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Cyclic tensile strain and cyclic hydrostatic pressure differentially regulate expression of hypertrophic markers in primary chondrocytes

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Abstract

Endochondral ossification is regulated by many factors, including mechanical stimuli, which can suppress or accelerate chondrocyte maturation. Mathematical models of endochondral ossification have suggested that tension (or shear stress) can accelerate the formation of endochondral bone, while hydrostatic stress preserves the cartilage phenotype. The goal of this study was to test this hypothesis by examining the expression of hypertrophic chondrocyte markers (transcription factor Cbfa1, MMP-13, type X collagen, VEGF, CTGF) and cartilage matrix proteins under cyclic tension and cyclic hydrostatic pressure. Chondrocyte-seeded alginate constructs were exposed to one of the two loading modes for a period of 3 h per day for 3 days. Gene expression was analyzed using real-time RT-PCR. Cyclic tension upregulated the expression of Cbfa1, MMP-13, CTGF, type X collagen and VEGF and downregulated the expression of TIMP-1. Cyclic tension also upregulated the expression of type 2 collagen, COMP and lubricin, but did not change the expression of SOX9 and aggrecan. Cyclic hydrostatic pressure downregulated the expression of MMP-13 and type I collagen and upregulated expression of TIMP-1 compared to the unloaded controls. Hydrostatic pressure may slow chondrocyte differentiation and have a chondroprotective, anti-angiogenic influence on cartilage tissue. Our results suggest that cyclic tension activates the Cbfa1/MMP-13 pathway and increases the expression of terminal differentiation hypertrophic markers. Mammalian chondrocytes appear to have evolved complex mechanoresponsive mechanisms, the effects of which can be observed in the histomorphologic establishment of the cartilaginous skeleton during development and maturation.
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Keywords: Mechanobiology; Chondrocyte; Gene expression; Hypertrophic markers; Mechanical loading; ECM genes

Introduction

Chondrocyte differentiation follows a series of maturational stages which include proliferation, pre-hypertrophy, hypertrophy and terminal differentiation. Each stage is characterized by the expression of specific markers. For example, indian hedgehog is uniquely expressed in the pre-hypertrophic zone, type X collagen, alkaline phosphatase and MMP-13 are markers of hypertrophic chondrocytes, and VEGF and osteocalcin are markers of terminal differentiation. Several hypertrophic and terminal differentiation markers provide important functions in endochondral ossification, which include the breakdown of calcified cartilage matrix, inducing invasion of endothelial and mesenchymal cells into the calcified cartilage and regulating mineralization. The chondrocyte maturational process is generally thought to be under suppression, mainly by the inhibitory influence of TGFβ and PTHrP. The removal of one of these inhibitory pathways or a shift in the dominance towards an enhancing pathway (i.e., via BMPs) could be sufficient to accelerate chondrocyte maturation towards endochondral ossification. For example, chondrocyte maturation is considered arrested in normal, healthy articular cartilage, whereas the onset of osteoarthritis may be construed as a loss of constraints on chondrocyte maturation.

In addition to signaling by regulatory molecules, it has been suggested that mechanical loading can influence the rate of chondrocyte maturation. In fact, the histomorphologic development of articular cartilage may be correlated

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with distributions of pressure, fluid flow, and deformation created during in vivo loading [1]. The evidence for this hypothesis comes from numerous cell loading experiments showing the expression of many factors involved in endochondral ossification (VEGF [2,3], CTGF [4], MMPs [5], Cbfa1 [6]) is sensitive to mechanical loading, and from continuum models of skeletal development which suggest that endochondral ossification can be arrested by cyclic hydrostatic pressure and accelerated by cyclic shear [7,8]. The goal of this study was to test this latter hypothesis by determining the differential effects of hydrostatic pressure and shear-related tensile strain on the expression of cartilage specific ECM proteins and markers of the hypertrophic phenotype. The expression of Cbfa1/Runx2 and Sox9, which are considered master genes of osteogenesis and chondrogenesis respectively [9,10], were of particular interest. Cbfa1, in addition to its direct role in bone formation, is involved in the expression of its targets MMP-13, osteocalcin, and alkaline phosphatase in hypertrophic chondrocytes as well osteoblasts. Sox9, on the other hand, regulates the expression of types II, IX and XI collagen, aggrecan, and COMP in developing cartilage. We hypothesized that the genes associated with hypertrophy and ossification would be upregulated by cyclic tension and that genes associated with chondrogenesis would be upregulated by hydrostatic pressure.

