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In vitro expansion affects the response of chondrocytes to mechanical stimulation[1]

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Summary

Objective: Expansion of autologous chondrocytes is a common step in procedures for cartilage defect repair. Subsequent dedifferentiation can alter cellular response to mechanical loading, having major consequences for the cell's behavior *in vivo* after reimplantation. Therefore, we examined the response of primary and expanded human articular chondrocytes to mechanical loading.

Method: Primary and expanded chondrocytes were stretched at either 0.5% or 3.0% at 0.5 Hz, 2 h per day, for 3 days. Gene expression levels of matrix components (aggrecan (AGC1), lubricin (PRG4), collagen type I (COL1), type II (COL2) and type X (COL10)) as well as matrix enzymes (matrix metalloproteinase 1 (MMP1), MMP3, MMP13) and SOX9 were compared to unstretched controls. To evaluate the effect of a chondrogenic environment on cellular response to stretch, redifferentiation medium was used on expanded cells.

Results: In primary chondrocytes, stretch led to mild decreases in AGC1, COL1 and COL10 gene expression (maximum of 3.8-fold) and an up-regulation of PRG4 (2.0-fold). In expanded chondrocytes, expression was down-regulated for AGC1 (up to 21-fold), PRG4 (up to 5.0-fold), COL1 (10-fold) and COL2 (2.9-fold). Also, expression was up-regulated for MMP1 (20-fold) and MMP3 (up to 4-fold), while MMP13 was down-regulated (2.8-fold). A chondrogenic environment appeared to temper effects of stretch.

Discussion: Our results show that expansion alters the response of human chondrocytes to stretch. Expanded chondrocytes greatly decrease gene expression of matrix constituents and increase expression of MMPs, whereas primary chondrocytes hardly respond. Our data could be a reference for optimization of cell sources or expansion protocols for reimplanted chondrocytes.

Key words: Cell culture, Mechanical loading, Chondrocyte phenotype, Dedifferentiation, Gene expression, Cell deformation, Stretch, Tissue engineering.

Introduction

In autologous chondrocyte implantation (ACI) procedures, cartilage is harvested from an autologous donor site and isolated chondrocytes are expanded *in vitro* to obtain sufficient cell numbers before implantation into the defect site. However, during expansion culture, chondrocytes lose their specific chondrocytic phenotype and become more fibroblast-like[1,2]. This phenotypical change, called dedifferentiation, is accompanied by a decreased gene expression of cartilage specific markers like collagen type II (COL2)[3]. This process might also alter the response of chondrocytes to extracellular stimuli. The current work studied the response of chondrocytes to mechanical stimulation after dedifferentiation resulting from monolayer expansion.

In their natural environment, chondrocytes are constantly deformed as a result of loading due to normal daily activities. Guilak *et al.*[4] estimated the loss of cell height of chondrocytes resulting from physiological loading to be approximately 20%. *In vivo* deformation will also occur in reimplanted chondrocytes after ACI. Normal physiological loading is generally regarded as a prerequisite for the maintenance of proper articular joint functioning, while injurious loading can lead to cartilage degeneration. Dynamic compression of bovine explants or three-dimensional scaffold cultures has indeed shown a stimulatory effect *in vitro*, not only on load bearing matrix components[5–11], but recently also on lubricin (PRG4)[12]. Other forms of mechanical stimulation like fluid flow induced shear stress[13,14] and mechanical stretch[15,16] also elicit a response in primary bovine chondrocytes. In human normal, healthy chondrocytes Millward-Sadler *et al.*[17] found that cyclic stretch has an anabolic effect, as was shown by an increase in aggrecan (AGC1) expression and decrease in matrix metalloproteinase 3 (MMP3) expression. This effect was not seen in osteoarthritic (OA) chondrocytes, where no

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change in AGC1 or MMP gene expression was observed. This difference might be attributed to a change in mechanotransduction pathways between normal and OA chondrocytes[18–20]. In another study with human cartilage, Plumb and Aspden[21] also showed that cyclic loading was not stimulatory in cartilage explants from human femoral heads. These results are contradictory to those found for young bovine chondrocytes, where loading was stimulatory[5–7].

