into chondrocytes[2]. Thus, the current model is that canonical Wnt signaling opposes a chondrogenic cell differentiation program in

* Address correspondence and reprint requests to: S. Monteagudo, Laboratory of Tissue Homeostasis and Disease, Skeletal Biology and Engineering Research Center, KU Leuven, Leuven, Belgium. Tel: 32-16-342542; Fax: 32-16-346200.
E-mail addresses: Xiangdong.Wang@kuleuven.be (X. Wang), Frederique.Cornelis@kuleuven.be (F.M.F. Cornelis), Rik.Lories@kuleuven.be (R.J. Lories), Silvia.Monteagudo@kuleuven.be (S. Monteagudo).

https://doi.org/10.1016/j.joca.2019.07.007
1063-4584/

early chondroprogenitor cells. Mouse embryos expressing a constitutively active mutant form of β-catenin in developing cartilage under the control of collagen 2 promoter/enhancer, thereby increasing active Wnt signaling in cells that have acquired a chondrocyte identity, exhibit severe dwarfism and skeletal deformity. In these mice, synthesis of cartilage matrix molecules is strongly reduced and the growth plate structure fails to organize properly[7,9,10]. Thus, excessive activation of this signaling can disturb the normal endochondral bone formation process and lead to skeletal disorders. Therefore, Wnt/β-catenin signaling should be tightly controlled for normal cartilage development.

The Wnt/β-catenin pathway demonstrates an extraordinary degree of complexity and fine-tuning, and many aspects of its regulation remain uncharacterised[2,11]. Although the interactions of Wnt and other signaling molecules during endochondral ossification have been intensively studied, mechanisms that control the extracellular distribution of Wnt molecules and subsequent signaling strength during chondrogenesis are less well understood. In this context, increasing evidence supports that heparan sulfate (HS)-carrying proteoglycans (HSPGs) that are present at the cell surface and in the extracellular matrix play a critical role in controlling the distribution and activity of secreted growth factors. Many growth factors and morphogens, including those that play a role in skeletal development, exhibit affinities for the HS chains of HSPGs[12,13]. HSPGs are composed of a core protein with several attached chains of HS glycosaminoglycans (GAG), and include syndecans, glypicans and perlecan[13]. The HS chains consist of repeating D-glucuronic acid and N-acetyl-D-glucosamine residues that are assembled into linear polysaccharides by glycosyltransferases of the exostosin family (EXTs)[12]. Within the Golgi-associated EXT1/EXT2 protein complexes, EXT1 protein has a major catalytic function in HS polymerization, while EXT2 −though required− may have structural or supportive roles[14]. The physiological importance of EXTs and HS in skeletal development is illustrated by the severe limb defects demonstrated in conditional *Ext1* deficient mice[15] and also by the human bone disorder Multiple Hereditary Exostoses, caused by mutations in EXT enzymes, with the most severe phenotypes associated with EXT1 mutations[14]. Of note, postnatal conditional ablation of β-catenin in cartilage induces ectopic periosteal chondroma-like cartilage formation in mice[16]. How EXT1 activity and associated HS levels may affect Wnt/β-catenin signaling activation during chondrogenesis remains unexplored.

In the present study, we aimed to define the impact of EXT1 and HS content in determining the strength of canonical Wnt signaling activation during chondrogenesis. To this end, we knocked-down or overexpressed HS-synthetizing enzyme EXT1 in ATDC5 cells cultured as micromasses. The ATDC5 cell line, derived from mouse teratocarcinoma cells and characterized as a chondrogenic cell line, exhibits a multistep process of chondrogenic differentiation analogous to that observed during endochondral bone formation. Thus, it is a well-established *in vitro* model for skeletal development[17].

Material and methods

Materials

CHIR99021 and XAV939 were purchased from Sigma and Selleckchem, respectively. Sodium chlorate was obtained from Sigma, human recombinant WNT3A from R&D systems.

ATDC5 micromass cultures

ATDC5 cells were cultured in growth medium (1:1 Dulbecco's modified Eagle's medium (DMEM): Ham's F-12 mix (Gibco)) containing 1% (vol/vol) antibiotic−antimycotic (Gibco), 5% fetal bovine serum (FBS) (Gibco), 10 μg/ml human transferrin (Sigma) and 30 nM sodium selenite (Sigma). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. High-density micromass cultures of ATDC5 cells were grown to study chondrogenic differentiation[18]. Cells were trypsinized, washed and resuspended at 2×10^7 cells/ml in a chondrogenic medium made of DMEM-F12 enriched by 1% (vol/vol) antibiotic−antimycotic, 5% FBS, 5 μg/ml human transferrin and 1 × ITS (Insulin, Transferrin, Selenite) premix (resulting in 10 μg/ml insulin, 5 μg/ml human transferrin and 30 nM sodium selenite) (Life Technologies). One droplet (10 μl) was carefully placed in the centre of each well of a 24-well plate. Cells were allowed to adhere for 2 h at 37°C, followed by addition of 500 μl chondrogenic medium. Micromasses and supernatants were collected at time points 1, 7 and 14 days for functional assays.

Generation of stably knocked-down ATDC5 cell line

Silencing of *Ext1* was performed using a GIPZ-shRNA construct directed against mouse *Ext1* (Dharmacon); and a non-interfering GIPZ vector (Dharmacon) was used as a control. ATDC5 cells were transfected using Lipofectamine LTX (Thermo Fisher) and after 24 h, selection with 3 μg/ml puromycin (Thermo Fisher) was initiated and continued for 14 days. Three different antibiotic-resistant clonal colonies of each condition were isolated and grown independently. Knock-down efficiency was assessed by quantitative RT-PCR.

Generation of stably overexpressing ATDC5 cell line

ATDC5 cells were transfected with control pCMV6-entry vector, pCMV6-*Ext1* ORF clone (PS100001; RC200644; Origene) using Lipofectamine LTX reagent (Thermo Fisher). Selection was initiated 24 h after transfection by supplementing the maintenance medium with 500 ng/ml Geneticin (Thermo Fisher). Clones were picked after 14 days of selection. Three different antibiotic-resistant clonal colonies of each condition were isolated and grown independently. Overexpression levels were assessed by quantitative RT-PCR. Antibiotic pressure was maintained for the whole duration of the experiments.

Human articular chondrocyte culture

Human articular chondrocytes were isolated from the hip of one non-osteoarthritis patient (male, 68 years old) undergoing total hip replacement surgery, as previously described[19]. The University Hospitals Leuven Ethics Committee and Biobank Committee approved the study and specimens were taken with patients' written consent.

