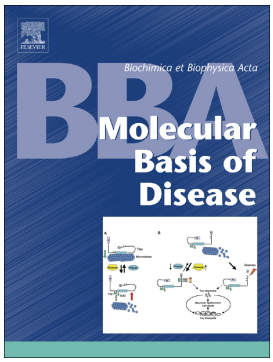

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Extracellular matrix content and WNT/β-catenin levels of cartilage determine the chondrocyte response to compressive load

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ABSTRACT

During osteoarthritis (OA)-development extracellular matrix (ECM) molecules are lost from cartilage, thus changing gene-expression, matrix synthesis and biomechanical competence of the tissue. Mechanical loading is important for the maintenance of articular cartilage; however, the influence of an altered ECM content on the response of chondrocytes to loading is not well understood, but may provide important insights into underlying mechanisms as well as supplying new therapies for OA. Objective here was to explore whether a changing ECM-content of engineered cartilage affects major signaling pathways and how this alters the chondrocyte response to compressive loading.

Activity of canonical WNT-, BMP-, TGF-β- and p38-signaling was determined during maturation of human engineered cartilage and followed after exposure to a single dynamic compression-episode. WNT/β-catenin- and pSmad1/5/9-levels declined with increasing ECM-content of cartilage. While loading significantly suppressed proteoglycan-synthesis and ACAN-expression at low ECM-content this catabolic response then shifted to an anabolic reaction at high ECM-content. A positive correlation was observed between GAG-content and load-induced alteration of proteoglycan-synthesis. Induction of high β-catenin levels by the WNT-agonist CHIR suppressed load-induced SOX9- and GAG-stimulation in mature constructs. In contrast, the WNT-antagonist IWP-2 was capable of attenuating load-induced GAG-suppression in immature constructs.

In conclusion, either ECM accumulation-associated or pharmacologically induced silencing of WNT-levels allowed for a more anabolic reaction of chondrocytes to physiological loading. This is consistent with the role of proteoglycans in sequestering WNT-ligands in the ECM, thus reducing WNT-activity and also provides a novel explanation of why low WNT-activity in cartilage protects from OA-development in mechanically overstressed cartilage.

Keywords: Mechanical loading, chondrocyte, SOX9, cell signaling, osteoarthritis

1. INTRODUCTION

The mechanical environment of articular cartilage plays an important role in regulating the development and maintenance of the tissue. Dynamic compressive loading can support extracellular matrix (ECM) synthesis of cartilage [1], whereas abnormal loading, such as disuse, static loading or altered joint biomechanics, can disrupt the ECM, soften the cartilage matrix [2,3], suppress the biosynthetic activity of chondrocytes [4] and lead to osteoarthritis (OA) [5]. Full thickness cartilage defects that are habitually loaded during the regeneration period, synthesize and deposit more proteoglycans into the new tissue. This demonstrates the benefit of mechanical challenge in tissue regeneration [6]. However, to better understand the mechanisms of OA development and to effectively utilize mechanical signals in the clinic as a non-drug-based intervention to improve cartilage regeneration in joint diseases, it is vital to better understand chondrocyte mechanotransduction. ECM molecules, especially proteoglycans, are lost from degenerating cartilage during OA-development. Furthermore, mechanical disuse is a major risk factor for disease progression. Thus, it is important to unravel how the ECM content of cartilage affects the response of chondrocytes to physical challenge. A better understanding of the role of the ECM in chondrocyte mechanotransduction could also enable the development of novel therapies for joint diseases like OA.

The mechanical compression of engineered cartilage holds great potential to study the response of human articular chondrocytes (HAC) to loading. *In vitro* studies established that chondrocytes respond to repetitive loading regimes by altering gene expression and matrix biosynthesis [7-13]. Importantly, in agarose constructs, the biological response to dynamic compression evolved as a function of time in culture where postponed loading appeared beneficial [4,8,14,15]. Whereas dynamic compressive loading of intact cartilage explants can stimulate proteoglycan synthesis immediately [1,16], the response of agarose-embedded chondrocytes not only depended upon the day of culture but was also enhanced when cells were surrounded by a pericellular ECM [8]. Thus, the presence of cartilaginous ECM as well as its content in the tissue is believed to play a critical role in the response of chondrocytes to mechanical signals, although the underlying mechanisms still remain unclear.

The association of 3D-culture with chondrocyte redifferentiation and accumulation of a proteoglycan-rich ECM is well established. However, how this affects the balanced activity of important signaling pathways in chondrocytes is largely unknown. The proliferation, differentiation as well as homeostasis of chondrocytes strongly depends upon the connection of structural matrix macromolecules with a broad range of growth factors. The common growth factors FGFs, TGF-βs and BMPs are stored in the ECM of healthy articular cartilage [17] and their release as well as interaction

with receptors is often influenced by a number of ECM molecules. For example, the proteoglycans aggrecan, biglycan and fibromodulin regulate TGF-β activity by sequestering TGF-β within the ECM [18]. Also BMPs, like BMP2, contain an N-terminal heparin-binding site whose interaction with immobilized heparin modulates the biological activity of BMP2 [19]. FGFs bind to heparin proteoglycans of the ECM which facilitate receptor interaction [20] and proteoglycans of the cartilage matrix like heparan sulfate can interact with WNT-ligands and inhibit its presentation to frizzled receptors [21]. Proteoglycans also contribute to strain shielding of the chondrocytes [22] as well as the conversion of mechanical loading into changes in interstitial osmolarity [23]. Thus, loading of diseased cartilage with an insufficient ECM content may evoke an unwanted cell response which may aggravate cartilage degeneration or damage immature engineered cartilage which was implanted in patients to treat cartilage defects. Immature engineered cartilage with low proteoglycan content is considered an *in vitro* model for OA cartilage which has lost part of its proteoglycans during disease progression. This study intends to address the open question of how changes in ECM content of cartilage may affect the functional response of chondrocytes to mechanical challenge.