Not only the source of chondrocytes determines the cell's response to mechanical loading. Wiseman *et al.*[22] showed that bovine articular chondrocytes in agarose constructs exhibited decreased proliferation and proteoglycan synthesis after monolayer expansion upon mechanical stimulation compared to primary chondrocytes. Since expansion and the associated dedifferentiation of human chondrocytes is an essential step in ACI-like procedures, the effect of expansion on the matrix-forming capacities warrants further investigation.

Therefore, we investigated, through real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis, how human articular chondrocytes, after monolayer expansion, respond to stretch depending on their expansion and corresponding differentiation state. In addition, we examined whether a specific chondrogenic environment, which leads to redifferentiation to the chondrogenic phenotype, alters the response of expanded chondrocytes to stretch in terms of gene expression.

Methods

CELL CULTURE

Cartilage was obtained from patients undergoing total knee replacement surgery (after approval by the local ethical committee; MEC2004-322). Full thickness cartilage was harvested, treated with 0.2% protease in physiological saline solution (Sigma, St. Louis, MO, USA) for 90 min and subsequently digested overnight in basal medium [Dulbecco's modified eagle medium (DMEM), 4.5 g/l glucose with 10% Fetal Calf Serum (FCS), 0.1% gentamicine and 0.6% fungizone (all Invitrogen, Scotland, UK)] supplemented with 0.15% collagenase B (Roche Diagnostics, Mannheim, Germany). The following day, the harvested cell number was determined using a heamacytometer. The primary chondrocytes were then either seeded at a density of 7500 cells/cm^2 in a T175 culture flask for expansion culture or seeded at a density of 300,000 cells/well in collagen type I (COL1) coated Flexcell six-well plates (Flexercell, McKeesport, PA, USA). The cells plated for expansion were cultured for three passages. These expanded chondrocytes were then seeded at a density of 300,000 cells/well in the Flexcell COL1 coated six-well plates (Fig. 1).

MECHANICAL STIMULATION

Cells were left to adhere firmly to the flexible membrane of the six-well plate during a 5 day pre-culture with basal medium. On day 5, cells were stretched using a modified Flexcell set-up (Flexercell, McKeesport, PA, USA) inside an incubator (37°C, 5% CO$_2$). This set-up was previously described[23]. Briefly, a low pressure created under the six-well plates pulls the flexible membrane over a loading post, resulting in homogenous biaxial strain. The size of the loading post and the level of the pressure correlate to the amount of stretch applied to the adherent cells. Loading posts of 25 mm and 30 mm diameter were used, resulting in applied strains of 3.0% and 0.5%, respectively. Cyclic stretch at a frequency of 0.5 Hz was applied twice daily for 1 h with a 1 h rest period. This protocol was repeated for 3 days. Unstretched controls were placed in the device without stretching the membranes.

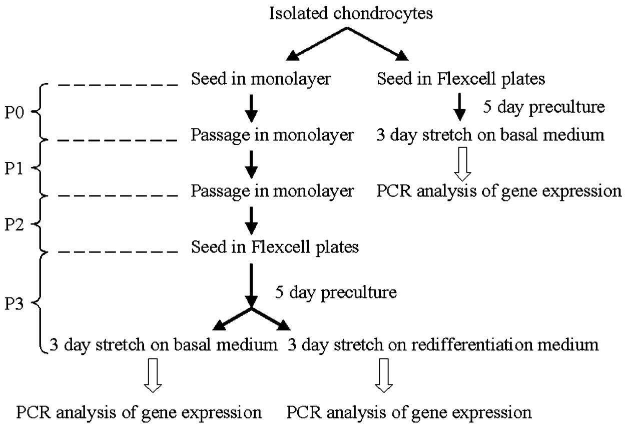

Fig. 1. Layout of experimental set-up.