Quantitative RT-PCR

Total RNA was isolated using the NucleoSpin RNAII kit (Machery-Nagel) following the manufacturer's protocol. Complementary DNA was synthesized from 0.5 to 1 μg of total RNA using the RevertAid H minus First Strand cDNA synthesis kit (Fermentas GmbH). SYBR green analyses were performed as described previously using Maxima SYBRgreen qPCR master mix system (Fermentas)[19]. Gene expression levels were calculated following normalization to housekeeping gene *Hprt* messenger RNA (mRNA) levels using the comparative Ct (Cycle threshold) method. Primers used for RT-qPCR analysis are listed in Supplementary Table I.

Heparan sulfate ELISA

Supernatant, cell lysates and pellet rest fraction from *Ext1* knocked-down or overexpressing cells were collected at day 7 of micromass culture. The HS Quantikine ELISA Kit (Amsbio) was used according to the manufacturer's instructions. Data were normalized to the total protein concentration measured by BCA protein assay (Pierce).

Alcian blue staining

ATDC5 micromasses were washed with PBS and fixed with 95% ice-cold methanol for 30 min at 4°C. After rinsing three times with water, micromasses were stained with Alcian Blue (AB) (0.1% AB 8GX, Sigma), washed three times with water and air dried. Quantification of the staining was performed by dissolving the micromasses with 6 M guanidine (Sigma) and measuring the absorbance at 595 nm with a spectrophotometer (BioTek Synergy).

Dimethylmethylene blue (DMMB) assay

Biochemical analysis of DNA and glycosaminoglycan (GAG) amount was determined by Dimethylmethylene Blue (DMMB) assay, as previously described[18]. Harvested micromasses were digested at 56°C overnight with 0.1 M proteinase K in PBE buffer (1 mM EDTA, 10 mM Tris, pH 6.5). DNA amount was measured using the nanodrop (nanodrop 2000). Glycosaminoglycan amount was measured at 525 nm with a spectrophotometer (BioTek Synergy) using DMMB dye added to 50 μl of pellet digestion or undiluted supernatant and bovine chondroitin sulfate (Sigma) as a standard.

Luciferase assay

Stably *Ext1* knocked-down or overexpressing ATDC5 cells (7500/well) were seeded in 96-well plates for 24 h and transiently transfected with Super8X TOPFlash or Super8X FOPFlash (TOPFlash mutant control) (Addgene plasmids #12456 and #12457, respectively) for canonical Wnt signaling reporter using Lipofectamine LTX Reagent with PLUS Reagent (Life Technologies) according to the manufacturer's protocol. After 24 h, cells were treated with 100 ng/ml recombinant WNT3A protein or vehicle and collected 24 h later. Luciferase assay was performed using Luciferase Assay system (Promega) as previously described[19].

Western Blot analysis

Cells were harvested and resuspended in RIPA buffer (Thermo Scientific) supplemented with 1 mM phenylmethanesulfonyl (Sigma), 5% protease inhibitor cocktail (Sigma), 2.3 mM Na_3VO_4 (Sigma) and 5 mM NaF (Merck Millipore). Cell lysates were sonicated (two cycles of 7 s) and centrifuged at 18.000 g for 10 min, and supernatant containing proteins was collected. Protein concentration of the extracts was determined by Pierce BCA Protein Assay Kit (Thermo Scientific). Immunoblotting analyses were performed as previously described[19]. Antibodies against Actin (Sigma, A2066; dilution 1:4.000), active β-catenin (Merck Millipore, 05–665, clone 8E7; dilution 1:1.000) and total β-catenin (BD biosciences, 610154, clone 14; dilution 1:2.000) were used. Blotting signals were detected using the SuperSignalWest Femto Maximum Sensitivity Substrate system (Thermo Scientific).

Statistical analysis

Data are presented as mean and SD, representing the mean of technical replicates as indicated in figure legends. For the ATDC5 cells, experimental data reported in the manuscript are established from technical replicates of one representative colony. Replicates can therefore not be assumed to be strictly independent. The results obtained from other two colonies showed the same difference in a direction, but with different magnitude between the control and silencing or overexpression groups. In this hypothesis-finding exploratory study, no formal power analysis was performed. Statistical analyses were performed with GraphPad Prism software. Data distribution was evaluated on the basis of parameter characteristics, quantile–quantile (QQ) and residuals plots. *T*-tests or ANOVA tests were applied. Estimates of differences of means between groups (95% confidence intervals (CI)) are reported in the Results section. When different groups were compared by one-way ANOVA tests or when interaction was presented in two-way ANOVA tests, pair-wise *t*-tests were eventually performed applying a Bonferroni correction for multiple comparisons to control for type I errors in rejecting the null hypothesis as indicated in figure legends.

Results

Ext1 knock-down increases markers of chondrogenesis and proteoglycan deposition in ATDC5 cells

To study how EXT1 activity affects chondrogenic differentiation, we generated ATDC5 cells in which endogenous *Ext1* was knocked-down. ATDC5 cells are mouse chondrogenic precursor cells that differentiate and undergo chondrogenesis when cultured in micromasses. Following stable transfection, relative *Ext1* mRNA levels were determined in control cells transfected with a non-interfering short hairpin micro (shmi) RNA (Gipz) and in cells with stable knock-down (KD) generated by shmiRNA against *Ext1* (shExt1), at baseline (day 1) and after 7 and 14 days in the differentiation culture. In the presented cell line, representative of at least 3 distinct colony-expanded clones (see [Fig. S1]), *Ext1* mRNA levels were on average 66% (95%CI: 23–89) down-regulated in shExt1 cells compared to Gipz cells [Fig. 1(A)]. This down-regulation was sustained during the differentiation process. We then assessed whether *Ext1* KD resulted in reduced HS biosynthesis. Effectively, the concentration of HS was decreased in shExt1 compared to Gipz cells (on average 16.69 ng/mg (95%CI: 4.6–28.77) in supernatant, 43.7 ng/ml (95%CI: −21.2–107.8) in cell lysate, and 34.01 ng/mg (95%CI: 4.09–63.92) in rest fraction) [Fig. 1(B)]. These results indicate that *Ext1* KD leads to reduced biosynthetic activity of HS proteoglycans during chondrogenesis.