Mechanotransduction involves the integration of multiple biophysical and biochemical signals including the changes in cellular strain, hydrostatic pressurization, fluid shearing, and tissue osmolarity [24]. Ion channels, integrin molecules, primary cilia and matrix-bound growth factors have all been implicated in transducing the extracellular biophysical signals into a complex intracellular response [25,26]. TGF-β-, FGF-2, intracellular calcium and ERK1/2-signaling pathways have been implicated in mechanotransduction in chondrocytes [27-29]. We recently undertook a genome-wide characterization of load-induced differential changes in gene-expression in mature engineered cartilage and observed a stimulation of TGF-β, BMP, ERK1/2, calcium-signaling and WNT-signaling response genes in association with enhanced glycosaminoglycan (GAG) synthesis [12]. The objective here was to explore how changing the ECM content of engineered cartilage affects major signaling pathways and how this alters the chondrocyte response to compressive loading. Of special interest was to identify molecular pathways relevant for stimulation of cartilage matrix synthesis in response to loading. This knowledge will foster our understanding into the role of the surrounding ECM on chondrocyte function and may provide novel insights into the mechanisms of OA disease progression. Additionally, it is has the potential to optimize the response of engineered or diseased cartilage to mechanical challenges to further the therapeutic potential that dynamic compression holds in stimulation of cartilage anabolism.

2. MATERIALS AND METHODS

2.1. Cell cultivation

Following written informed consent, articular cartilage was resected from tibia plateaus of patients undergoing total knee replacement. The study has been approved by the local ethics committee (Medical Faculty of Heidelberg). Macroscopically normal cartilage was minced and digested overnight with 1.5 mg/ml collagenase-B and 0.1 mg/ml hyaluronidase. Chondrocytes were washed, plated at 5700 cells/cm^2 and expanded for 2 passages in DMEM low-glucose, 10% fetal calf serum (FCS), 10 U/ml penicillin, 100 mg/ml streptomycin at 37°C, 6% CO_2. Medium was changed twice a week.

2.2. Dynamic compression

HAC (5 x 10^5) were seeded into a collagen carrier (Optimaix, Matricel GmbH, Herzogenrath, Germany: 4 mm diameter, 1.5 mm high) and then attached via a fibrin gel to β-TCP (RMS Foundation, Bettlach, Switzerland: 4 x 4 x 11 mm, 70% porosity) which was used as a bone replacement material to imitate vicinity to subchondral bone [12]. Biphasic constructs were prepared and cultured in chondrogenic medium containing 10 ng/ml TGF-β-1 as described previously [30] for the indicated time points. Constructs were first compressed by 10% of their thickness (= offset) to guarantee that the contact to the piston was never lost throughout cyclic compression. Starting from this 10% static offset, the amplitude of cyclic compression was 25% during the applied loading intervals (10 min) while a 10% static offset was applied during the break intervals (10 min) during which cyclic compression was interrupted, this was repeated nine times. A strain of 25% was selected according to Mosher et al. which describe 20-30% as being physiological strain magnitudes which occur during dynamic loading of human articular cartilage [31]. Controls were kept in the same loading device however no load was applied. In defined experiments engineered cartilage was exposed to the MAPK inhibitor U0126 (Merck Millipore, Billerica, MA, USA), the WNT inhibitor IWP-2 (Tocris Bioscience, Bristol, UK), the WNT/β-catenin inhibitor Dickkopf (recombinant human Dickkopf-1, R&D Systems, Minneapolis, MN, USA) the WNT/β-catenin agonist CHIR99021 (Tocris Bioscience, Bristol, UK) or the corresponding solvent (DMSO, PBS) before and during loading.

2.3. Metabolic labeling

ECM synthesis in mechanically challenged constructs was measured directly after loading. Engineered cartilage was detached from β-TCP by a scalpel and constructs were posed on a nylon mesh in a 48 well plate to allow labeling from all sides. Samples were immersed in 500 µl chondrogenic medium containing either 4 µCi $^{35}SO_4$ to measure glycosaminoglycan synthesis or 5 µCi

^{3}H-L-proline (ARC, St. Louis, MO, USA) to detect synthesis of proline-containing proteins. By this mostly the collagens will be labeled since they contain 38% of proline while other proteins contain only 5% of proline. Label incorporation was performed under standard conditions for 24 hours. After 5 washing steps in either 500 µl 1 mM Na_2SO_4 or 1 mM L-proline respectively in PBS for 20 min while shaking, samples were digested in 0.5 mg/ml Proteinase-K at 60°C and shaking with 800 rpm overnight. The incorporated label was quantified by β-scintillation counting using the program Winspectral. Radioactive label referred to the DNA content determined as described below.

2.4. Histology

Constructs were fixed in Bouin's solution for 2 days, dehydrated in graded 2-propanol series, embedded in paraffin, microsectioned in 5 µm slices and stained with Safranin O (0.2% in 1% acetic acid) and fast green (0.04% in 0.2% acetic acid) as described previously [30]. For collagen-type II immunohistology, antigen retrieval was performed with 4 mg/ml hyaluronidase (Merck, Darmstadt, Germany) and 1 mg/ml pronase (Roche Diagnostics, Mannheim, Germany). Blocking was performed with 5% BSA and sections were incubated overnight at 4°C with monoclonal mouse-anti-human collagen-type II antibody. Reactivity was detected using biotinylated goat anti-mouse secondary antibody, streptavidin-alkaline phosphatase (30 min, 20°C; Dako, Glostrup, Denmark) and fast red (Sigma-Aldrich, St. Louis, MO, USA).

2.5. Gene expression

Samples were harvested at the termination of loading, quick frozen in liquid nitrogen and total RNA was isolated from disintegrated samples by a phenol/guanidine isothiocyanate extraction (pegGOLD TriFast™, peqlab, Erlangen, Germany) and purified using Zymoclean™ Gel DNA Recovery Kit (ZymoResearch, Irvine, CA, USA). Samples were mixed with 3-fold volume of agarose dissolving buffer (ADB), loaded in Zymo-Spin columns, rinsed with washing-buffer and eluted in DNase/RNase-free H_2O. cDNA was synthesized from 500 ng total RNA using oligo(dT) primers and Omniscript reverse transcriptase (Qiagen, Hilden, Germany). Changes in gene expression were monitored by LightCycler® 96 (Roche Diagnostics, Mannheim, Germany) via detection of Absolute qPCR SYBR-Green-Mix (Thermo Fischer Scientific, Waltham, MA, USA). The PCR products were quality-checked by agarose gel electrophoresis and examination of melting curves. Primer sequences are summarized in Suppl. Table 1. Gene expression levels were normalized to the indicated housekeeping genes (HKGs) in order to minimize the putative gene expression alterations caused by differential expression of one specific HKG. The crossing point of the HKGs was determined for each cDNA sample, their mean was calculated and used to normalize all other genes of interest tested for the same cDNA sample. The number of cDNA copies was correlated with the apparent threshold cycle

(CT). Building the difference between CT of a gene of interest and CT of HKGs from one sample gives ∆CT values which were expressed as percentage of HKGs.