REDIFFERENTIATION MEDIUM

To examine the effects of a chondrogenic environment, experiments were also conducted with redifferentiation medium[2]. This medium consisted of DMEM high glucose, 1:100 insulin-transferrin-selenium A supplement (ITS) + (BD Biosciences, Bedford, MA, USA), 10 ng/ml transforming growth factor-β2 (TGF-β2) (recombinant human, R&D Systems, Abington, UK), 10 ng/ml insulin-like growth factor-1 (IGF-1), 25 μg/ml L-ascorbic acid 2-phosphate (both from Sigma, St. Louis, MO, USA), 0.1% gentamicine and 0.6% fungizone (both from Invitrogen, Scotland, UK). The redifferentiation medium was added at the onset of stretch.

PCR

Directly after the last stretch cycle total RNA was isolated using the Nucleospin II kit according to the manufacturer's instructions (Machery-Nagel, Düren, Germany) and nucleic acid content was determined spectrophotometrically (NanoDrop® ND1000, Isogen Life Science, The Netherlands). For cDNA synthesis and real-time quantitative PCR (qPCR) methods see Uitterlinden *et al.*[24]. An ABI7000 was used for cycling.

Taqman™ or SybrGreen™ I assays were performed on AGC1, proteoglycan 4 (PRG4, alias lubricin or superficial zone protein), COL1, COL2 and COL10, MMP1, MMP3, MMP13 and transcription factor (sex determining regionY)-box 9 (SOX9). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. All primer and probe nucleotide sequences for gene amplifications are listed in Table I.

DATA ANALYSIS

Expression was normalized to GAPDH and expressed relatively using the $2^{-(\Delta\Delta Ct)}$ method of Livak[25]. Subsequently, expression levels of unstretched control conditions were set to 1 and stretched conditions were plotted relative to controls.

Results are means plus standard deviation. Statistical significance was determined using a Kruskal–Wallis test (SPSS Inc., Chicago, IL, USA) prior to testing stretched vs unstretched conditions by Mann–Whitney test. Differences were considered significant when $P < 0.05$.

For every experiment with primary cells, six control wells were used for each donor, while three wells were used for 0.5% and three wells for 3.0% strain. The first experiment with expanded cells had the same set-up as the experiments with primary cells. For the other experiments with expanded cells, three wells were used for unstretched controls on basal medium and three wells were used for unstretched controls with redifferentiation medium. For both stretched conditions (0.5% and 3.0%), three wells per plate were used with basal medium and three wells were used with redifferentiation medium. Table II summarizes experimental details: some wells were lost due to low cell yield after harvest.

Results

EFFECT OF EXPANSION CULTURE ON THE LEVELS OF GENE EXPRESSION

Upon expansion in monolayer culture, gene expression of COL1 was up-regulated while SOX9 expression was down-regulated, typical for dedifferentiation toward a more fibroblast-like phenotype (Fig. 2). At the same time, COL2 is hardly expressed and COL10 expression is completely absent in dedifferentiated chondrocytes, also consistent with the shift toward a fibroblast-like state. Also, expression levels of MMP1, MMP3 and MMP13 were considerably lower after expansion.