Then, we measured mRNA expression of molecular markers of chondrogenic differentiation, in particular *Sox9*, *Col2a1* and *Aggrecan*, by RT-qPCR. Gene expression of the master regulator of chondrogenic cell fate *Sox9* was enhanced in shExt1 cells, on average 0.8 difference between the means (95%CI: 0.14–1.476) [Fig. 1(C)] in the presented cell line, representative of at least 3 distinct colony-expanded clones. Similarly, expression of *Col2a1* and *Aggrecan* was up-regulated in shExt1 compared to control cells (10.46 difference between the means (95%CI: 5.9–15) and 712 difference between the means (95%CI: 561–862) [Fig. 1(C)]. Next, we evaluated the levels of total proteoglycans in the extracellular matrix of the micromasses, using AB staining. We observed an increase in total proteoglycans in shExt1 cells compared to controls (0.084 difference in absorbance (95%CI: 0.070–0.098) in the presented cell line, representative of at least 3 distinct colony-expanded clones) [Fig. 1(D)]. To validate these observations, we

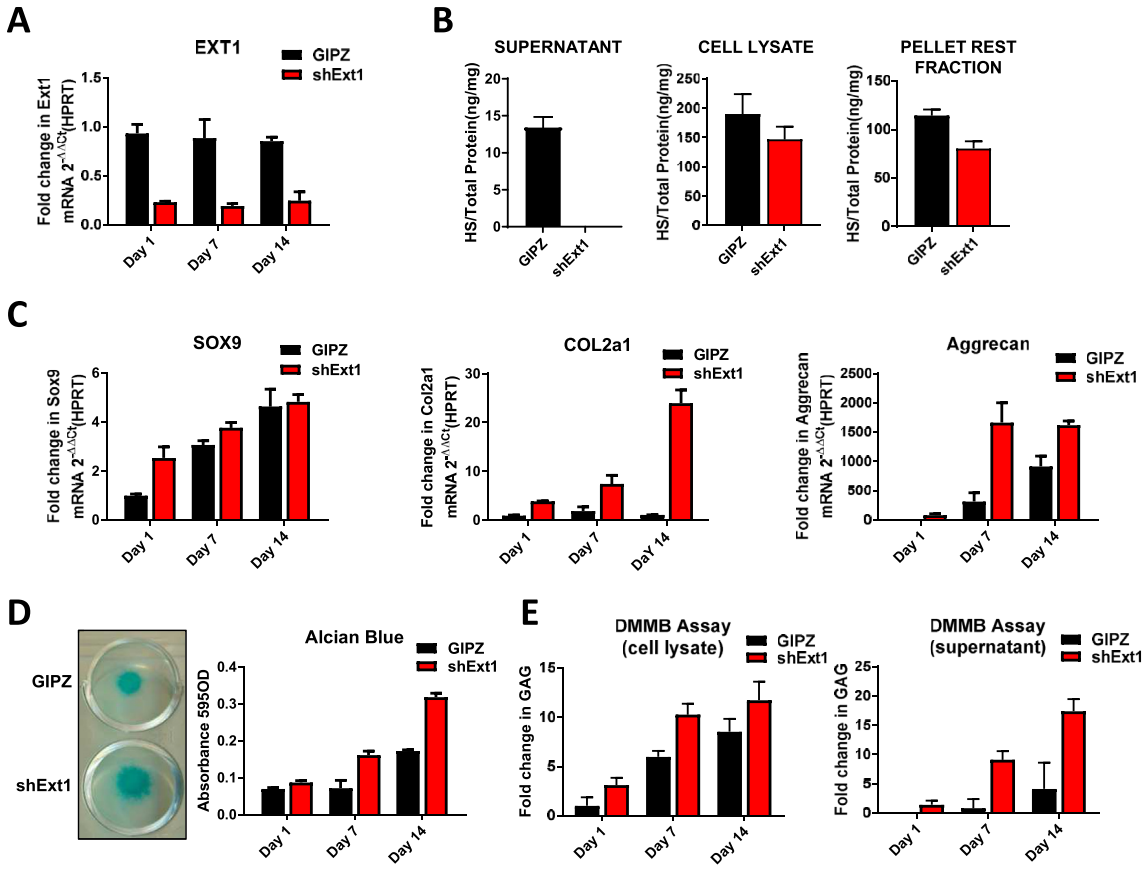

Fig. 1. *Ext1* knock-down enhances chondrogenic differentiation in ATDC5 cells. (A) Quantitative RT-PCR analysis in control cells transfected with a non-interfering short hairpin micro (shmi) RNA (GIPZ) and in cells transfected with shmiRNA against *Ext1* (shExt1), showing successful knock-down (KD) of *Ext1* at day 1, 7 and 14 in shExt1 cells ($P = 0.015$ in two-way ANOVA for silencing vs control). (B) Heparan Sulfate (HS) ELISA assay showing reduced HS production in shExt1 cells in supernatant samples ($P = 0.019$ by *t*-test), cell lysate ($P = 0.136$ by *t*-test) and pellet rest fraction ($P = 0.039$ by *t*-test). (C) *Ext1* KD increases mRNA expression of chondrogenic markers *Sox9* ($P = 0.028$ in two-way ANOVA for silencing vs control), *collagen 2a1* (*Col2a1*) ($P = 0.004$ by two-way ANOVA for interaction between silencing and time) and *aggrecan* ($P = 0.014$ by two-way ANOVA for interaction between silencing and time). (D) Alcian blue staining showing increased total proteoglycan deposition in shExt1 cells during chondrogenesis ($P < 0.0001$ by two-way ANOVA for interaction between silencing and time). Left panel: representative image of staining at day 14 is shown. Right panel: quantification of staining by colorimetry. (E) Dimethylmethylene blue (DMMB) assay showing increased total glycosaminoglycan (GAG) content in shExt1cells, in both supernatants and cell lysates ($P = 0.015$ and 0.0004 by two-way ANOVA for silencing vs control, values are fold change compared to control day 1 cell lysates). Data shown are from one established cell line representative of at least three independent biological replicates, except for 1B ($n = 1$ independent clone). Error bars indicate mean ± SD of two (1A) or three (1B-E) technical replicates per condition.

performed a DMMB assay. Again, we detected an enhancement in total proteoglycan content in shExt1 cells compared to controls (3.199 difference between the means (95%CI: 1.037–5.36) and 10.77 difference between the means (95%CI: 7.98–13.56) for cell lysate and supernatant, compared to the lysate of control cells at day 1 in the presented cell line, representative of at least 3 distinct colony-expanded clones) [Fig. 1(E)]. Collectively, these results indicate that *Ext1* deficiency and resulting HS reduction promotes chondrogenic differentiation.

Ext1 knock-down decreases canonical Wnt signaling activation during chondrogenesis

We then investigated the impact of *Ext1* loss-of-function on the activation of canonical Wnt signaling. In Wnt reporter-transfected cells, *Ext1* KD reduced luciferase activity in basal conditions (on average 0.23 unit decrease (95%CI: 0.10–0.36) in the presented cell line, representative of 3 distinct colony-expanded clones) [Fig. 2(A)]. In line with these observations, shExt1 cells showed lower active β-catenin protein levels than control cells during chondrogenic differentiation, assessed by Western Blot analysis [Fig. 2(B)]. Also, mRNA levels of direct Wnt target gene *Axin2* were decreased in shExt1 cells compared to controls (0.44 difference between means (95%CI: 0.14–0.75) in the presented cell line, representative of 3 distinct colony-expanded clones) [Fig. 2(C)]. Altogether, these findings indicate that, in basal conditions, canonical Wnt signaling activation is impaired in *Ext1*-deficient cells during chondrogenesis.

