PHYSIOLOGICAL STRETCHING INDUCES A DIFFERENTIAL EXTRACELLULAR MATRIX GENE EXPRESSION RESPONSE IN ACETABULAR LABRUM CELLS

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Abstract

The acetabular labrum is a fibrocartilaginous ring surrounding the acetabulum and is important for hip stability and contact pressure dissipation through a sealing function. Injury of the labrum may contribute to hip-joint degeneration and development of secondary osteoarthritis. Understanding how extracellular matrix (ECM) production and remodelling is regulated is of key importance for successful tissue restoration. The present study hypothesised that physiological stretching enhanced the metabolic activity and altered the ECM gene expression in labrum cells. Primary bovine labrum cells were physiologically stretched for up to 5 d. 24 h after the last stretch cycle, changes in metabolic activity were measured using the PrestoBlue™ HS Cell Viability Reagent and ECM gene expression was examined using the quantitative polymerase chain reaction method. Targets of interest were further investigated using immunofluorescence and enzyme-linked immunosorbent assay. Metabolic activity was not affected by the stretching (0.9746 ± 0.0614, $p > 0.05$). Physiological stretching upregulated decorin (*DCN*) (1.8548 ± 0.4883, $p = 0.002$) as well as proteoglycan 4 (*PRG4*) (1.7714 ± 0.6600, $p = 0.029$) and downregulated biglycan (*BGN*) (0.7018 + 0.1567, $p = 0.008$), cartilage oligomeric matrix protein (*COMP*) (0.5747 ± 0.2650, $p = 0.029$), fibronectin (*FN1*) (0.5832 ± 0.0996, $p < 0.001$) and spondin 1 (*SPON1*) (0.6282 ± 0.3624, $p = 0.044$) gene expression. No difference in PRG4 and DCN abundance or release could be measured. The here identified mechanosensitive targets are known to play relevant roles in tissue organisation. Therefore, physiological stretching might play a role in labrum tissue homeostasis and regeneration.

Keywords: Labrum, hip, tissue remodelling, regeneration, extracellular matrix organisation, mechanical loading, stretching, cyclic tensile strain, mechanobiology, gene expression.

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List of abbreviations

ACAN	aggrecan
ACTB	actin beta
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
ASPN	asporin
BGN	biglycan
COL10A1	collagen type X alpha 1 chain
COL1A1	collagen type I alpha 1 chain
COL2A1	collagen type II alpha 1 chain
COL3A1	collagen type III alpha 1 chain
COL9A1	collagen type IX alpha 1 chain
COMP	cartilage oligomeric matrix protein
DAPI	4',6-diamidino-2-phenylindol
DCN	decorin
DMEM	dulbecco's modified eagle medium
DPT	dermatopontin
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
FMOD	fibromodulin
FN1	fibronectin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
MMP	matrix metalloproteinase
MYOC	myocilin
OGN	osteoglycin
OMD	osteomodulin
PRG4	proteoglycan 4 (or lubricin)

RGD	arginine-glycine-aspartate
SLRP	small leucine-rich proteoglycan
SOX9	sry-box transcription factor 9
SPARC	secreted protein acidic and cysteine rich (or osteonectin)
SPON1	spondin 1
SPP1	secreted phosphoprotein 1 (or osteopontin)
TGFB1	transforming growth factor β
TIMP	metalloproteinase inhibitor
TNC	tenascin C
TNXB	tenascin XB
VCAN	versican
YWHAZ	tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein zeta

Introduction

The fibrocartilaginous labrum is a horseshoe-shaped structure that provides an essential sealing and stabilising function to the human hip joint (Ferguson *et al.*, 2000; Ferguson *et al.*, 2003; Smith *et al.*, 2011). Any injury to this tissue structure may result in hip instability and concomitant cartilage damage and have direct consequences for the subsequent degeneration of the joint (Crawford *et al.*, 2007; Ferguson *et al.*, 2003; Leunig-Ganz, 2008; McCarthy *et al.*, 2003; Smith *et al.*, 2011).

The creation of novel (tissue-) engineered grafts requires profound knowledge of the tissue being replaced in order to fully restore its function. The mechanical properties of a labrum graft should ideally match those of the healthy labrum tissue to restore the mechanical as well as the mechanobiological functions (Ferguson *et al.*, 2001; Ishiko *et al.*, 2005; Kawamura *et al.*, 2021; Smith *et al.*, 2009). During certain daily activities, the labrum tissue is subjected to loads that result in substantial tissue deformation (Dy *et al.*, 2008; Safran *et al.*, 2011). In the case of degradable graft solutions, initial mechanical support is provided by the graft material itself. Long-term mechanical integrity, however, requires the cells to be metabolically active and to produce an ECM that resembles the native tissue structure and is able to sustain the local mechanical loads. Therefore, understanding how ECM production and remodelling is regulated is of key importance for successful tissue restoration.