EFFECT OF STRETCH ON PRIMARY CHONDROCYTES

Gene expression of matrix components (AGC1, PRG4, COL1, COL2 and COL10) was moderately altered by

Table I
Primer and probe nucleotide sequences for all gene amplifications

Gene	Acc. no.	Primer	Nucleotide sequences
GAPDH	BC_083511	HsGAPD_F	ATGGGGAAGGTGAAGGTCG
		HsGAPD_R	TAAAAGCAGCCCTGGTGACC
		HsGAPD_FAM	CGCCCAATACGACCAAATCCGTTGAC
AGC1	NM_001135	HsAGC1_F	TCGAGGACAGCGAGGCC
		HsAGC1_R	TCGAGGGTGTAGCGTGTAGAGA
		HsAGC1_FAM	ATGGAACACGATGCCTTTCACCACGA
MMP1	NM_002421	HsMMP1_F	CTCAATTTCACTTCTGTTTTCTG
		HsMMP1_R	CATCTCTGTCGGCAAATTCGT
		HsMMP1_FAM	CACAACTGCCAAATGGGCTTGAAGC
MMP3	NM_002422	HsMMP3_F	TTTTGGCCATCTCTTCCTTCA
		HsMMP3_R	TGTGGATGCCTCTTGGGTATC
		HsMMP3_FAM	AACTTCATATGCGGCATCCACGCC
MMP13	NM_002427	HsMMP13_F	AAGGAGCATGGCGACTTCT
		HsMMP13_R	TGGCCCAGGAGGAAAAGC
		HsMMP13_FAM	CCCTCTGGCCTGCTGGCTCA
SOX9	NM_000346	HsSOX9_F	CAACGCCGAGCTCAGCA
		HsSOX9_R	TCCACGAAGGGCCGC
		HsSOX9_FAM	TGGGCAAGCTCTGGAGACTTCTGAACG
COL1	NM_000088	HsCOL1_F	CAGCCGCTTCACCTACAGC
		HsCOL1_R	TTTTGTATTCAATCACTGTCTTGCC
		HsCOL1_FAM	CCGGTGTGACTCGTGCAGCCATC
COL2	NM_033150	HsCOL2_F	GGCAATAGCAGGTTCACGTACA
	NM_001844	HsCOL2_R	CGATAACAGTCTTGCCCCACTT
		HsCOL2_FAM	CCGGTATGTTTCGTGCAGCCATCCT
COL10	NM_000493	HsCOL10_F	CAAGGCACCATCTCCAGGAA
		HsCOL10_R	AAAGGGTATTTGTGGCAGCATATT
		HsCOL10_FAM	TCCAGCACGCAGAATCCATCTGA
*PRG4**	NM_005807	HsPRG4_F	TTGCGCAATGGGACATTAGTT
		HsPRG4_R	AGCTGGAGATGGTGGACTGAA
		—	—

*SYBRGreen I assay.

stretch in P0 chondrocytes (Fig. 3). AGC1 and COL1 were down-regulated in a response to mechanical stimulation of both 0.5% and 3.0% strain levels. Gene expression of AGC1 was only slightly altered, a 1.8-fold down-regulation was found at 0.5% strain and 1.6-fold change at 3.0% strain. COL1 showed a 3.8-fold decrease in gene expression level compared to control at 0.5% strain and a 2.1-fold decrease at 3.0% strain. COL10 was also marginally down-regulated (2-fold) at both strain levels. Gene expression levels of COL2 remained unaltered when loaded with either 0.5% or 3.0% strain, while levels of PRG4 were slightly up-regulated compared to control, 1.8-fold at 0.5% and 1.9-fold at 3.0%.

Stretch did neither statistically significantly alter the gene expression of MMP1, MMP3 and MMP13, nor did it change SOX9 expression levels.

Table II
Number of donors and technical repetitions for every experimental condition

Condition	Stretch level	*n*	Number of donors
Primary chondrocytes	Control	19	4
	0.5%	11	4
	3.0%	12	4
Expanded chondrocytes on basal medium	Control	12	3
	0.5%	9	3
	3.0%	9	3
Expanded chondrocytes on redifferentiation medium	Control	6	2
	0.5%	6	2
	3.0%	6	2