Then, we aimed to evaluate how *Ext1* KD affects canonical Wnt signaling activation upon exogenous Wnt ligand stimulation. ShExt1 cells exhibited lower luciferase activity than control cells when the Wnt signaling pathway was activated by recombinant WNT3A protein (difference of the means 11.21 units (95%CI: 10.04–12.38) in the presented cell line, representative of 3 distinct colony-expanded clones) [Fig. 2(D)]. Notably, in control cells, WNT3A stimulation led to a down-regulation in chondrogenic differentiation markers *Col2a1* (77.76 difference between means, 95%CI = 17.21–7138.3) and *Aggrecan* (71 difference between means, 95%CI: 16.6–128, both in the presented cell line, representative of 3 distinct colony-expanded clones) [Fig. 2(E)], in line with previous reports[20,21]. However, in shExt1 cells, WNT3A treatment did not negatively affect the expression levels of chondrogenic markers. These data suggest that *Ext1* deficiency reduces canonical Wnt signaling activation and abolishes subsequent effects on chondrogenic differentiation.

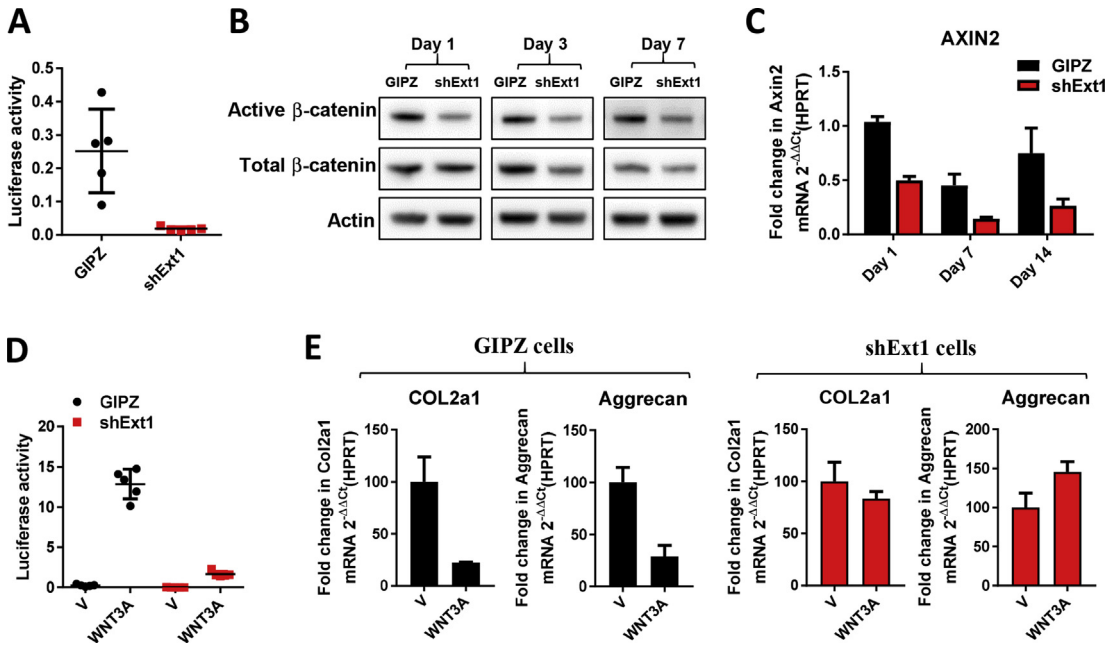

Fig. 2. *Ext1* knock-down limits canonical Wnt signaling activation in ATDC5 cells. (A) TOP/FOP luciferase reporter assay showing reduced Wnt/β-catenin activation in shExt1 cells ($P = 0.0032$ by *t*-test). (B) Western Blot analysis showing decreased active-β-catenin expression in shExt1cells at 3 different time points compared to control cells. Actin was set as loading control. (C) mRNA expression of Wnt target gene *Axin2* ($P = 0.0247$ for silencing vs control by two-way ANOVA). (D) TOP/FOP luciferase reporter assay in shExt1 cells showing less response than control cells upon exogenous Wnt signaling stimulation with recombinant WNT3A ($P < 0.0001$ Bonferroni-corrected for 6 tests by two-way ANOVA). (E) Loss of chondrogenic markers *Col2a1* and *aggrecan* upon recombinant WNT3A treatment in control cells (left panel) is prevented in shExt1cells (right panel) ($P = 0.0154$ and $P = 0.0164$ Bonferroni-corrected for two tests by two-way ANOVA). Vehicle (V) expression was set as 100 %. Data shown are from one established cell line representative of at least three independent biological replicates except for 2E ($n = 1$ cell line). Error bars indicate mean ± SD of five (2A-2D), two (2C) and three (2E) technical replicates per experiment.

Ext1 overexpression reduces chondrogenic markers and proteoglycan deposition in ATDC5 cells

Next, we explored the effects of *Ext1* gain-of-function during chondrogenic differentiation. To this end, we established stable transfectants of ATDC5 cells where *Ext1* was overexpressed (*Ext1*+). Relative *Ext1* mRNA levels were determined in control cells expressing pCMV6-entry vector compared to *Ext1*+ cells after 1, 7 and 14 days of differentiation culture [Fig. 3(A)]. *Ext1* mRNA levels were increased in *Ext1*+ compared to control cells (difference between means 1.092 (95%CI: 0.2059–1.977) at day 1, 1.376 (95%CI: 0.9304–1.821) at day7, 4.005 (95%CI: 3.312–4.698) at day 14 in the presented cell line, representative of 3 distinct colony-expanded clones). Then, we evaluated the impact of *Ext1* overexpression on HS content. As expected, we detected enhanced HS concentration in *Ext1*+ compared to control cells (114.6 ng/mg (95%CI: 76.9–152.2) and 133.9 ng/mg (95%CI: 103–164) for cell lysate and rest fraction) [Fig. 3(B)]. As opposed to shExt1, *Ext1*+ cells displayed a slight reduction in chondrogenic differentiation markers *Sox9* (0.09 difference between means (95%CI: 0.02–0.17) in the presented cell line, representative of 3 distinct colony-expanded clones) and *Col2a1* (4.9 difference between means (95%CI: 3.3–6.5) in the presented cell line, representative of 3 distinct colony-expanded clones) [Fig. 3(C)]. No changes were detected in *Aggrecan* expression in *Ext1*+ cells compared to controls in these conditions. Total proteoglycan content appeared to be reduced in *Ext1*+ cells, assessed by AB staining (on average 0.06 units (95%CI: 0–0.11)) [Fig. 3(D)] and by DMMB assay (0.32 difference between the means (95%CI: 0.20–0.43)) [Fig. 3(E)] in the presented cell line, representative of 2 and 3 distinct colony-expanded clones, respectively. Interestingly, *Ext1*+ cells treated with sulfate synthesis inhibitor sodium chlorate ($NaClO_3$), which prevents effective HS biosynthesis[22–24], did not exhibit reduced total proteoglycan content [Fig. 3(F)]. Overall, these results indicate that excessive EXT1 function impairs chondrogenic differentiation.