2.6. Mechanical testing

Unconfined indentation testing was done using the very-low-rubber hardness (VLRH)-test method at DIN ISO27588 (Digitest II, Bareiss, Oberdischingen, Germany) as described previously [30]. Sample hardness, given in VLRH units, was calculated from the difference in penetration depths that a ball indenter covers between an initial low contact force held for 5 s and a high total force held for 30 s. Several day 3 samples were too soft to obtain a hardness value above the detection threshold.

2.7. Biochemical analysis

For determination of proteoglycan and DNA content constructs were digested with 0.5 mg/ml Proteinase-K at 60°C while shaking at 800 rpm overnight. The GAG content within the digests was quantified by the 1,9-dimethylmethylene blue (DMMB) assay [32] with values deduced from a chondroitin-6-sulphate standard curve. DNA was quantified by Quant-iT™-PicoGreen® (Life Technologies, Carlsbad, California, USA). The GAG content of the sample was normalized to its DNA-level.

2.8. MTT-Assay

For determination of the cell viability in loaded and unloaded constructs, the engineered cartilage layer was separated from β-TCP at the end of the loading episode and transferred into a new 24-well plate. To determine mitochondrial cell activity samples were immediately labeled with MTT (Thermo Fischer Scientific, Waltham, MA, USA) at 37°C according to [33]. Extracted color was read at 530 nm.

2.9. Western blotting

After loading, constructs were harvested and flash frozen in liquid nitrogen before the cartilage layer was separated from β-TCP. Proteins were extracted in PhosphoSafe™ Extraction-Reagent (Merck Millipore, Billerica, MA, USA) containing 1% Pefabloc® SC (Sigma-Aldrich, St. Louis, MO, USA). 30-75 µg protein was separated on 10% polyacrylamide gels, blotted and stained as described [34]. Primary antibodies (Suppl. Table 2) were detected with HRP-conjugated goat-anti-mouse-IgG or HRP-conjugated goat-anti-rabbit-IgG (1:5000; Jackson ImmunoResearch, Suffolk, UK) using Lumi-Light Western Blotting substrate (Roche, Mannheim, Germany) or WesternBright ECL-HRP (Advansta, Menlo Park, CA, USA) substrate.

2.10. Statistical analysis

Data were analyzed with SPSS-22. Statistical tests were performed assuming non-parametric, non-paired samples. Values for corresponding unloaded control samples were set as 1. Comparison of compressed vs. uncompressed groups was performed using Mann-Whitney U and Student's T-test (unpaired). For time comparisons Kruskal-Wallis-test and Mann-Whitney U test were applied. Time lines were presented as mean ± standard deviation. Correlations were determined recording the Pearson's coefficient (r^2).

3. RESULTS

3.1. Pathway activity during maturation of engineered cartilage

Engineered cartilage with distinct ECM content was produced by pre-culturing constructs under differentiation conditions for either 3, 21 or 35 days. Tissue morphology, gene expression and GAG/DNA content were determined to confirm increasing matrix deposition and HAC re-differentiation over the pre-culture period. Histology demonstrated that in immature engineered cartilage (day 3) HAC were surrounded by little pericellular cartilage-like matrix and no interterritorial matrix was evident within the tissue. In contrast, by day 21, ample cartilage ECM had already been deposited and by day 35 a proteoglycan- and collagen-type-II-rich ECM had developed, which was nearly uniformly distributed throughout the tissue (Fig. 1A). Accumulation of GAG/DNA increased significantly (4.1-fold) from day 3 to day 21 and up to 5.3-fold from day 3 to day 35 (Fig. 1B). The raising of COL2A1 and ACAN gene expression (Fig. 1C) documented increasing redifferentiation of HAC. Engineered cartilage increased in hardness during maturation culture in a similar time course like the GAG/DNA content (Fig. 1D).

Samples were subjected to Western blot analysis in order to assess which signaling pathways changed in association with HAC redifferentiation. Interestingly, active WNT/β-catenin-levels increased during serial passaging of HAC in expansion cultures, while chondrocyte redifferentiation produced a steady decline in active β-catenin levels during tissue maturation (Fig. 2A). Remarkably, the chondrogenic master transcription factor SOX9 showed inverse protein levels to active β-catenin, with a decline during expansion and an upregulation over 3D maturation and ECM deposition (Fig. 2A). Levels of canonical BMP signaling also declined slowly during maturation cultures according to Western blot analysis for pSmad1/5 (Fig. 2B), while canonical TGF-β signaling (pSmad2/3; Fig. 2C) remained high, as expected for this TGF-β-containing differentiation medium. In contrast, the activity of phospho P38, associated with non-canonical TGF-β-signaling, was high during expansion and

remained thus unaltered during HAC dedifferentiation, however it dropped rapidly with a shift to re-differentiation conditions (Fig. 2D). Altogether, canonical WNT and canonical TGF-β/BMP signaling appear to be interesting parameters to follow during tissue maturation-dependent mechanical challenge.