EFFECT OF STRETCH ON EXPANDED CHONDROCYTES

P3 cells showed a much larger response to stretch (Fig. 4) than primary cells. Gene expression of most matrix proteins (AGC1, PRG4, COL1 and COL2) was severely down-regulated after stretching of the cells. AGC1 and COL1 showed the most significant change in gene expression. Expression levels of AGC1 were 15.6-fold lower at 0.5% when compared to unstretched controls, while 3.0% resulted in a 11-fold decrease. COL1 was down-regulated approximately 10-fold for both 0.5% (11.9-fold) and 3.0% strains (8.0-fold). Gene expression of PRG4 was also lower when cells were stretched at 0.5% (5.0-fold decrease) or 3.0% (2.5-fold decrease). After expansion, COL2 expression was absent in chondrocytes from one donor. In those cases where COL2 was still expressed, stretching down-regulated its expression levels (up to 2.9-fold for 0.5% stretch). COL10 was not expressed in any donor after expansion.

In expanded chondrocytes, MMP1 and MMP3 were both up-regulated after stretching. MMP1 showed a 20-fold up-regulation while MMP3 was up-regulated 3.5-fold (at 0.5% strain) or 2.3-fold (at 3.0% strain). MMP13 was down-regulated in response to cell straining at both 0.5% (2.8-fold) and 3.0% (1.9-fold). Again, no effect of stretch on *SOX9* gene was found.

EFFECT OF STRETCH ON EXPANDED CELLS IN A CHONDROGENIC ENVIRONMENT

On redifferentiation medium, expanded chondrocytes re-expressed COL2 and COL10, indicating a return to a more

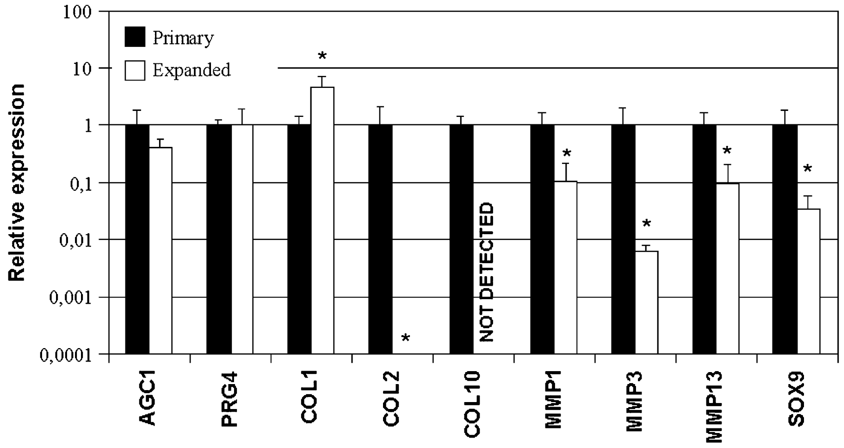

Fig. 2. Relative gene expression levels of unstretched primary chondrocytes vs expanded (P3) chondrocytes. * Indicates significant difference ($P < 0.05$).

chondrogenic phenotype. COL1 and PRG4 expression was also higher on redifferentiation medium. Gene expression of MMP1 and MMP13 was also up-regulated on redifferentiation medium.

This chondrogenic environment did not significantly change the alterations in gene expression levels of matrix components by expanded chondrocytes associated with stretch (Fig. 5). AGC1 and COL1 were still severely down-regulated, while PRG4 was again only moderately down-regulated. COL2 was re-expressed on redifferentiation medium, but here stretch also appeared to down-regulate gene expression. No effect of stretch was found on mRNA levels of COL10 gene expression. MMP1 expression was still up-regulated, but to a lesser extent compared to basal medium. MMP3 expression was still significantly up-regulated in stretched conditions compared to unstretched controls on redifferentiation medium. MMP13 was no longer significantly down-regulated. Overall an expression pattern was found that was similar to that found with basal expansion medium, but the effects seemed somewhat tempered.

General trends for all conditions are summarized in Table III.