The present study centred on the response of acetabular labrum cells to long-term stretching, with a specific focus on metabolic activity and expression of genes coding for ECM structural and remodelling proteins. The study hypothesised that physiological stretching (1) enhanced the metabolic activity and (2) altered the ECM gene expression in labrum cells. An anabolic gene-expression response to physiological stretching was expected and the study aimed to identify novel targets with mechanosensitive gene expression in labrum cells. The cyclic tensile strain was applied at a frequency of 1 Hz, simulating gait frequency, and a maximum strain of 10 %. Regarding mechanical properties of the labrum tissue, 10 % strain lies in the non-linear (toe region) or linear elastic region of the stress-strain curve (Ferguson *et al.*, 2001; Ishiko *et al.*, 2005) and is not expected to induce any tissue damage. Recent studies have suggested that local strains of around 10 % lie within the range of physiological loading of the labrum tissue. Manoeuvring of human hip cadaver specimens through 12 physiological positions axially loaded with 445 N revealed an average tensile strain of 5 % in the anterior labrum in both the axial and circumferential directions, with maximum strains of 13.6 % and 8.4 %, respectively (Dy *et al.*, 2008). A subsequent descriptive laboratory study extended the scope of the loading manoeuvres to 36 movements and measured the circumferential strain in the anterior, posterior and lateral acetabular labrum using differential variable reluctance transducers (Safran *et al.*, 2011). The findings emphasise the important role that strain levels as high as 10 % could play during normal activity.

Human labrum cells sense and respond to 10 % cyclic tensile strain by changes in gene expression levels (Kawamura *et al.*, 2021). Several *in vitro* and *in vivo* studies on the articular cartilage and the meniscus (morphologically similar fibrocartilaginous tissue to that of the labrum) have demonstrated that mechanical factors play a critical role in the tissue development, homeostasis, degeneration and regeneration (Carter *et al.*, 2004; McNulty and Guilak, 2015). The genes investigated in the present study have previously been shown to be mechanically regulated in cartilage, meniscus or labrum (*e.g. SOX9*, *PRG4*, *COMP*, *FN1*, *COL2A1*, *COL1A1*, *MMP1*, *MMP3*, *MMP9*) (Bleuel *et al.*, 2015b; Kawamura *et al.*, 2021; McNulty and Guilak, 2015), to be associated with chondrogenesis (*e.g. SOX9*, *COL2A1*, *COL9A1*, *COMP*, *ACAN*) (Bleuel *et al.*, 2015b; De Crombrugghe *et al.*, 2000), to be involved in fibrillogenesis (*e.g. COL1A1*, *DCN*, *BGN*, *FMOD*) (Mead *et al.*, 2018; Schaefer and Iozzo, 2008), to prevent fibrocartilaginous tissue from heterotopic ossification (*e.g. ADAMTS-12*) (Mead *et al.*, 2018) and/or to be dysregulated in osteoarthritis (*e.g. ADAMTS-4*, *ADAMTS-5*) as well as osteoarthritic labrum cells (*e.g. OMD*, *OGN*, *ASPN*, *TIMP-1*, *MMP1*) (Juchtmans *et al.*, 2015; Schon *et al.*, 2020; Verma and Dalal, 2011). Some of these targets are further known to modulate mechanical tissue properties. For instance, *ACAN* has been shown to increase the cartilage tissue resistance to dynamic compressive mechanical loading (Kiani *et al.*, 2002), *DCN* to increase the tensile stiffness of collagen type I-rich tissues (Reese *et al.*, 2013) and *PRG4* to reduce frictional properties of articular surfaces (Swann *et al.*, 1985). Therefore, changes in the expression of selected genes might indicate an adaptation of the tissue composition, structure and mechanical properties to stretching. Thereby, the present study provides

Table 1. TaqMan primer list. Most of the selected genes are coding for matrix proteins, including collagens, proteoglycans and glycoproteins. Other genes are coding for matrix-protein-degrading enzymes, including ADAMTs and MMPs as well as MMP inhibitory TIMPs.

Gene symbol	Gene name	TaqMan Assay ID
COL1A1	Collagen type I alpha 1 chain	Bt01463861_g1
COL2A1	Collagen type II alpha 1 chain	Bt03251861_m1
COL3A1	Collagen type III alpha 1 chain	Bt03249906_m1
COL9A1	Collagen type IX alpha 1 chain	Bt07108096_g1
COL10A1	Collagen type X alpha 1 chain	Bt03215582_m1
FN1	Fibronectin 1	Bt00415008_m1
SPARC	Secreted protein acidic and cysteine rich (or osteonectin)	Bt03214620_m1
SPP1	Secreted phosphoprotein 1 (or osteopontin)	Bt03213107_m1
SPON1	Spondin 1	Bt03259574_m1
MYOC	Myocilin	Bt03230953_m1
TNC	Tenascin C	Bt03253327_m1
TNXB	Tenascin XB	Bt03215806_m1
ASPN	Asporin	Bt03210983_m1
BGN	Biglycan	Bt03244532_m1
DCN	Decorin	Bt03230914_m1
FMOD	Fibromodulin	Bt03212664_m1
OGN	Osteoglycin	Bt03259135_m1
OMD	Osteomodulin	Bt03211983_m1
ACAN	Aggrecan	Bt03212186_m1
PRG4	Proteoglycan 4 (or Lubricin)	Bt04299827_m1
VCAN	Versican	Bt03217632_m1
COMP	Cartilage oligomeric matrix protein	Bt04299192_g1
DPT	Dermatopontin	Bt03237981_m1
TIMP1	Metalloproteinase inhibitor 1	Bt03223721_m1
TIMP2	Metalloproteinase inhibitor 2	Bt03231007_m1
TIMP3	Metalloproteinase inhibitor 3	Bt03237487_m1
ADAMTS12	A disintegrin and metalloproteinase with thrombospondin motifs 12	Bt04293260_m1
ADAMTS4	A disintegrin and metalloproteinase with thrombospondin motifs 4	Bt03224697_m1
ADAMTS5	A disintegrin and metalloproteinase with thrombospondin motifs 5	Bt04230785_m1
MMP1	Matrix metalloproteinase 1	Bt03212825_m1
MMP13	Matrix metalloproteinase 13	Bt03214050_m1
MMP2	Matrix metalloproteinase 2	Bt03216009_m1
MMP3	Matrix metalloproteinase 3	Bt04259490_m1
MMP9	Matrix metalloproteinase 9	Bt03215999_m1
TGFB1	Transforming growth factor beta-1 proprotein	Bt04259484_m1
TGFB2	Transforming growth factor beta-2 proprotein	Bt03276349_m1
TGFB3	Transforming growth factor beta-3 proprotein	Bt03272217_m1
SOX9	SRY-box transcription factor 9	Bt07108872_m1
ACTB	Actin beta	Bt03279174_g1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Bt03210913_g1
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	Bt01122444_g1