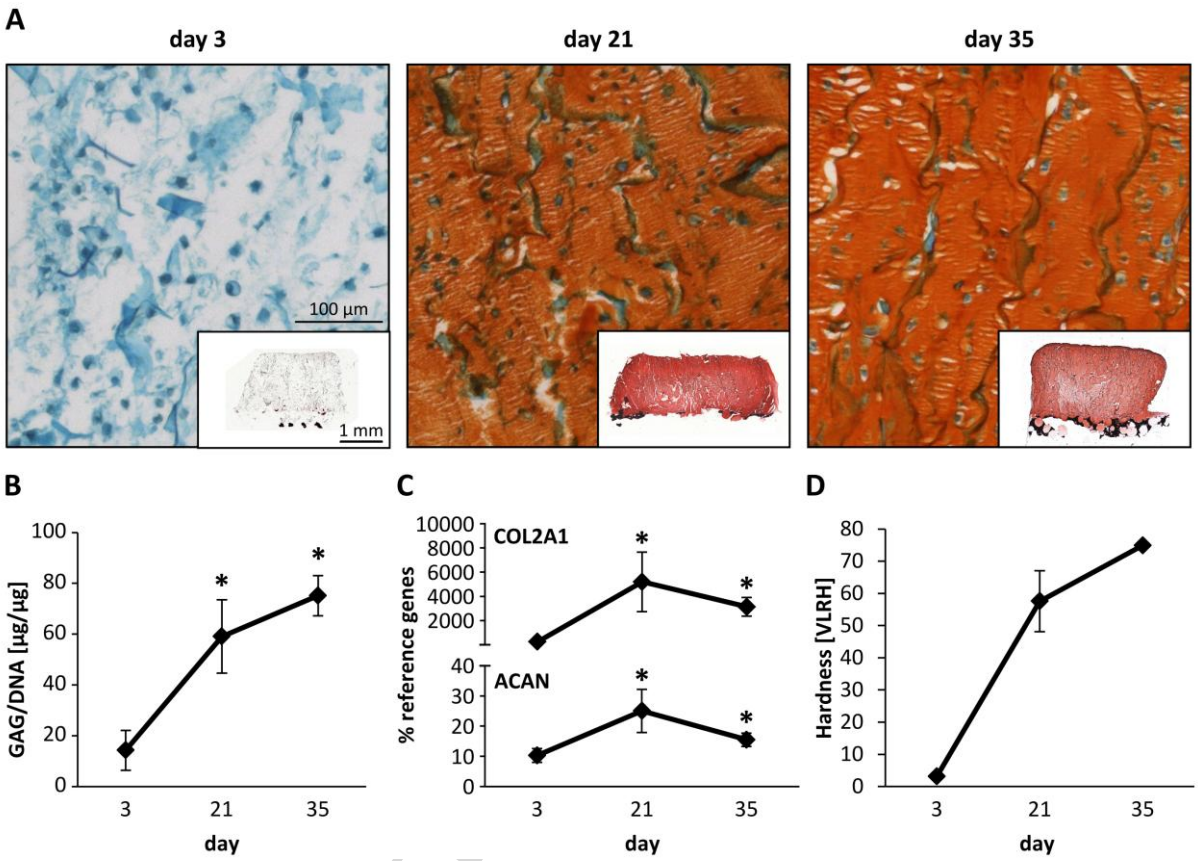

Figure 1 The influence of pre-culture time on matrix content, gene expression and hardness. Biphasic tissue engineering constructs seeded with $5x10^5$ human articular chondrocytes were cultured for 3, 21 and 35 days under chondrogenic conditions. (A) Standard paraffin sections were stained with Safranin O/Fast Green to visualize GAG deposition, inlay: total collagen type 2 staining. Black: left-over β-TCP; (representative pictures from n=3 donors each). (B) Mean GAG content ± SD of constructs measured by DMMB assay refers to the DNA content (n=3 donors). C) Gene expression ± SD is given as percentage of the mean levels of reference genes RPL13, HNRPH1 and CPSF6 (n=3 donors). Two-tailed, unpaired t-test, *$p < 0.05$, in comparison to day 3. D) Construct hardness over maturation culture in VLRH units (n = 7 samples, two donors).

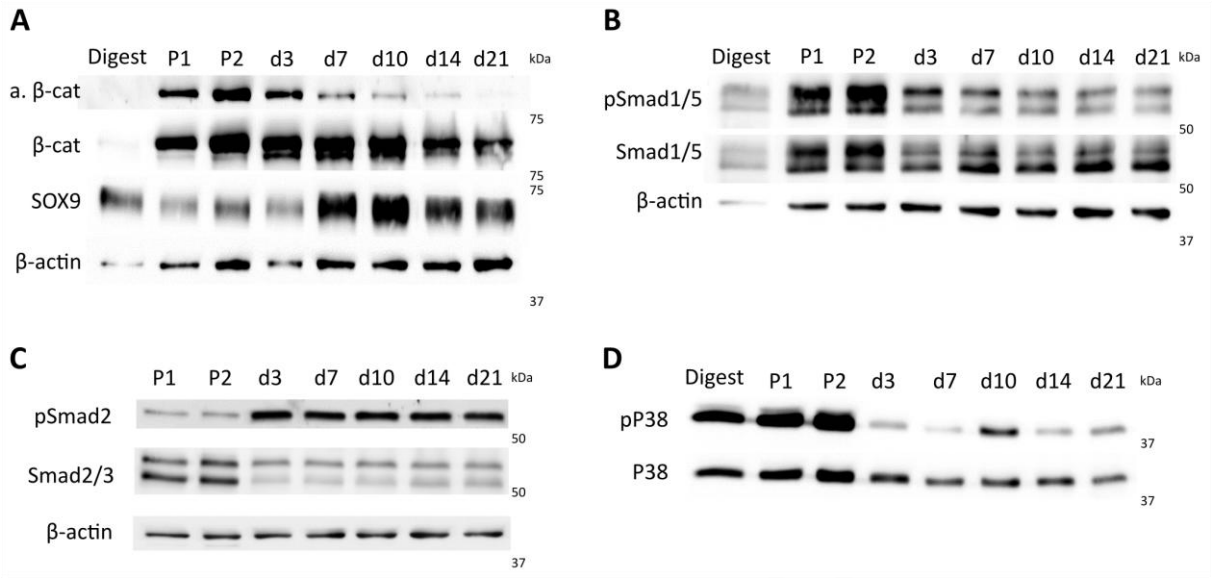

Figure 2 Western blot analysis of expanded chondrocytes up to passage 2 (P2) as well as during redifferentiation within constructs up to day 21 (d21) stained for (A) active (a.) and total β-catenin (β-cat), SOX9 and for β-actin used as a loading control. (B) Staining for phospho Smad1/5, total Smad1/5 and β-actin as loading control. (C) Staining for phospho Smad2, total Smad2/3 and β-actin as loading control. (D) Staining for phosphorylated P38 and total P38 used as a loading control. (n=3, 1 donor each).