Discussion

Our results indicate that *in vitro* expansion affects the response of chondrocytes to a mechanical stretch protocol. Real-time RT-PCR analysis revealed a decrease in expression of genes encoding for matrix components as well as a rise in expression of matrix degrading enzymes after stretching of expanded chondrocytes. In primary chondrocytes the response was markedly less substantial and significant. We also studied the effect of a chondrogenic environment that is known to direct dedifferentiated chondrocytes back toward a chondrogenic phenotype. These partially redifferentiated chondrocytes showed similar effects as the expanded, dedifferentiated chondrocytes, however, the effects of stretch appeared to be tempered. This is consistent with the shift toward the primary phenotype, since primary chondrocytes reacted only marginally to stretch.

The observation of up-regulation of the matrix degrading enzymes MMP1 and MMP3 after stretch is consistent with the notion that chondrocytes assume a more fibroblast-like

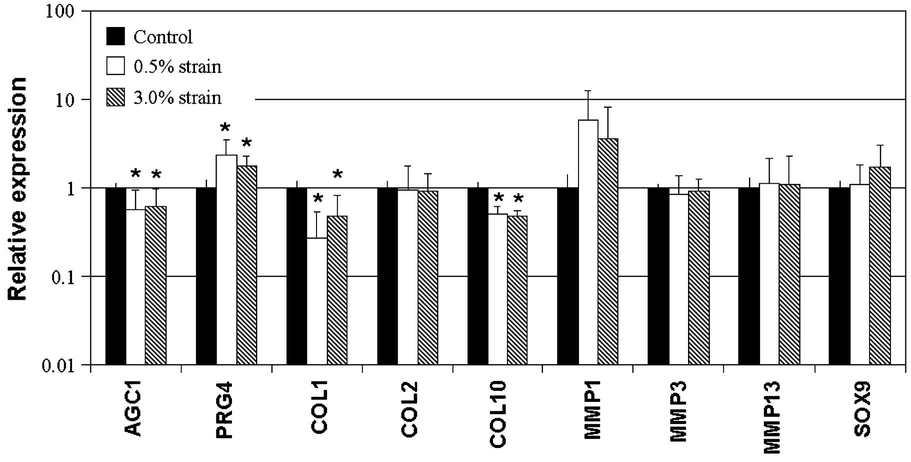

Fig. 3. Gene expression of primary chondrocytes under strain (0.5% and 3.0%) relative to unstrained controls. * Indicates statistically significant difference with control ($P < 0.05$).

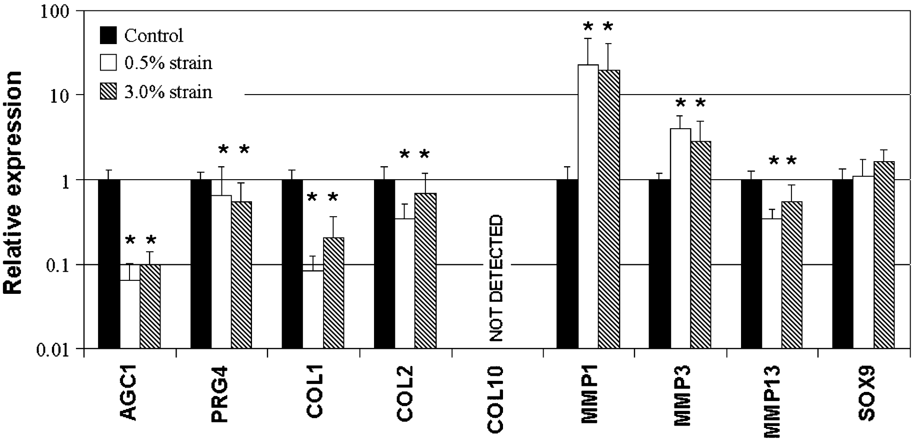

Fig. 4. Gene expression of expanded, dedifferentiated chondrocytes under strain (0.5% and 3.0%) relative to unstrained controls. * Indicates statistically significant difference with control ($P < 0.05$).