preliminary insights into the mechanoregulation of the labral ECM.

Materials and Methods

Labrum cell isolation and culture

A portion of the superior labrum from bovine hip joints (N = 6; 410-511 d old) was collected and minced into small tissue fragments. Minced tissue was digested using a two-step enzymatic digestion. Briefly, minced labrum tissue was incubated for 90 min at 37 °C with DMEM/F12 (DMEM; 11330032, Gibco) supplemented with 2 % Gibco™ Antibiotic-Antimycotic and 0.4 % pronase (53702-250KU, Merck Millipore), then centrifuged and washed using PBS, followed by the second digestion for 15-18 h at 37 °C in DMEM/F12 with 2 % Antibiotic-Antimycotic and 0.3 % collagenase type II (17101015, Gibco). Both digestion steps were carried out in a standard cell culture incubator (37 °C, 5 % CO_2, 95 % humidity). A single-cell suspension was obtained by filtration through a 100 µm cell strainer (542000, Greiner Bio-One). Cells were centrifuged, washed and seeded into polystyrene tissue culture flasks (90026, TPP, Trasadingen, Switzerland) at a density of 1,200 cells/cm^2 and cultured using DMEM/F-12, supplemented with 10 % FCS and 1 % Antibiotic-Antimycotic at 37 °C and 5 % CO_2. Cells at passage 1-4 were used for the stretching experiments.

Stretching experiments

Polydimethylsiloxane stretch chambers (Strex, Osaka, Japan) were coated with 100 µg/mL rat tail type I collagen (Collagen, Type I solution from rat tail, C3867, Sigma-Aldrich). After complete evaporation overnight (18-20 h), chambers were washed with PBS and cells were seeded (3,000 cells/cm^2) and cultured using low serum culture medium (DMEM/F-12, 1 % Antibiotic-Antimycotic, 2 % FCS, 1 % ITS-G). After 3 d, chambers were assigned to two groups: static culture and 4 h stretching per day for 4-5 d (10 % uniaxial cyclic tensile strain, 1 Hz). 4 h of cyclic tensile strain was reported to be sufficient to induce a gene expression response in meniscus-derived cells (Kanazawa *et al.*, 2012; Upton *et al.*, 2006). To apply the stretching, the chambers were mounted on a commercial stretching bioreactor (STB-140-10, STREX, Osaka, Japan) placed in a standard incubator. For both conditions (stretched and static), 6 biological replicates with 2 technical replicates were used per outcome measure and were analysed 24 h after completion of the last stretching cycle to investigate the long-term response to repeated stretching.

Metabolic activity assay

24 h after completion of the last stretching cycle, the cells in the chambers were incubated with PrestoBlue™ HS Cell Viability Reagent (P50200, Thermo Fisher Scientific) diluted 1:10 in culture medium for 1.5 h at 37 °C in a standard cell culture incubator, according to manufacturer's instructions. Fluorescence intensity of the collected supernatant was measured using a plate reader (Infinite M200 PRO, TECAN) and fluorescence was quantified at 560/590 nm (excitation/emission).

Gene expression analysis

24 h after completion of the last stretching cycle, the cells were rinsed with cold PBS and lysed in 1 mL GENEzol (GZR100, Geneaid, New Taipei City, Taiwan). RNA was extracted using a combined GENEzol and PureLink RNA Mini Kit (12183025, Invitrogen) approach. Briefly, phenol-chloroform extraction was performed to separate RNA from DNA and proteins, following the manufacturer's recommendations. The RNA-containing phase was transferred to a PureLink RNA Mini Kit column and the manufacturer's protocol was followed to wash and elute the RNA. RNA yield and purity were measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). RNA extracts from technical replicates were pooled. Then, the TaqMan Reverse Transcription kit (N8080234, Applied Biosystems) was used to reverse transcribe the total RNA (500-1,000 ng) into cDNA in 60 µL volumes. Gene expression of stretched and non-stretched bovine labrum cells was assessed using custom TaqMan® Array Fast Plates (4413257, Thermo Fisher) and following the manufacturer's recommendations (Table 1). Briefly, 5 µL of cDNA (15 ng diluted in RNAse-free water) was combined with 5 µL of the TaqMan Fast Universal PCR Master Mix (2×) (#4352042, Thermo Fisher). The CFX96 Touch Detection System (Bio-Rad) was used to measure the gene expression.