3.2. Load-induced alterations in proteoglycan synthesis correlates with GAG content

Next, we tested the response of HAC to cyclic compressive loading in engineered cartilage of distinct ECM content. The experimental setup is depicted in Suppl. Fig. 1. When a single 3h dynamic loading episode was applied on day 3, GAG synthesis declined significantly (0.85-fold, $p < 0.05$), while a trend to more ^{35}S-sulfate-incorporation was obtained on day 21 (Fig. 3A). After 35 days, loading significantly enhanced ^{35}S-sulfate incorporation (1.45-fold, $p < 0.05$). Overall the GAG content of constructs positively correlated with load-induced stimulation of GAG synthesis ($r^2 = 0.84$; $p \leq 0.001$; Fig. 3B). ACAN gene expression was significantly downregulated by loading (mean 46%) at day 3 (Fig. 3C) while expression of the proteoglycan-synthesizing enzyme CHSY1 was significantly stimulated by loading at day 21 and 35. In contrast to proteoglycan production, synthesis of proline-rich proteins like collagens was barely affected at either pre-culture time, according to ^{3}H-prolin-incorporation (Suppl. Fig. 2A). COL2A1 gene expression dropped significantly at day 3, although it appeared to be less load-sensitive later on (Fig. 3C). An MTT assay confirmed that HAC experienced no general damage in the less ECM-protected HAC in day 3 constructs (Suppl. Fig. 2B). This same loading episode produced a downregulation of proteoglycan production and thus a catabolic response in engineered

cartilage with low ECM content, however in mature cartilage an anabolic reaction with stimulation of proteoglycan synthesis and CHSY1 expression occurred. Thus, ECM content appears to pre-determine whether a catabolic or anabolic cell response is produced with the same loading regime. This demonstrated that loading at low proteoglycan/ECM content can have negative consequences on cartilage matrix production.

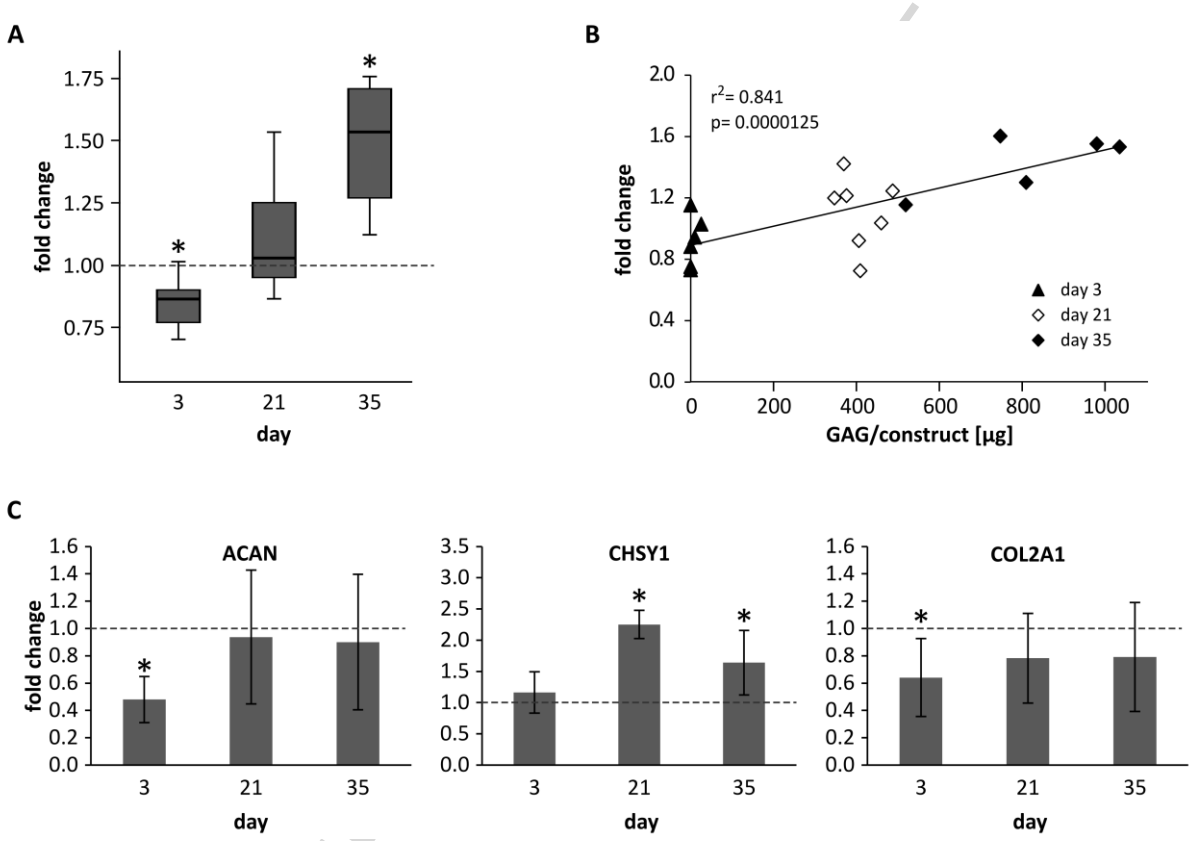

Figure 3 Load-induced alterations in proteoglycan synthesis and gene expression. (A) Changes in ^{35}S-sulfate incorporation into constructs over 24 hours following termination of loading. Values were normalized to the DNA content. Data of the compressed samples are shown relative to the uncompressed group indicated by dashed line set at 1. Boxes represent the interquartile range (IQR) and the median is shown as a horizontal line within each box. Whiskers extend to a maximum of 1.5 IQR. *p < 0.05 compressed vs. control, Kruskal-Wallis test with Mann-Whitney-U test post-hoc (n=6-8 samples, 3-4 donors). (B) A Pearson correlation between the fold-change of ^{35}S-sulfate incorporation in response to mechanical loading and the mean GAG content. Shown are the single values (n=18 samples, 3 donors) with pre-culture times indicated. (C) For gene expression analysis RNA was isolated from control and compressed samples at termination of loading and mRNA levels were determined by real-time qPCR. Data of the compressed samples (grey bars) are shown as mean ± SD relative to the uncompressed group indicated by dashed line set at 1. Two-tailed, unpaired t-test (n=3-5 samples, 3 donors). *p < 0.05.