phenotype upon dedifferentiation in respect that some studies report increased (pro-)MMP expression following (injurious) loading. For example, increased (pro-)MMP expression and activation was found in ligament fibroblasts[26], patellar tendon fibroblasts[27], scleral fibroblasts[28], uterine cervical fibroblasts[29] and cardiac fibroblasts[30] after loading with stretch. However, this up-regulation was not found in all types of fibroblast. Sambajon *et al.*[31] found no difference in proteinase activity of synovial fibroblasts after stretch.

However, matrix degradation is also part of the remodeling process and it cannot be excluded that the rise in expression of MMP1 and MMP3 after short-term stretch follows from a remodeling attempt by the cells. But one might expect a concurrent elevation in matrix components in case of remodeling, which is not seen in our experiments. Obviously, the translation of changes in gene expression to expression on protein level is not straightforward and short-term effects might differ from long-term (*in vivo*) effects. Therefore, to be able to interpret our results from a practical viewpoint, protein expression and enzymatic activity should be assessed and the consequences for long-term protein expression need to be established.

Interestingly, expression levels of MMP13, the collagenase whose affinity for COL2 is the greatest[32], were down-regulated after cyclic stretch. This difference in response might be attributed to the fact that the collagenases MMP1 and MMP13 differ in their spatial distribution[33]. MMP1 is mainly expressed in the superficial cartilage layer, while MMP13 is chiefly expressed in the deep zone, where different deformation is experienced by the chondrocytes.

Other than very marginal changes in the expression of AGC1 and COL1 and COL10, primary chondrocytes did not show a marked response to stretch. This is in line with the findings of Millward-Sadler *et al.*[17], who found that expression levels of AGC1, MMP1 and MMP3 were unchanged in primary chondrocytes from OA patients after application of short-term cyclic stretch. The discrepancy with primary chondrocytes from healthy cartilage, which showed an increase in AGC1 expression and a decrease in MMP3 expression, was attributed to phenotypical alterations in OA chondrocytes. In OA chondrocytes, these changes might include altered expression of integrins, cytokines and growth factors. Indeed, integrins, and especially the fibronectin receptor integrin $\alpha5\beta1$, are involved in mechanotransduction[34] of both normal and OA chondrocytes. Although the exact mechanisms by which this transduction occurs are not yet fully understood, they appear to include initiation of integrin-dependent signaling cascades. Expression of

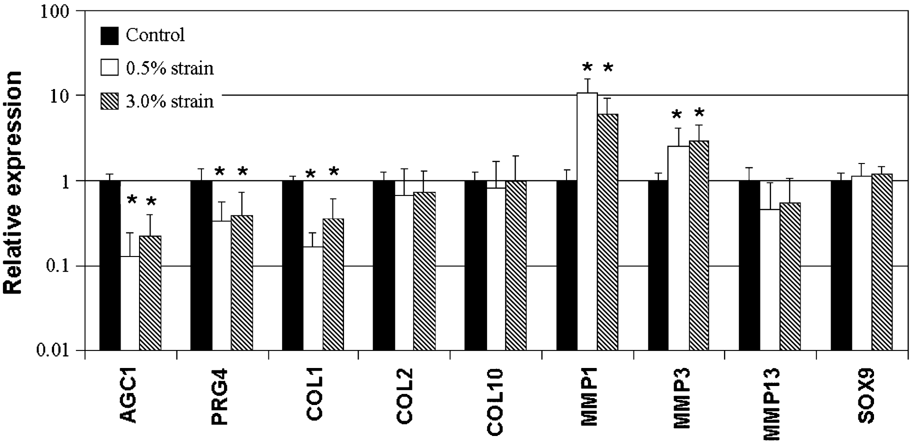

Fig. 5. Gene expression of expanded, dedifferentiated chondrocytes in a chondrogenic environment under strain (0.5% and 3.0%) relative to unstrained controls. * Indicates statistically significant difference with control ($P < 0.05$).