DCN and PRG4

24 h after the last load cycles, cells were washed with sterile PBS and fixed in ice-cold methanol for 10 min at – 20 °C. Cells were blocked and stained using the goat anti-mouse Alexa Fluor™ 488 Tyramide SuperBoost™ Kit (B40912, Thermo Fisher Scientific) according to manufacturer's instructions. Incubation

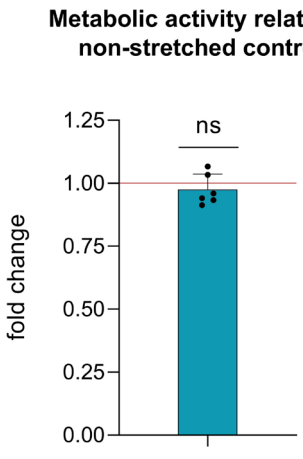

Fig. 1. Metabolic activity of stretched compared to non-stretched labrum cells. A fold change of 1 indicates no change in metabolic activity.

with 1:1,000 mouse monoclonal IgG anti-proteoglycan 4 antibody (clone 5C11, MABT400, EMD Millipore Corporation, USA) or 1:200 mouse monoclonal [DCN/6289] anti-decorin antibody (antibodies.com, # A278161) was carried out overnight at 4 °C. Cells were imaged using an Olympus IX51 microscope fitted with a 20× objective lens.

The collected culture medium was centrifuged for 15 min at 1,000 ×*g* and the supernatants were used for analysis. Cells were collected by trypsinisation, centrifuged for 15 min at 1,000 ×*g*, re-suspended in PBS and used for analysis. Commercially available ELISA kits (MBS744269 and MBS1605212, MyBioSource, Inc., San Diego, CA, USA) were used for the quantitative detection of DCN and PRG4 by strictly following the manufacturer's protocol.

Data analysis

GraphPad Prism 8 (GraphPad Software Inc.) for data visualisation and statistical analysis. All data are presented as fold change of stretched with respect to non-stretched controls and presented as mean ± standard deviation. Gene expression data were analysed by the 2ΔΔCt method (Livak and Schmittgen, 2001). Results are shown as $2^{-\Delta\Delta Ct}$, representing gene expression fold changes relative to the housekeeping genes and to the non-stretched control condition. The selected housekeeping genes *GAPDH*, *ACTB* and *YWHAZ* were confirmed to be unaffected by the treatment condition. ΔCt data distribution was assessed using the two-sided Shapiro-Wilk test and QQ-plots. Differences between conditions in each gene (ΔCts) were assessed using either two-sided paired *t*-tests or non-parametric Wilcoxon matched-pairs signed rank test. Statistical significance was defined as $p < 0.05$.

Results

Metabolic activity

Overall metabolic activity was not affected by stretching, with a fold change of 0.9746 ± 0.0614 in stretched compared to non-stretched cells ($p > 0.05$) (Fig. 1).

Gene expression analysis

Gene expression analysis showed that all the selected ECM genes were expressed in labrum cells, with the highest expression found for *COL1A1* and *COL3A1* and the lowest expression for *COL9A1* and *COL10A1* (Fig. 2). Stretching induced a differential ECM gene expression response, including an increase in *DCN* (1.8548 ± 0.4883, $p = 0.002$) and *PRG4* (1.7714 ± 0.6600, $p = 0.029$) and a decrease in *BGN* (0.7018 ± 0.1567, $p = 0.008$), *COMP* (0.5747 ± 0.2650, $p = 0.029$), *FN1* (0.5832 ± 0.0996, $p < 0.001$) and *SPON1* (0.6282 ± 0.3624, $p = 0.044$) expression levels (Fig. 3).

Protein analysis of PRG4 and DCN

PRG4 and DCN proteins were detected intracellularly in both stretched and non-stretched controls (Fig. 4**a**). There was no significant difference in intracellular content and total release of PRG4 and DCN measured between stretched and non-stretched cells ($p > 0.05$) (Fig. 4**b**).

Discussion

Understanding how matrix production and remodelling is regulated is of key importance for successful labrum tissue restoration. The present study investigated the effects of long-term stretching