Methods

Chondrocyte culture

Primary chondrocytes were isolated from the middle and deep zones of calf bovine humeral head cartilage using a sequential pronase/collagenase digestion. Chondrocytes were suspended in a 2% alginate, 0.9% NaCl solution at a density of 4×10^6 cells/ml. The alginate was polymerized in 22 mm diameter $\times$ 1.5 mm high casts by soaking in 50 mM CaCl2 in 0.9% NaCl for 30 min. The cells were cultured in DMEM with 10% fetal bovine serum, 2 mM L-glutamine, 0.01% BSA, 0.35 mM proline, 50 μg/ml gentamycin, 20 μg/ml ascorbate, 1 mM cysteine and 1 mM pyruvate for 3 days to allow the cell to reach equilibrium. The alginate was then solubilized in 55 mM sodium citrate in 0.9% NaCl for 30 min at 37°C and the cells were recast in alginate and punched into cylinders for hydrostatic pressure (5.8 mm diameter, 1.5 mm high) and cast into rectangular beams for tensile experiments. Alginate beams (14 mm $\times$ 8 mm $\times$ 1.5 mm) were made using a rectangular mold into which two porous polyethylene end pieces were embedded. The tensile strain and hydrostatic pressure loading experiments were performed eight separate times, each time with cells from a different animal. For each digest, the cells were divided into four groups, from which two loaded (hydrostatic pressure, tension) and two controls (hydrostatic pressure control, tension control) were taken.

Mechanical stimulation

Labview software was used to control movement of the two microtesting systems used in this study. The tensile apparatus consisted of a load cell mounted in series with a PM500 actuator (Newport Corp, Irvine, CA, USA) (Fig. 1A). Since shear stress is always associated with tension in some direction, it was thought to approximate some aspects of cyclic shear loading. Fixtures attached to the actuator and load cell had three sets of paired pins which allowed attachment of the alginate specimens via the polyethylene end pieces. The pins assured proper alignment. Sinusoidal strains of 9% peak-to-peak were applied to the alginate specimens. Control groups consisted of specimens of the same geometry which were cultured in a vessel in the absence of loading.

The hydrostatic pressure system consisted of a fluid-filled chamber which was pressurized via the movement of a piston (Fig. 1B). A second unloaded chamber was used for control specimens. Prior to hydrostatic pressure, both the loaded and control samples were placed into plastic hybridization bags containing temperature- and CO_2-equilibrated media. The bags were sealed with no air pockets and placed into a temperature-equilibrated chamber which was filled with 37°C water. A $+/-$ 10V output from a NB-MIO-16x data acquisition card running on a Macintosh G4 computer was amplified to $+/-$ 24V. The voltage controlled the position of a valve (Tiger 2000, F-Reihe, Festo AG) letting 8 bar pressurized air into one or the other end of a double cylinder (Type ADVU, Festo AG). The inward movement of the cylinder was controlled by an adjustable stop, assuring that the pressure did not rise above 5 MPa. The magnitude of the pressure was visually confirmed by the manometer mounted in the pressurized chamber. Both loading modes were performed at 0.5 Hz, 3 h per day for 3 days. Load was applied for 3 days based on the findings in the literature which show that the biosynthetic rate is higher when hydrostatic loading is applied for several days in succession, compared to just one day [11]. The resulting peak cyclic stress and strain for each loading mode are given in Table 1.

Gene expression analysis

After the last day of loading, the specimens were cultured for one additional day and processed as follows. Alginate specimens were solubilized in 55mM sodium citrate in 0.9% NaCl for 30 minutes at 37°C and centrifuged at 2,000 rpm for 5 min. Total RNA was isolated using an RNeasy mini kit (Quiagen AG, Switzerland) and the concentration and purity of each sample was assayed spectrophotemetically. RNA was reversed transcribed using Multiscribe reverse transcriptase in the presence of dNTPs and random hexamers following the manufacturer's protocol (Taqman Reverse Transcription Reagents, Applied Biosystems, Cham, Switzerland). TAQman probe and primer sets