Table III
Summary of results

Gene	Condition and trend of relative expression			
	No stretch, P3[1]	Stretch, P0[2]	Stretch, P3[3]	Stretch, P3, redifferentiation medium[4]
AGC1	=	↓	↓↓	↓
PRG4	=	↑	↓	↓
COL1	↑	↓	↓↓	↓
COL2	↓↓↓	=	↓	=
COL10	ND	↓	ND	=
MMP1	↓	=	↑↑	↑
MMP3	↓↓↓	=	↑	↑
MMP13	↓↓	=	↓	=
SOX9	↓↓	=	=	=

[1] Basal gene expression in expanded chondrocytes relative to primary controls (no strain, Fig. 2) and strain-related changes in expression in primary[2], expanded chondrocytes[3] and expanded chondrocytes stretched on redifferentiation medium[4]. Relative trends are indicated by symbols (↓, down-regulation; ↑, up-regulation; =, unchanged) with multiple arrows indicating stronger effects. Single arrow: 0–10-fold change, double arrow: 10–100-fold change, triple arrow: 100–1000-fold change. ND, not detected.

integrins and integrin-associated proteins is altered in chondrocytes upon expansion[35], which could account for the differences in response between primary and expanded chondrocytes found in this study.

Our model system utilizes monolayer culture with stretching (elongation) in the lateral direction, whereas the three-dimensional *in situ* loading involves compression of the cells embedded in a matrix that includes solid, water and charges that control deformation upon loading. Knight *et al.*[36] showed that compression (of alginate) leads to contralateral elongation, although the exact deformation of the cell depends on the mechanical properties of the cell relative to its surrounding[37]. Plumb and Aspden[21] found that, contrary to healthy bovine cartilage, cyclic compressive loading was not stimulatory in cartilage biopsies from human femoral heads. Lee *et al.*[38] found decreased mRNA levels of COL2 and AGC1 following shear stress loading of chondrocytes of patients suffering from OA. Also, Wiseman *et al.*[22] showed that, after three to four passages, healthy bovine articular chondrocytes seeded in agarose showed reduced glucosaminoglycan (GAG) synthesis after dynamic compressive loading. Nugent *et al.*[12] showed, with a shear deformation model, an up-regulation of PRG4 with the same order of magnitude as the primary chondrocytes in our stretch model system. Despite these consistent responses, the model systems never accurately represent the *in vivo* situation, where conditions such as deformation and environmental parameters are actively controlled. This limits the interpretation for the *in vivo* situation, where other factors, including cell attachment, molecular environment with different serum conditions and a complex loading situation might all influence the cell's response.

In conclusion, this study clearly shows that expanded human chondrocytes respond differently to stretch than primary chondrocytes. The down-regulation of both major components in articular cartilage, AGC1 and COL2, as well as the up-regulation of matrix degradative MMPs in the expanded chondrocytes after stretch might be regarded as degradative, although the effects of this altered gene expression on protein level still remain to be studied. If expanded chondrocytes in ACI-like procedures have a similar expression response after *in situ* loading, this cell source might not be the optimal choice for such a procedure. Consequently, it might be that other cell sources, redifferentiation protocols prior to implantation or limiting deformation (e.g., by movement restricting post-surgical therapy or use of a rigid scaffold) improve the cell's capacity to form a functional extracellular matrix and reduce enzymatic activity. However, implanted chondrocytes should also become involved in remodeling of the matrix, starting with degradation that might lead to better incorporation with the host matrix. From the current study, providing short-term RNA-level responses, we have no information regarding the long-term consequences for the matrix and its *in situ* incorporation potential. Our findings may therefore be regarded as a reference point for future studies that aim to optimize protocols for tissue formation by expanded chondrocytes.

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