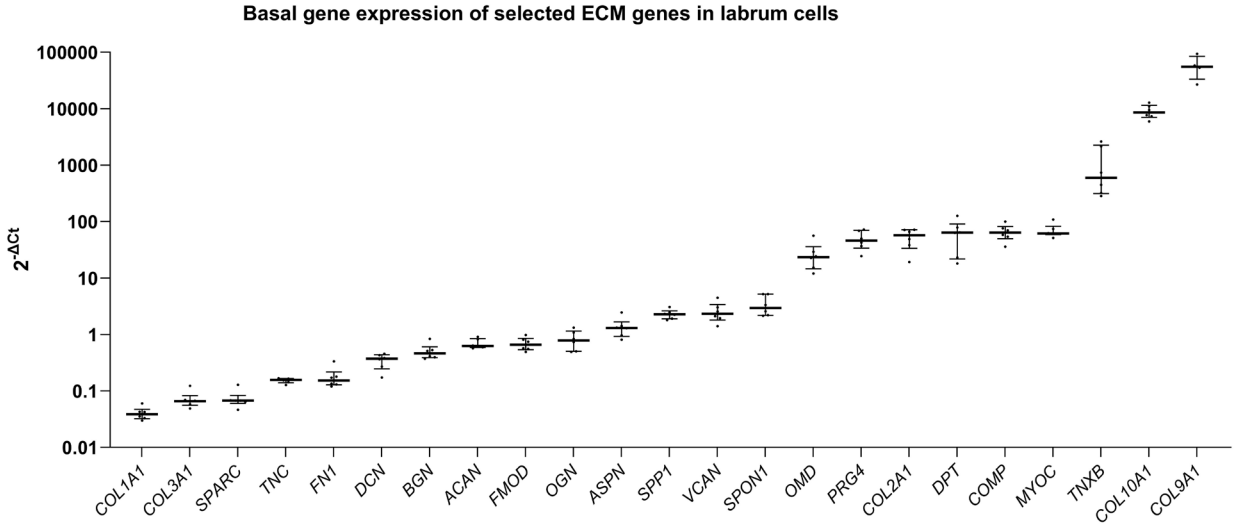

Fig. 2. Basal gene expression of selected ECM genes in labrum cells (non-stretched control), represented by median and interquartile range. Small values indicate high gene expression levels and large values indicate low gene expression levels. Values below 1 indicate a gene expression below the housekeeping gene expression and values above 1 indicate a higher gene expression compared to the housekeeping gene.

on metabolic activity and ECM gene and protein expression. 6 ECM-related genes were identified with a mechanosensitive gene expression (Fig. 3) that have not been reported in labrum cells previously.

Metabolic activity

Recent findings have suggested a highly active phenotype in human labrum cells, similar to meniscus cells and higher than in chondrocytes (Dhollander *et al.*, 2012). Physiological dynamic long-term stretching did not have any significant effects on the overall cell metabolic activity.

Basal ECM gene expression and mechanical influence on chondrogenic genes

Fibrocartilaginous tissues consist of a mixture of hyaline cartilage and dense fibrous connective tissue. Recent findings have suggested that healthy or non-degenerated labrum tissue is more closely related to tough fibrous tissue rather than to articular cartilage tissue (Kawamura *et al.*, 2021). In line with the literature on human labrum cells (Dhollander *et al.*, 2012), high levels of *COL1A1* and low levels of *COL2A1* were found (Fig. 2), indicating that the labrum is populated with fibro-chondrocytes or fibroblast-like cells rather than with chondrocytes. This was further supported by the finding that chondrogenic genes were expressed at lower levels than *COL1A1*, with especially low levels of *COL9A1* (Fig. 2). Therefore, not only cartilage but also more specific labrum ECM markers should be considered when evaluating novel (tissue-) engineering applications for labrum reconstruction.

A balance of pro-chondrogenic and pro-fibrogenic factors might be required to maintain the fibrocartilaginous state of the labrum tissue. A recent study suggested that stretching of human labrum cells might promote cartilage matrix metabolism, indicated by an increase in *SOX9* and *COL2A1* expression (Kawamura *et al.*, 2021). In contrast, the present study results did not show a strong effect of long-term cyclic stretching on chondrogenic gene