3.3. Load-induced upregulation of SOX9 depends upon construct maturity

SOX9 is a master regulator for many cartilage-relevant ECM molecules including collagen type II and aggrecan, which is also induced by TGF-β and BMPs [35,36]. We next examined whether the differential regulation of SOX9 or the canonical TGF-β and BMP-signaling may explain the catabolic versus anabolic response of chondrocytes to loading in engineered cartilage of varying ECM content. Although SOX9 protein levels varied slightly on day 3 between different HAC donor populations, SOX9 was either unaltered or tended toward decreasing following loading in all immature constructs (n=4; Fig. 4A). In contrast, in the ECM-rich day 21 and 35 constructs, loading strongly enhanced SOX9 protein (n=3; Fig. 4B) and gene expression levels (Fig. 4C). Independent of construct maturity, BMP2 and BMP6 expression were significantly stimulated by compression, albeit with a lower mean amplitude at day 3 (3.1-fold) compared to day 21 (11.4-fold BMP2 / 41.2-fold BMP6; Fig. 4C). However, Western blotting analysis demonstrated no consistent load-induced changes of canonical BMP-signaling pSmad1/5/9 or of total Smad1/5 protein (n=3, Fig. 4D) but confirmed the decline of canonical BMP-signaling with increasing cartilage ECM deposition. Furthermore, canonical TGF-β signaling via pSmad2/3 was not altered after mechanical challenge (data not shown). P38-phosphorylation, involved in non-canonical TGF-β-signaling, was also not or very weakly affected by loading (Suppl. Fig. 2C). This suggests that the load-induced and ECM-dependent increase of SOX9 was not determined by changes in canonical/noncanonical TGF-β/BMP-signaling. Given that BMPs, TGF-β and pSmad2/3 respective pSmad1/5/9-signaling are known to induce SOX9 and stimulate cartilage matrix synthesis in HAC [30], we considered it unlikely that the lack of an early SOX9 response and the suppression of proteoglycan synthesis after loading at day 3 is due to the higher constitutive pSmad1/5/9 levels in constructs with low ECM content. Thus, we next focused on the WNT signaling activity.

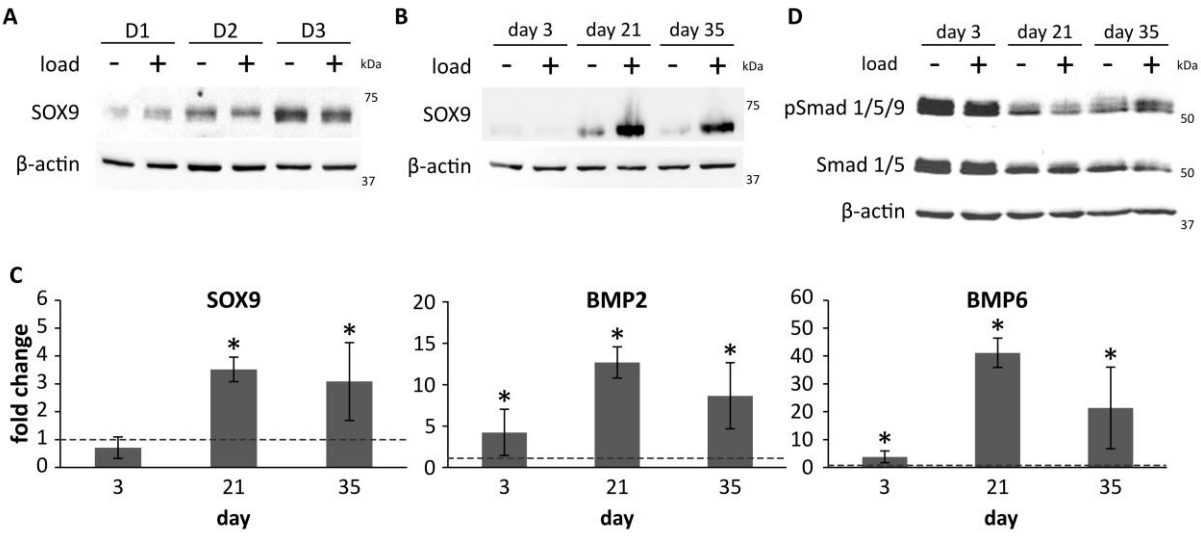

Figure 4 Regulation of SOX9 and canonical BMP signaling in response to loading. (A) Day 3 samples from three different donors (D1-D3) were exposed to loading, used for Western blot analysis and stained for SOX9, with β-actin as a loading reference. (B) Western blot analysis of samples exposed to loading after different pre-culture times. Control and compressed samples were stained for SOX9 with β-actin as a reference, (n=3, 3 donors). (C) For gene expression analysis RNA was isolated from control and compressed samples at the termination of loading and mRNA levels were determined by real-time qPCR. Data of the compressed samples (grey bars) are shown as mean ± SD relative to the uncompressed group indicated by dashed line set at 1. Two-tailed, unpaired t-test (n=3-5 samples, 3 donors), *$p < 0.05$. (D) Control and compressed samples were subjected to Western blot analysis, stained for phosphorylated Smad1/5/9, total Smad1/5 with β-actin as a reference. (n=2, 1 donor each).