Ext1 overexpression enhances canonical Wnt signaling activation during chondrogenesis

Then, we determined whether *Ext1* overexpression affects Wnt/β-catenin signaling activation. First, we evaluated luciferase activity in Wnt-reporter transfected cells, both in basal conditions and upon recombinant WNT3A stimulation. In non-stimulated conditions, we detected no differences in Wnt/β-catenin signaling activation between *Ext1*+ and control cells [Fig. 4(A)]. After WNT3A treatment, *Ext1*+ cells showed on average 14.18 units higher luciferase activity than controls (95%CI: 6.84–21.2) in the presented cell line, representative of 2 distinct colony-expanded clones. *Ext1*+ cells treated with $NaClO_3$ to block effective HS biosynthesis, did not show higher Wnt-reporter activity than control cells [Fig. 4(A)], indicating that differences in Wnt/β-catenin signaling activation rely on changes in HS content. Consistent with these findings, we detected higher protein levels of β-catenin in *Ext1*+ than control cells, assessed by Western Blot analysis [Fig. 4(B)]. We also determined higher mRNA levels of Wnt direct target gene *Axin2* in *Ext1*+ cells than in controls (0.15 difference between the means (95%CI: 0.06–0.25) in the presented cell line, representative of 3 distinct colony-expanded clones) [Fig. 4(C)]. To investigate whether changes in Wnt/β-catenin signaling activation in *Ext1*+ cells contribute to impaired chondrogenesis, we treated these cells with Wnt antagonist XAV939 and determined total proteoglycan content as indicator of chondrogenic differentiation status [Fig. 4(D)]. XAV939 is a specific tankyrase inhibitor that stimulates β-catenin degradation by stabilizing axin, the concentration-limiting component of the destruction complex[25]. Wnt inhibition prevented the reduction in total proteoglycan content in *Ext1*+ cells.

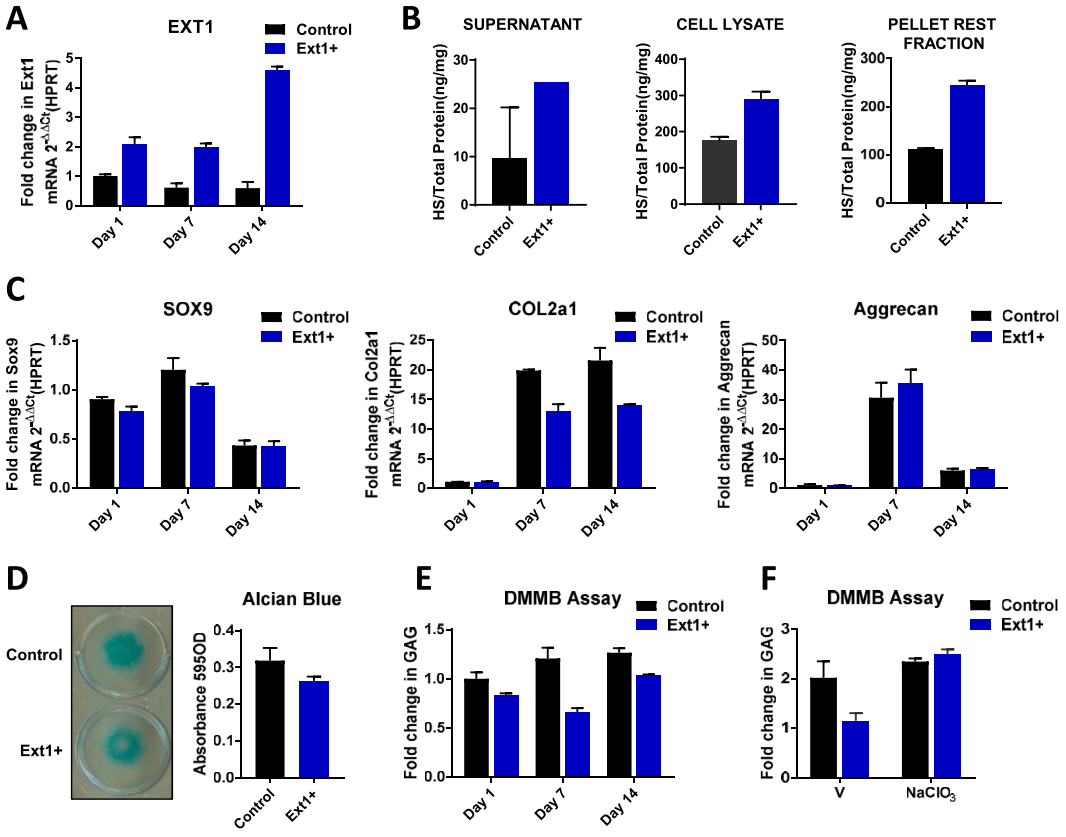

Fig. 3. *Ext1* overexpression reduces chondrogenic differentiation in ATDC5 cells. (A) Quantitative RT-PCR analysis of ATDC5 clones stably-transfected with control pCMV6-entry vector (controls) or with pCMV6-*Ext1* ORF (Ext1+) showing successful overexpression of *Ext1* in Ext1+ cells at day 1, 7 and 14 ($P < 0.0001$ for interaction between overexpression and time by two-way ANOVA). (B) Heparan Sulfate ELISA assay showing HS production in Ext1+ cells and in controls (in cell lysate ($P = 0.001$ by *t*-test) and in pellet rest fraction ($P = 0.0027$ by *t*-test). (C) mRNA expression of chondrogenic markers *Sox9* ($P = 0.0107$ for overexpression vs control in two-way ANOVA), *collagen 2a1* (*Col2a1*) ($P = 0.0150$ for interaction between overexpression and time in two-way ANOVA) and *aggrecan* ($P = 0.32$ and 0.31 for overexpression vs control and for interaction), in Ext1+ cells compared to controls. (D) Alcian blue staining showing reduced total proteoglycan deposition in Ext1+ cells during chondrogenesis ($P = 0.059$ by *t*-test). Left panel: representative image of staining at day 14 is shown. Right panel: quantification of staining by colorimetry at day 14. (E) DMMB assay showing reduced total glycosaminoglycan content normalized to DNA content in Ext1+ cell lysates ($P < 0.0001$ for interaction between overexpression and time in two-way ANOVA). (F) Treatment with sodium chlorate ($NaClO_3$), a selective chemical inhibitor of HS biosynthesis, prevents depletion of glycosaminoglycans in Ext1+ cells, assessed by DMMB assay of the cell lysates ($P = 0.0012$ Bonferroni-correct for two tests in two-way ANOVA). Data shown are from one established cell line representative of at least three independent biological replicates, except for 3B ($n = 1$ independent clone), 3D and 3F ($n = 2$ independent clones). Error bars indicate mean ± SD of three technical replicates per condition.