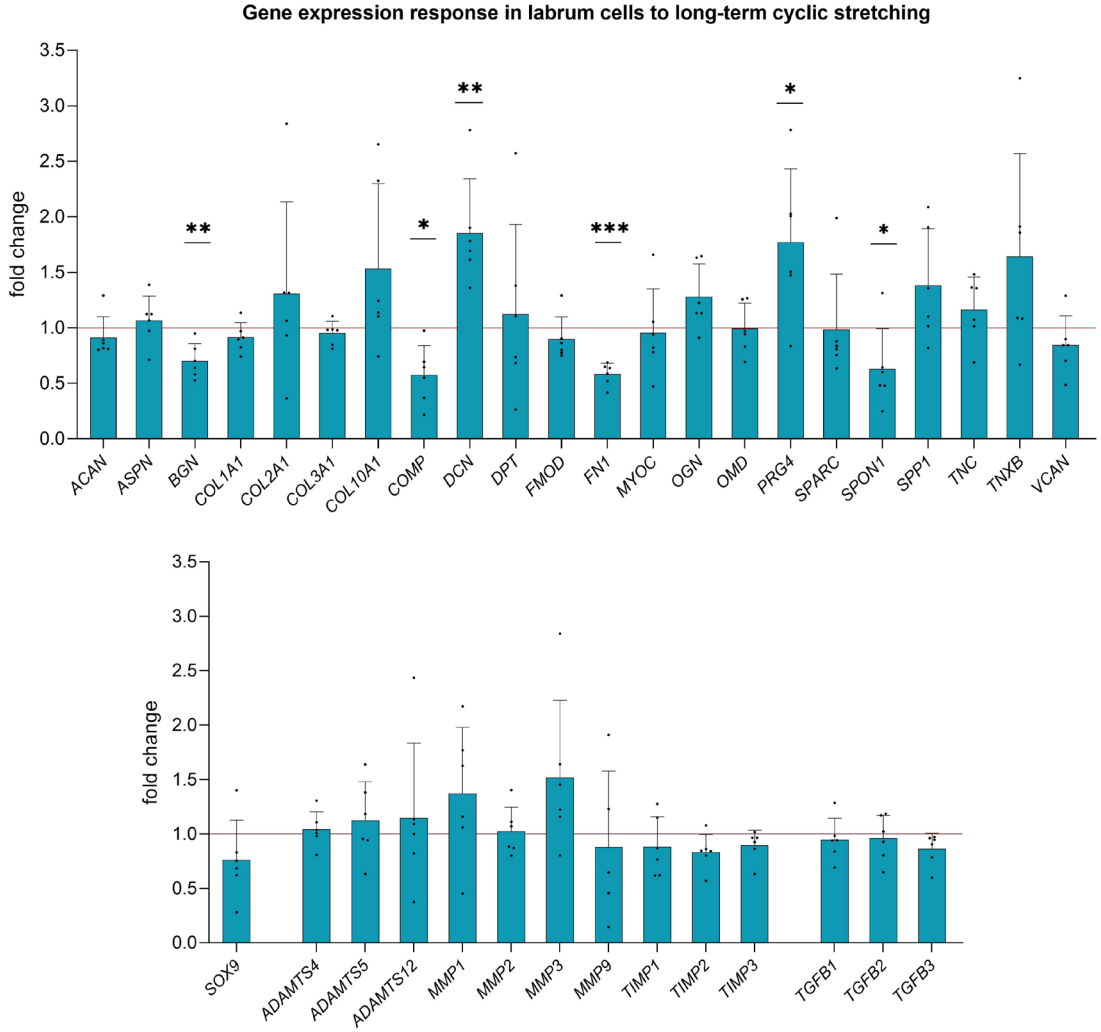

Fig. 3. Gene expression response in labrum cells to long-term stretching. Values below 1 indicate a gene expression downregulation and values above 1 indicate a gene expression upregulation in stretched compared to non-stretched cells. ECM-related genes are shown in the upper panel (anabolic). The lower panel from left to right shows genes coding for the transcription factor SOX9 (chondrogenesis), ECM-degrading enzymes of the ADAMTS and MMP family (catabolic), tissue inhibitors of MMPs and members of the TGFβ family (mechanobiological mechanism).

expression in labrum cells. Among the chondrogenic genes analysed (*COL2A2*, *ACAN*, *SOX9*, *COMP*, *COL9A1*), a significant downregulation of *COMP* and a non-significant downregulation of *SOX9* were found in 5 out of 6 bovine tissue explants. *COL9A1*, *COL2A1* and *ACAN* were not affected by the long-term stretching (Fig. 3). Similarly to studies investigating the response of chondrocytes to stretching, differences in the mechanical loading applied and the time point of analysis might explain the different finding (Bleuel *et al.*, 2015b). Another reason for the different findings might be that different cell populations were studied. Cells populating the meniscus, another fibrocartilaginous tissue, show different phenotypes and a different response to stretching depending on the meniscus region. Short-term stretching increases *COL2A1* and *SOX9* expression in cells derived from the inner meniscus, whereas cells derived from the outer meniscus region do not show any response (Kanazawa *et al.*, 2012; Upton *et al.*, 2006).

Mechanosensitive ECM genes

Studies on the biology of the labrum cells are scarce. In the present study, mechanosensitive ECM genes were identified in labrum cells, indicating that mechanical loading might play a role in labrum tissue homeostasis and regeneration. In the following section, the newly identified mechanosensitive targets are introduced with respect to mechanosensitivity and relevant functions.

Cyclic long-term stretching significantly downregulated *COMP* expression (Fig. 3). COMP is an highly abundant ECM protein which binds other structural ECM proteins. Therefore, a decrease in COMP might reduce tissue integrity (Acharya *et al.*, 2014; Clark *et al.*, 1999; Hamodat, 2020). Previous studies, mainly on cartilage, have demonstrated the mechanosensitivity of COMP. Increased COMP gene and protein expression are identified in cartilage explants following cyclic compression, whereas, under static compression, *COMP* and other ECM gene expression levels are decreased (Giannoni *et al.*, 2003; Wong *et al.*, 1999). Moderate long-term stretching of chondrocyte-seeded constructs and of 2D-cultured chondrocytes significantly increases COMP expression and its deposition in the ECM (Bleuel *et al.*, 2015a; Wong *et al.*, 2003).

A decrease in *FN1* expression was found in labrum cells following the application of long-term cyclic tensile strain (Fig. 3). FN1, a highly abundant glycoprotein of the ECM that connects collagen fibres and other ECM proteins, has been shown to transmit forces through RGD-binding integrins from the ECM to chondrocytes (Chevalier, 1993; Marques *et al.*, 2008). Cyclic compression of adult bovine articular cartilage explants at varying amplitudes and frequencies for 45 h significantly increases FN1 expression and synthesis (Wong *et al.*, 1999). Interestingly, moderate stretching of 2D-cultured chondrocytes increases FN1 levels (Bleuel *et al.*, 2015b), whereas the present study identified a decrease in FN1 levels in labrum cells following physiological stretching.

Long-term stretching significantly increased *DCN* and decreased *BGN* expression levels in labrum cells (Fig. 3). DCN and BGN both belong to the growing