3.4. β-catenin levels decline with increasing ECM deposition and determines the loading response

To assess whether compressive loading may affect canonical WNT-signaling and whether this depends upon construct maturity, active and total β-catenin levels were examined in all groups. No consistent load-dependent changes in total or active β-catenin-levels were observed (Fig. 5A), although constitutive β-catenin-levels declined with increasing ECM deposition as observed previously in Fig. 2. To evaluate whether differential WNT activity between ECM-poor and ECM-rich constructs may be a relevant reason for their distinct loading response, day 35 constructs were treated with the WNT-agonist CHIR prior to loading. Treatment strongly enhanced total and active β-catenin levels in chondrocytes (Fig. 5B). Remarkably, the load-induced upregulation of SOX9 was suppressed by this treatment (Fig. 5B) and GAG synthesis was no longer stimulated (Fig. 5C) with enhanced WNT/β-catenin levels indicating the regulation of SOX9 occurred downstream of WNT signaling.

To assess whether, on the opposite, WNT-suppression may rescue the load-induced drop of GAG synthesis at day 3, immature constructs were exposed to the WNT antagonists dickkopf or IWP-2. Commonly used antagonist doses applied over day 0 - 3 of the differentiation culture partially suppressed active β-catenin (Fig. 5D). When loading was then applied in the presence of IWP-2, it reverted the previously negative SOX9 protein response obtained after loading under control conditions (Fig. 5E). Remarkably, under IWP-2-treatment, the ^{35}S-sulfate incorporation/DNA was no longer suppressed by loading (Fig. 5F), suggesting that reduction of WNT-activity allowed for a higher GAG-synthesis in response to loading. Importantly, CHIR and IWP-2 treatment alone did not alter the

proteoglycan synthesis in the absence of mechanical challenge (Suppl. Fig. 2D). In conclusion, increased ECM deposition correlated with a silencing of WNT-activity in chondrocytes and the balance of WNT/β-catenin activity determined the degree by which loading was translated in the stimulation of matrix synthesis.

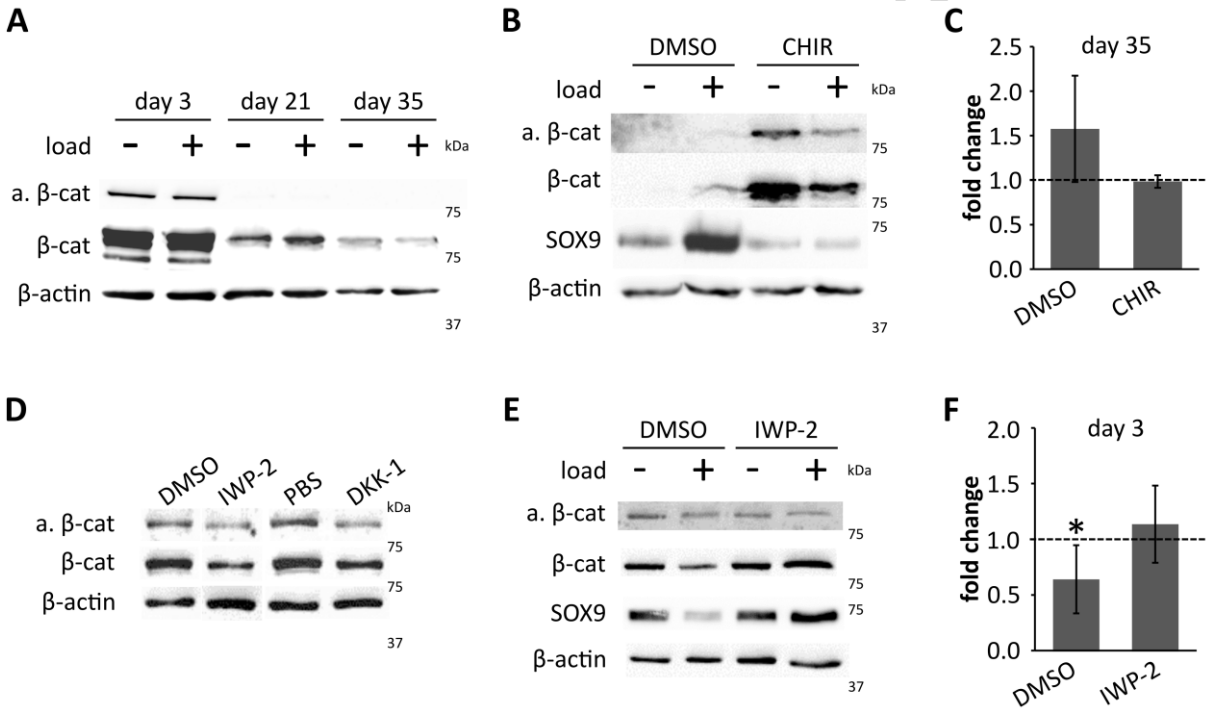

Figure 5 Canonical WNT signaling and load-induced SOX9 stimulation. (A) Western-blot analysis of samples exposed to loading after different pre-culture times. Control and compressed samples were stained for active (a.) and total β-catenin, β-actin was used as a reference, (n=4, 4 donors). (B) Constructs were exposed to 5 µM CHIR or 0.1% DMSO 24 hours prior to dynamic loading at day 35. Western-blot analysis of control and compressed samples stained for active (a.) and total β-catenin and SOX9; β-actin was used as a reference, (n=3, 3 donors). (C) Alteration of ^{35}S-sulfate-incorporation into day 35 constructs over 24 hours following termination of loading. Values were normalized to the DNA content. Control samples were set as 1. (D) Constructs were exposed to 2 µM IWP-2 or 0.1% DMSO or Dickkopf-1 (250 ng/ml solved in PBS) from day 0-3. Western blot analysis of uncompressed samples, with β-actin used as a reference. (E) Constructs were exposed to 2 µM IWP-2 or 0.1% DMSO from day 0-3 and subjected to dynamic loading on day 3. Western blot analysis with β-actin used as a reference, (n=3, 3 donors). (F) Alteration of ^{35}S-sulfate incorporation over 24 hours into treated day 3 constructs following termination of loading (n=4, 3 donors). Two-tailed, unpaired t-test (n=4, 3 donors), *$p < 0.05$.