Thus, all these data demonstrate that EXT1 gain-of-function enhances the strength of canonical Wnt signaling and negatively affects chondrogenic differentiation.

Canonical Wnt signaling regulates *Ext1* expression

Next, we investigated whether Wnt/β-catenin signaling reciprocally regulates *Ext1* expression. Pharmacological activation of Wnt/β-catenin signaling using CHIR99021, a GSK3 inhibitor, resulted in a concentration-dependent down-regulation of *Ext1* expression in ATDC5 cells (difference between means 0.32 (95% CI:0.073–0.566) and 0.39 (95%CI:0.145–0.6387) for dosage 3 and 10 μM respectively) in the presented cell line, representative of 2 distinct colony-expanded clones [Fig. 4(E)]. Conversely, Wnt inhibitor XAV939 up-regulated *Ext1* expression (difference between means 0.25 (95%CI: 0.01–0.5)). These data, in combination with results reported above, suggest the existence of a regulatory loop between EXT1 and Wnt/β-catenin signaling during chondrogenesis [Fig. 4(F)].

Finally, we determined whether Wnt/β-catenin signaling also regulates EXT1 expression in human articular cartilage. Recombinant WNT3A treatment down-regulated *Ext1* mRNA expression, in a concentration-dependent manner, in human articular chondrocytes (difference between means 0.445 (95%CI: 0.09367–0.7978), 0.5080 (95%CI: 0.1143–0.9016), 0.6022 (95%CI: 0.2501 to 0.9543) for 10, 100, 1000 ng/ml WNT3A respectively [Fig. 4(G)]. These insights suggest that Wnt/β-catenin pathway also regulates Ext1 in human articular cartilage.

Discussion

In this study, we report that EXT1, by modulating HS-proteoglycan content, affects the differentiation potential of ATDC5 chondroprogenitor cells, and these effects are in part attributable to modifications in the strength of Wnt/β-catenin pathway activation. Reciprocally, Wnt/β-catenin signaling regulates the expression of *Ext1*. Thus, our data suggest the existence of a regulatory loop between EXT1 and the Wnt/β-catenin signaling during chondrogenesis [Fig. 4(F)].

EXT1 and HS content affect chondrogenic differentiation potential of precursor cells

The importance of EXT1 and HS content in skeletal development and chondrogenic differentiation has been shown in several previous studies. In 2005, Hilton *et al.* analyzed full heterozygous *Ext1*-