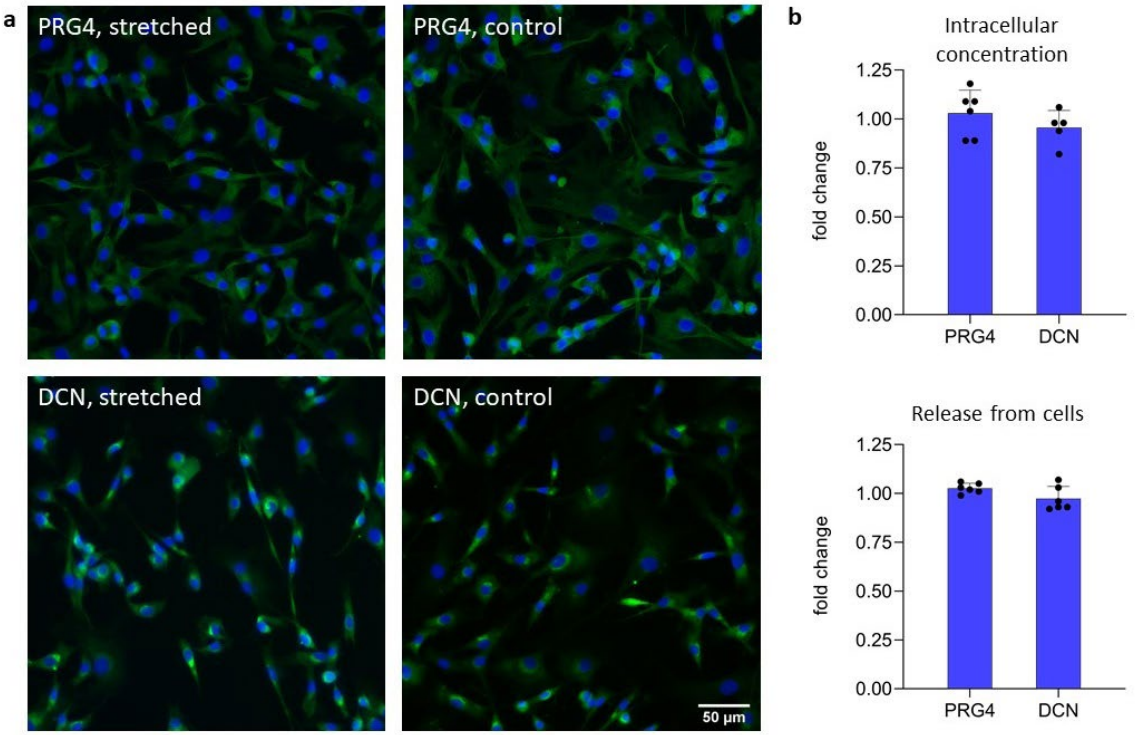

Fig. 4. PRG4 and DCN protein expression response in labrum cells to long-term stretching. (**a**) Immunofluorescence with DAPI counterstain and (**b**) quantification of released and intracellular DCN and PRG4 in stretched relative to non-stretched condition.

family of SLRPs and share a regulatory function of tissue organisation and remodelling (Schaefer and Iozzo, 2008). Among the SLRPs investigated in the present study (*ASPN*, *BGN*, *DCN*, *FMOD*, *OGN*, *OMD*), gene expression of two SLRPs was regulated by the long-term stretching and the gene expression of three other SLRPs (*OMD*, *OGN*, *ASPN*) was previously reported to be strongly downregulated in degenerated osteoarthritic labrum (Juchtmans *et al.*, 2015). These findings indicated that SLRPs could play an important role in the regulation of the labrum tissue organisation and maintenance. Further, DCN upregulation might increase the stiffness of the labrum tissue (Reese *et al.*, 2013). Interestingly, a divergent regulation of DCN and BGN, as identified in the present study, was in line with the previously proposed critical concept of compensation of one SLRP function over another. DCN upregulation in the absence of BGN has been specifically described in various tissues (Schaefer and Iozzo, 2008). Both SLPRs interact with ECM constituents, such as growth factors and structural proteins, modulate the cell response to mechanical loading and affect tissue organisation and tissue mechanical properties (Schaefer and Iozzo, 2008). The mechanical regulation of DCN and BGN expression has also been briefly studied in the meniscus. Biaxial stretching does not induce any change in DCN or BGN expression in meniscus cells, whereas dynamic compression upregulates *DCN* (but not *BGN*) expression (McNulty and Guilak, 2015; Upton *et al.*, 2006).

Relatively low *PRG4* expression levels were measured in the labrum cells, comparable to *COL2A1* levels (Fig. 2). However, long-term stretching significantly increased *PRG4* expression levels (Fig. 3). PRG4, also known as lubricin or superficial zone protein, is present in the synovial fluid and in the meniscus, cartilage and labrum tissue. It plays an important role in the lubrication of articulating joint surfaces by supporting low-friction motion (Swann *et al.*, 1985; Zhang *et al.*, 2012). The mechanosensitive regulation of PRG4 in cartilage has been widely studied in the past. Both moderate application of a dynamic shear compression or cyclic tensile strain on chondrocyte-seeded constructs increase *PRG4* expression levels (Grad *et al.*, 2006; Wong *et al.*, 2003). Elevated *PRG4* expression was also identified in 2D-cultured chondrocytes stretched at moderate (7 %) or high (21 %) strain. However, at high strain (21 %), *PRG4* expression levels decrease below non-stretched control levels after prolonged stretching (48 h) and, accordingly, PRG4 abundance is reduced (Bleuel *et al.*, 2015b).

In the present study, long-term stretching downregulated the expression levels of *SPON1* (Fig. 3). SPON1, also called f-spondin, was first identified as a highly expressed and secreted ECM protein in the floor plate and was primarily studied for its regulative role in the development of the nervous system (Burstyn-Cohen *et al.*, 1999; Debby-Brafman *et al.*, 1999; Klar *et al.*, 1992). Later, it was shown that *SPON1* regulates cartilage metabolism and is overexpressed in osteoarthritic cartilage and in the hip capsule and teres ligament of dogs affected by hip dysplasia (Attur *et al.*, 2009; Todhunter *et al.*, 2019). A mechanosensitive regulation of SPON1 has previously been identified in cells that are involved in tooth-tissue mineralisation (human cementoblasts) (Matsunaga *et al.*, 2016). Given its role in ECM regulation and in hip pathologies, SPON1 and encoded protein might be an interesting target for future studies investigating the regenerative mechanisms of labrum tissue.