4. DISCUSSION

The ECM of articular cartilage plays a critical role in physiological joint loading and the maintenance of articular cartilage function; however, the mechanisms by which mechanical loading is transduced into signals regulating chondrocyte homeostasis are not fully understood. In this study we show that WNT/β-catenin-levels are silenced with increasing ECM deposition and cartilage maturation in 3D cultures and have a critical influence on the response of chondrocytes to mechanical loading. HAC redifferentiation and the steadily increasing ECM deposition with time in 3D cultures dampened β-catenin-levels, while SOX9 protein levels rose. Early loading of ECM-poor constructs containing low basal SOX9 and high β-catenin levels provoked a suppression of proteoglycan synthesis. In contrast, the same loading episode in WNT/β-catenin poor mature constructs produced an anabolic response with significant stimulation of SOX9 and proteoglycan production. By manipulation of WNT/β-catenin activity levels via WNT agonists and antagonists prior to loading we provide here strong evidence that the balance of WNT-activity predetermines the biological response to loading regarding regulation of proteoglycan synthesis. High WNT activity prevented load-induced stimulation of SOX9 and allowed no stimulation of proteoglycan synthesis, thus, producing a response which is undesired not only in OA, but also for functional tissue engineering of cartilage and after chondrocyte-based cell therapy in patients. Thus, the WNT-balance in engineered cartilage correlated with ECM levels in the tissue and had the capacity to predetermine whether the complex changes during loading will or will not be orchestrated into an anabolic biological response.

Proteoglycans play a central role in governing poroelastic mechanics, electrically mediated swelling forces and stiffness of cartilage. While collagen remodeling is relatively slow in adult cartilage and requires years [37], proteoglycans are turned over much more rapidly within weeks [38]. Thus, proteoglycans represent a means of primary importance for the response to mechanical loads. This and the use of a collagen carrier for cartilage engineering may be the reason why the load-induced modulation of matrix synthesis was largely confined here to a proteoglycan response.

WNT signaling via β-catenin is one of the regulatory pathways that control chondrocyte formation during embryonic development. WNT gene expression was shown to inhibit chondrogenesis *in vivo* and *in vitro* [39,40]. Increased WNT-signaling caused cultured chondrocytes to decrease production of collagen type II, aggrecan and associated proteoglycans [41,42]. Negative effects of WNT-signaling on cartilage matrix production are thus well established. A link between WNT/β-catenin levels to HAC dedifferentiation, redifferentiation and ECM-deposition as well as an influence on the mechano-response of chondrocytes was not recognized prior to the current study. Recently it was reported that β-catenin limits SOX9 and inhibites chondrogenic differentiation during valve development and in aortic valve homeostasis, thus in a biomechanically challenged tissue [43]. Therefore, our observed

negative correlation between basal active β-catenin and SOX9 levels in dedifferentiating and redifferentiating chondrocytes is in line with such findings and altogether suggests location of canonical WNT-signaling upstream of SOX9, setting the stage for an anabolic versus catabolic loading response of chondrocytes depending upon the balance of WNT signaling.

WNTs are a family of 19 highly conserved morphogens. Engagement with their receptors results in signaling through several different pathways, of which the β-catenin-dependent or canonical WNT pathway is best understood. Which of the multiple WNT-proteins is serving as a signal during HAC dedifferentiation and whether antagonists are important for WNT/β-catenin up- and downregulation in this context is important to determine. This may be a complex task since the same WNT-ligand can have agonistic or antagonistic activity depending upon the presence of other WNT-ligands as shown for WNT16 [44]. We postulate that the main reason for higher active β-catenin in dedifferentiated chondrocytes may be the loss of ECM-production during monolayer expansion. Proteoglycans of cartilage matrix like heparan sulfate (HS) can interact with WNT-ligands and inhibit presentation to its frizzled receptors [21]. We postulate that at low HS concentrations, like those found in cartilage with low ECM content, the secreted WNT-ligands were insufficiently sequestered by cartilage matrix molecules and WNT-activity was therefore higher. Increasing deposition of GAG in the ECM during redifferentiation will then contribute to WNT silencing, fostering enhanced GAG synthesis in a positive feedback loop and would explain the observed positive correlation between GAG-content and anabolic loading response. Overall our data suggests that incoming signals from the ECM via mechanotransducers converge at some point on the WNT pathways whose delicate balanced activity is critical for cartilage maintenance and breakdown. This will decide whether an anabolic response to loading will be fostered or not.

The modulation of the mechanical loading response by WNT-activity levels is the most important novelty of this study. A delicate balance of WNT-activity is needed for cartilage homoeostasis as either repression or over activation of the β-catenin pathway leads to cartilage breakdown and OA [45,46]. Loss of function of the WNT inhibitor Frizzled-related-protein increased susceptibility to OA in humans [47] and mice [48]. Vice versa, antagonizing excessive canonical WNT-activation by deletion of WNT16 protected cartilage from breakdown in mice, in which OA was induced by destabilization of the medial meniscus and thus by exposing cartilage tissue to enhanced mechanical stress [44]. A decreasing expression of the WNT inhibitor Dkk-1 in joint tissues during development of OA has also linked WNT signaling to OA development [49]. Importantly, our study for the first time indicates that one explanation for the benefit of low WNT activity in mechanically overstressed cartilage tissue may be that it allows for a more anabolic loading response with enhanced proteoglycan synthesis while under higher WNT/β-catenin-activity suppressive effects may prevail.

Knowledge from our study can now enable designing optimal pre-conditioning strategies for engineered cartilage before implantation into cartilage defects to allow for early joint loading in patients and to develop novel therapies for joint diseases such as OA.

5. CONCLUSIONS:

This study demonstrates a correlation of WNT/β-catenin activity with HAC de- and re-differentiation and ECM deposition and establishes that the mechanical loading response of chondrocytes is modulated by WNT/β-catenin activity levels. High proteoglycan content in cartilage tissue as a potential cause for low WNT activity allowed for a beneficial loading response and this correlation provides a novel potential mechanism why low WNT activity in cartilage tissue may protect from OA development in overstressed cartilage tissue.

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COMPETING INTERESTS

The authors have no competing interests to declare.

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HIGHLIGHTS

- ECM content of engineered cartilage correlates with WNT/β-catenin activity
- High proteoglycan content in cartilage and low WNT-activity correlate with a beneficial loading response
- The mechanical loading response is determined by WNT/β-catenin activity levels
- Role of proteoglycans in sequestering WNT ligands as novel potential mechanism why low WNT-activity in cartilage may protect from OA