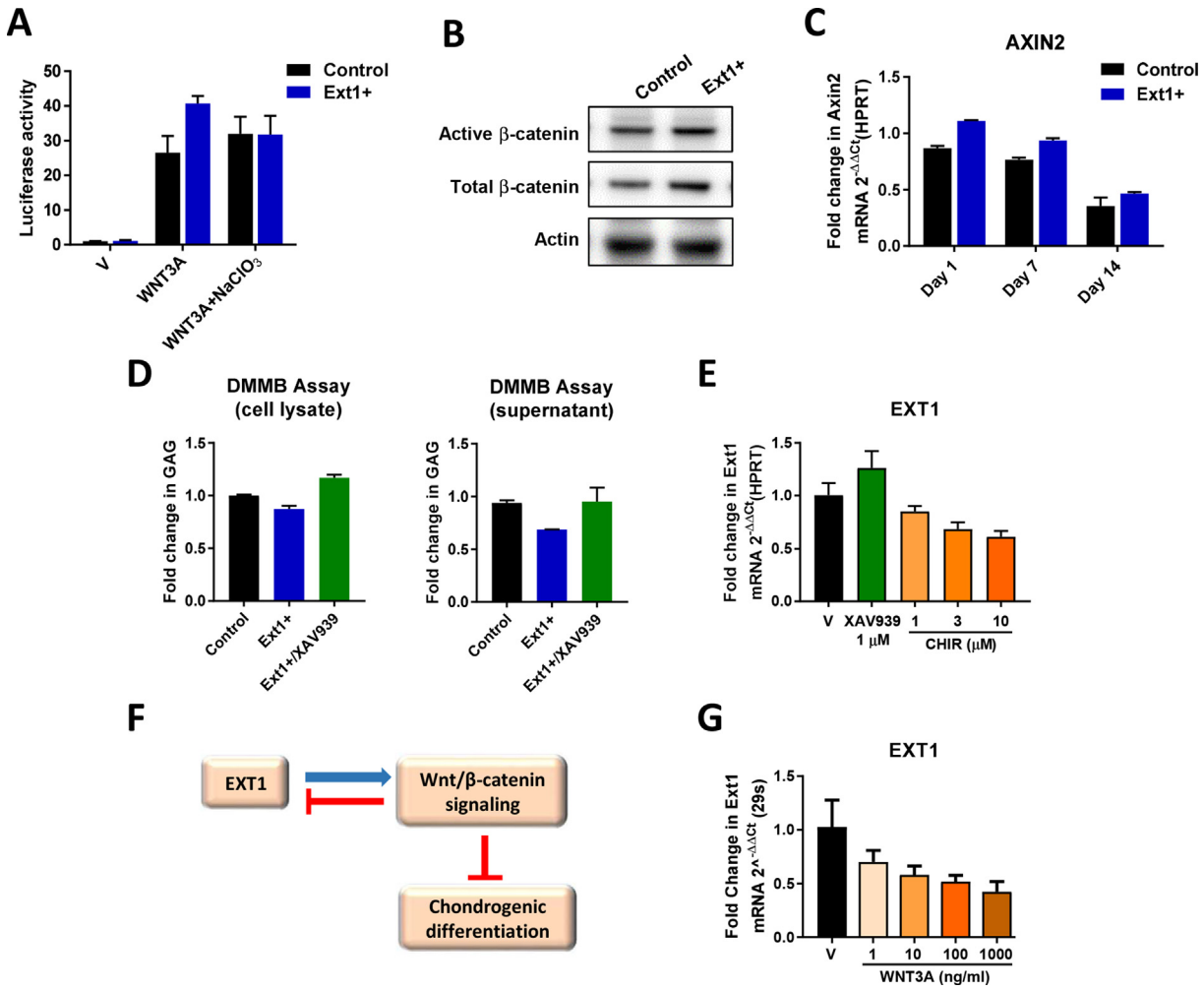

Fig. 4. ***Ext1*** **overexpression enhances canonical Wnt signaling activation in ATDC5 cells.** (A) TOP/FOP luciferase reporter assay in Ext1+ and control cells upon treatment with vehicle (V), recombinant WNT3A with or without $NaClO_3$ ($P = 0.0002$ Bonferroni corrected for three tests by two-way ANOVA). (B) Western Blot analysis showing higher β-catenin protein expression in Ext1+ cells compared to control cells. Actin was set as loading control. (C) mRNA expression of Wnt target gene *Axin2* ($P = 0.011$ for overexpression vs control by two-way ANOVA) in Ext1+ cells during chondrogenic differentiation. (D) Treatment with Wnt inhibitor XAV939 restores glycosaminoglycan content in Ext1+ cells, assessed by DMMB assay ($P = 0.0015$ and < 0.0001 Bonferroni-corrected for three tests in one-way ANOVA in cell lysate, $P = 0.075$ in one-way ANOVA for supernatant). (E) mRNA expression of *Ext1* upon treatment with V, Wnt activator CHIR99021 (CHIR) and XAV939 ($P = 0.04$, 0.0112 and 0.0028 for XAV939, CHIR 3 μM, CHIR 10 μM, Bonferroni-corrected for 4 tests by one-way ANOVA). (F) Model for the regulatory loop between EXT1 and Wnt/β-catenin signaling in chondrogenesis. (G) mRNA expression of *Ext1* in human articular chondrocytes upon treatment with vehicle (V) and recombinant WNT3A (1, 10, 100 and 1000 ng/ml) ($P = 0.075$, 0.014, 0.012 and 0.002 Bonferroni-corrected for 4 tests by one-way ANOVA). Data shown are from one established cell line representative of one (4B), two (4A, 4D and 4F), three (4C) independent biological replicates and from one non-osteoarthritis donor (4G). Error bars indicate mean ± SD of three technical replicates per condition.

deficient mouse embryos and found enhanced chondrocyte proliferation and delayed hypertrophic differentiation in the mutant long bones, together with alterations in gene expression and protein distributions of molecules of the Indian Hedgehog pathway[26]. In another study, Matsumoto *et al.* ablated *Ext1* in limb bud mesenchyme using the *Prx1-Cre* driver[15]. These conditional *Ext1* mutant mice displayed severe limb skeletal defects, including shortened and malformed limb bones, oligodactyly, and fusion of joints. In developing limb buds of these mutant mice, chondrogenic differentiation of mesenchymal condensations was delayed and impaired. In this case, alterations in BMP signaling were reported. In another study, Pacifici and co-workers analyzed conditional *Ext1* ablation in perichondrium and lateral chondrocytes flanking the epiphyseal region of mouse embryo long bones[27]. In line with our results, they observed increased chondrogenic potential in these *Ext1*-deficient mice, and they found enhanced BMP signaling. In 2017, Inubushi *et al.* generated a model of multiple hereditary exostoses in which EXT1 was ablated in progenitor cells in the perichondrium, but not in chondrocytes in the growth plate[28]. Again, they reported enhanced chondrogenic differentiation with development of osteochondromas in the mutant mice, in this case associated to changes in BMP signaling.

EXT1 and HS content impact the strength of the Wnt/β-catenin pathway

We found that defects in *Ext1* and the resulting reduction in HS lead to reduced Wnt/β-catenin pathway activation, and the opposite was observed when *Ext1* was overexpressed. To our knowledge, mechanistic evidence linking EXT1 and HS to Wnt/β-catenin signaling during chondrogenesis was missing. It is well established that the canonical Wnt signaling pathway regulates chondrogenesis in precursor cells[29]. Consistent with our data, low Wnt signaling activation promotes a chondrogenic differentiation program, while high Wnt signaling reduces chondrocyte differentiation[29].

In agreement with our results, mutations in *tout-velu*, the Drosophila orthologue of *Ext1*, disrupt Wingless signaling[30]. Some studies in *Drosophila* also revealed connections between HSPGs and

Wnt signaling. Genetic analyses of *Drosophila* mutants defective in the core proteins or in GAG biosynthesis indicated the requirement of HSPGs for Wnt signaling spreading[31]. Experiments showing that Wg (the best characterized *Drosophila* Wnt) can bind to heparin supports the idea that HSPGs regulate Wnt signaling possibly by maintaining the local concentration of Wnt proteins on the cell surface[32]. On the other hand, since Wg fails to move across a clone of cells defective in the synthesis of HSPGs, HSPGs also regulates the restrictive movement of Wnt in the extracellular space[31]. Here, we report that treatment with sodium chlorate that blocks N-sulfation of the GAG chains of HS, rescued the phenotype of *Ext1* overexpressing cells. In line with these findings, a *Drosophila* mutant defective in an enzyme required for N-sulfation of the GAG chains of HS exhibits impaired distribution and signaling of Wg[33]. Thus, HS modification by N-sulfation appears to be important in the regulation of Wnt activity during chondrogenesis.

Activation of Wnt signaling measured by a luciferase reporter assay without exogenous stimulation was very low in both *Ext1* overexpressing and control cells. Yet, a small increase was seen in total and active β-catenin levels. This apparent discrepancy could be partially explained by the different techniques applied, the level of analysis (reporter gene expression vs protein levels) but also because the overexpression model may have limited biological impact on endogenous Wnts as the amount of HSPGs may be sufficient to activate the cascade. This may not only apply to the reporter assay but also to other results in the overexpression studies where effect sizes were small compared to the knock-down experiments.

The regulatory loop between EXT1/HS and Wnt/β-catenin signaling that our study identified during chondrogenic differentiation may also play a role in osteoarthritis (OA) as this loop also appears to be present in human articular chondrocytes, but additional studies are required to provide clear evidence on this assumption. EXT1 may contribute to hyper-activation of the Wnt signaling pathway, a deleterious event for cartilage homeostasis and important mechanism in the pathogenesis of OA[11].

Our observations are currently restricted to an *in vitro* setting with several limitations. Overexpression and knock-down studies as presented here suffer from variation both within the same clone as in between cell lines. Hence, the biological relevance of the observed differences in the different *in vitro* experiments will need to be further assessed in more complex *ex* or *in vivo* models. Therefore, future studies are required to further demonstrate the presence of this regulatory mechanism in articular cartilage and its possible implications in OA.

Contributions

XW, SM and RJL were involved in study design, analysis of data and writing the manuscript. XW and FMFC were involved in data acquisition. All authors were involved in interpretation of data and revising the article and approved final content of the article.

Conflict of interest

Leuven Research and Development, the technology transfer office of KU Leuven, has received consultancy and speaker fees on behalf of RJL from Galapagos and Samumed, active in the field of osteoarthritis. All other authors declare that they have no competing interests.

Funding

XW received an unrestricted fellowship from UCB-Pharma to KU Leuven. The research was supported by a grant to RJL from the Flanders Research Foundation (FWO Vlaanderen).

Data and materials availability

The data supporting the findings of this study are available within the article and from the corresponding author on reasonable request.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.joca.2019.07.007.