Protein analysis of PRG4 and DCN

Immunofluorescence showed intracellular presence of PRG4 and DCN in both stretched and non-stretched cells. Protein deposition in the extracellular space could not be detected (Fig. 4**a**). Despite the significant upregulation of *DCN* and *PRG4* expression levels (Fig. 3), intracellular protein level and total release were not increased (Fig. 4**b**). Further research is required to understand the implications of the observed gene-expression response on protein level and in a broader scope on tissue homeostasis and mechanical tissue properties.

The study had some limitations. The labrum cells used were bovine labrum cells. It is ethically challenging to harvest non-degenerated healthy labrum specimens from human donors; therefore, bovine tissue offers an alternative model of the healthy labrum. The bovine model has been successfully used in the past to describe the mechanical properties of the labrum (Ferguson *et al.*, 2001; Ishiko *et al.*, 2005) and is an attractive model system for biomechanical and mechanobiological studies. Stretching of 2D cultured cells on a flexible substrate is a widely applied *in vitro* method to investigate cell mechanobiology. However, it must be noted that cells in their native ECM niche might respond differently to a mechanical stimulus compared to isolated cells seeded on a flexible substrate. Nevertheless, the present study provided novel insights into the mechanosensitive ECM gene expression of labrum cells, which may serve as a basis for future studies using more complex *in vitro* systems. Finally, it must be noted that gene and protein expression responses are transient and measurements at discrete time points might not allow to capture all the gene and protein expression changes induced.

Conclusion

The findings of the study demonstrated that bovine labrum cells were capable of sensing physiological stretching and responded by changes in gene expression of ECM proteins, including *DCN*, *BGN*, *PRG4*, *COMP*, *FN1* and *SPON1*. Therefore, physiological cyclic tensile strain might play a role in labrum tissue homeostasis and regeneration.

Author contributions

SH, SG, EC and SJF were involved in the design of the study. SH and SG performed the experiments and measurements and all authors contributed to the interpretation of the results. SJF and ML defined the broad project aims and created the funding. SH drafted the manuscript and all authors contributed to the final manuscript and have approved the submitted version.

The work was performed at ETH Zurich, Institute of Biomechanics. SJF and ML have received funding from the Swiss National Science Foundation (205321-176023). EC has received an Early Postdoc Mobility fellowship from the Swiss National Science Foundation. SJF is shareholder in an orthopaedic planning software and services company (CustomSurg AG).

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Discussion with Reviewers

Reviewer: Your experiments were peformed using bovine-tissue-derived labrum cells. Is there any knowledge regarding the physiological loading of the acetabular labrum in bovine specimens compared to human? Is this different in quadrupeds?

Authors: To the best of our knowledge, the physiological loading of the labrum has not been investigated in cows so far. Differences in joint kinematics between quadrupeds and human beings may exist, however, comparable mechanical properties (Ferguson *et al*., 2001; Ishiko *et al*, 2005) and similarities in tissue structure (unpublished data, under review) indicate a preserved biomechanical function.

Reviewer: Could the coating of the stretching chambers with collagen type I influence gene expression? Did you also analyse gene expression directly after cell isolation without the cells being in contact with the coated dishes?

Authors: Among the genes investigated in the study, *COL1A1* was the highest expressed. Fig. 1 shows low $2^{-\Delta\Delta Ct}$ values indicating high *COL1A1* expression levels compared to other genes measured. A high *COL1A1* expression is supported by literature on human-labrum-derived cells (Dhollander *et al*., 2012) and is further in line with measured gene expression of bovine labrum cells cultured in the absence of collagen type 1 coating (standard culture flask) as

well as with measured gene expression of bovine labrum tissue explants (unpublished data, currently under review).

Reviewer: Could the authors speculate on the effect of labrum degeneration on labral cell response to loading? Might there be frequency- and/or duration-dependent effects?
Authors: Kawamura *et al.* (2021) collected "healthy" and degenerated labrum tissues from patients with osteoarthritis undergoing total hip arthroplasty and identified differences in tissue morphology and a weaker gene expression response to physiological stretching in isolated healthy labrum cells compared to degenerated labrum cells. While the study identified mechanobiological differences between an healthy and a degenerated labrum, it remains unclear if the observed difference in mechanosensitive gene expression is a cause and/or a result of degeneration-associated morphological features.

Loading parameters may influence the stretching-induced gene expression of labrum cells. Studies on meniscus-derived fibrochondrocytes showed that magnitude, frequency and duration of cyclic tensile strain affect the mechanosensitive gene expression. Cyclic tensile strain stimulates the synthesis of the transcription factor SOX9 in a duration-dependent manner (Kanazawa *et al.*, 2012) and suppresses IL1-mediated catabolism in a magnitude- and frequency-dependent manner (Ferretti *et al.*, 2006, additional reference). However, it must be noted that fibrochondrocytes derived from the inner and outer meniscus showed differences in basal gene expression and in gene expression response to stretching, highlighting the broad spectrum of fibrocartilage-derived cells. Therefore, although both the meniscus and labrum are composed of fibrocartilage, tissue- and region-specific mechanobiological features may exist and additional research is required to understand the mechanobiological function of the unique labrum-derived cells in healthy and degenerated conditions.

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Editor's note: The Scientific Editor responsible for this paper was Mauro Alini.