References

1. Jochmann K, Bachvarova V, Vortkamp A. Heparan sulfate as a regulator of endochondral ossification and osteochondroma development. Matrix Biol 2014;34:55–63.
2. Lories RJ, Corr M, Lane NE. To Wnt or not to Wnt: the bone and joint health dilemma. Nat Rev Rheumatol 2013;9:328–39.
3. Usami Y, Gunawardena AT, Iwamoto M, Enomoto-Iwamoto M. Wnt signaling in cartilage development and diseases: lessons from animal studies. Lab Investig 2016;96:186.
4. Rudnicki JA, Brown AMC. Inhibition of chondrogenesis by Wnt gene expression in vivo and in vitro. Dev Biol 1997;185:104–18.
5. Hartmann C, Tabin CJ. Dual roles of Wnt signaling during chondrogenesis in the chicken limb. Development 2000;127:3141–59.
6. Wada N, Kawakami Y, Ladher R, Francis-West PH, Nohno T. Involvement of Frzb-1 in mesenchymal condensation and cartilage differentiation in the chick limb bud. Int J Dev Biol 1999;43:495–500.
7. Guo X, Day TF, Jiang X, Garrett-Beal L, Topol L, Yang Y. Wnt/β-catenin signaling is sufficient and necessary for synovial joint formation. Genes Dev 2004;18:2404–17.
8. Witte F, Dokas J, Neuendorf F, Mundlos S, Stricker S. Comprehensive expression analysis of all Wnt genes and their major secreted antagonists during mouse limb development and cartilage differentiation. Gene Expr Patterns 2009;9:215–23.
9. Akiyama H, Lyons JP, Mori-Akiyama Y, Yang X, Zhang R, Zhang Z, *et al.* Interactions between Sox9 and β-catenin control chondrocyte differentiation. Genes Dev 2004;18:1072–87.
10. Tamamura Y, Otani T, Kanatani N, Koyama E, Kitagaki J, Komori T, *et al.* Developmental regulation of Wnt/β-catenin signals is required for growth plate Assembly, cartilage integrity, and endochondral ossification. J Biol Chem 2005;280:19185–95.
11. Monteagudo S, Lories RJ. Cushioning the cartilage: a canonical Wnt restricting matter. Nat Rev Rheumatol 2017;13:670.
12. Esko JD, Selleck SB. Order out of Chaos: assembly of ligand binding sites in heparan sulfate. Annu Rev Biochem 2002;71:435–71.
13. Bernfield M, Götte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, *et al.* Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem 1999;68:729–77.
14. Pacifici M. The pathogenic roles of heparan sulfate deficiency in hereditary multiple exostoses. Matrix Biol 2018;71–72:28–39.
15. Matsumoto Y, Matsumoto K, Irie F, Fukushi J-i, Stallcup WB, Yamaguchi Y. Conditional ablation of the heparan sulfate-synthesizing enzyme Ext1 leads to dysregulation of bone morphogenic protein signaling and severe skeletal defects. J Biol Chem 2010;285:19227–34.
16. Cantley L, Saunders C, Guttenberg M, Candela ME, Ohta Y, Yasuhara R, *et al.* Loss of β-Catenin induces multifocal

periosteal chondroma-like masses in mice. Am J Pathol 2013;182:917–27.
17. Yao Y, Wang Y. ATDC5: an excellent in vitro model cell line for skeletal development. J Cell Biochem 2013;114:1223–9.
18. Guns L-A, Monteagudo S, Kvasnytsia M, Kerckhofs G, Vandooren J, Opdenakker G, *et al*. Suramin increases cartilage proteoglycan accumulation in vitro and protects against joint damage triggered by papain injection in mouse knees in vivo. RMD Open 2017;3, e000604.
19. Monteagudo S, Cornelis FMF, Aznar-Lopez C, Yibmantasiri P, Guns L-A, Carmeliet P, *et al*. DOT1L safeguards cartilage homeostasis and protects against osteoarthritis. Nat Commun 2017;8:15889.
20. Lee H-H, Lee J-Y, Shih L-H. Proper Fhl1 expression as Wnt signaling is required for chondrogenesis of ATDC5 cells. Anim Cell Syst 2013;17:413–20.
21. Miyamoto K, Ohkawara B, Ito M, Masuda A, Hirakawa A, Sakai T, *et al*. Fluoxetine ameliorates cartilage degradation in osteoarthritis by inhibiting Wnt/β-catenin signaling. PLoS One 2017;12. e0184388-e0184388.
22. Safaiyan F, Kolset SO, Prydz K, Gottfridsson E, Lindahl U, Salmivirta M. Selective effects of sodium chlorate treatment on the sulfation of heparan sulfate. J Biol Chem 1999;274:36267–73.
23. Holmes BB, DeVos SL, Kfoury N, Li M, Jacks R, Yanamandra K, *et al*. Heparan sulfate proteoglycans mediate internalization and propagation of specific proteopathic seeds. Proc Natl Acad Sci USA 2013;110:E3138–47.
24. Phamantu NT, Fagnen G, Godard F, Bocquet J, Bonnamy PJ. Sodium chlorate induces undersulfation of cellular proteoglycans and increases in FSH-stimulated estradiol production in immature rat Sertoli cells. J Androl 1999;20:241–50.
25. Huang S-MA, Mishina YM, Liu S, Cheung A, Stegmeier F, Michaud GA, *et al*. Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. Nature 2009;461:614.
26. Hilton MJ, Gutiérrez L, Martinez DA, Wells DE. EXT1 regulates chondrocyte proliferation and differentiation during endochondral bone development. Bone 2005;36:379–86.
27. Huegel J, Mundy C, Sgariglia F, Nygren P, Billings PC, Yamaguchi Y, *et al*. Perichondrium phenotype and border function are regulated by Ext1 and heparan sulfate in developing long bones: a mechanism likely deranged in Hereditary Multiple Exostoses. Dev Biol 2013;377:100–12.
28. Inubushi T, Nozawa S, Matsumoto K, Irie F, Yamaguchi Y. Aberrant perichondrial BMP signaling mediates multiple osteochondromagenesis in mice. JCI Insight 2017;2.
29. Day TF, Guo X, Garrett-Beal L, Yang Y. Wnt/β-Catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev Cell 2005;8:739–50.
30. Takei Y, Ozawa Y, Sato M, Watanabe A, Tabata T. Three *Drosophila* EXT genes shape morphogen gradients through synthesis of heparan sulfate proteoglycans. Development 2004;131:73–82.
31. Yan D, Lin X. Shaping morphogen gradients by proteoglycans. Cold Spring Harb Perspect Biol 2009;1.
32. Chakrabarti A, Matthews G, Colman A, Dale L. Secretory and inductive properties of Drosophila wingless protein in Xenopus oocytes and embryos. Development 1992;115:355–69.
33. Lin X, Perrimon N. Dally cooperates with Drosophila Frizzled 2 to transduce Wingless signalling. Nature 1999;400:281.