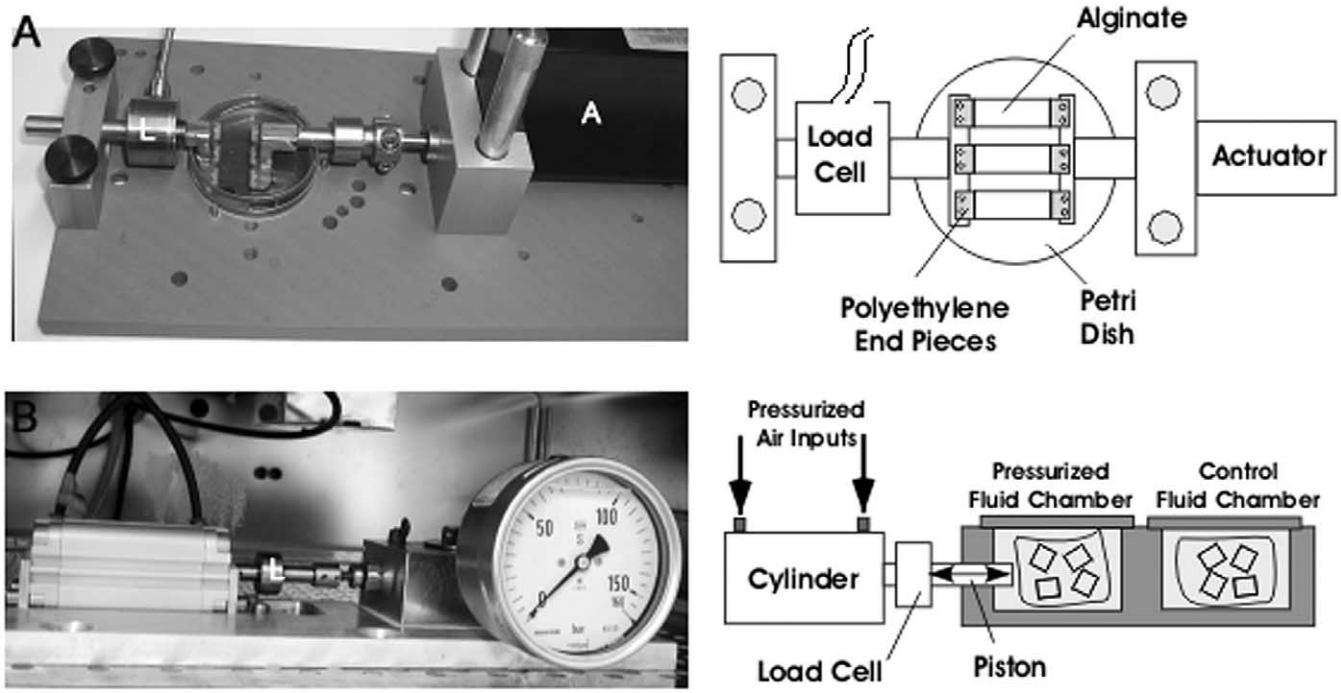


Fig. 1. Tensile (A) and hydrostatic pressure (B) testing configurations. In the tensile configuration, three alginate strips are exposed to a sinusoidal stretch via the computer- controlled actuator (A). A load cell (L) measures the corresponding change in load. In the hydrostatic pressure chamber, alginate disks are sealed in bags and placed in a fluid filled chamber. Compressed air is alternately pumped into the double cylinder to create cyclic pressures between 0–5 MPa.

were designed using the Primer Express software (Applied Biosystems, Cham, Switzerland) to have a melting temperature between 58–60C°. The sequences for bovine MMP-13, collagen X, TIMP-1, TIMP-2, CTGF, VEGF and GAPDH were available in the GenBank (Table 2). The primer/probe set for Cbfa1 was taken from the region of exon 4 which shows 100% identity between the mouse and human sequence. Bovine primer/probe sets for sox9, collagen 2, aggrecan, COMP, collagen 1 and lubricin (superficial zone protein) are shown in Table 3. All probes were labelled on the 5′ end with a FAM reporter fluorescent dye and on the 3′ end with a TAMRA quencher fluorescent dye. Cleavage of the probe and emission of the fluorescent reporter dye allows detection of the accumulation of the PCR product with each cycle. Gene-specific PCR products were continuously measured by means of an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) during 45 cycles (15 s at 95°C followed by 1 min at 60°C). Standard curves were performed for all probe/primer sets (Fig. 2) and the efficiency of the reactions was determined to be greater than 91%.

Table 1
Peak stress and strains in alginate culture

Loading mode	Cyclic stress/strain
Tension	0.004 MPa/9% ($n = 8$)
Hydrostatic pressure	5 MPa/~0% ($n = 8$)

Twenty ng cDNA/reaction and a primer and probe concentration of 900 nM and 300 nM were used, respectively. Each reaction was performed in duplicate. Expression was given as $2_T^{\Delta C_T}$, where C_T is the cycle number at which the fluorescence level reached a threshold value of 0.151 and ΔC_T is endogeneous control C_T value minus the C_T value of the target gene. Gene expression normalized to unloaded control was calculated as $2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T = \Delta C_T$ (load) $- \Delta C_T$ (control) (User Bulletin #2, Applied Biosystems). The difference in amplification efficiency between GAPDH and the gene of interest was less than 7.3%. The GAPDH values for the loaded and unloaded control varied by less than one tenth of a cycle (SD = 0.06 cycle), demonstrating that GAPDH was an excellent endogenous control.

Statistics

The data were analyzed using an ANOVA with repeated measures. In the case of a significant interaction term, a non-parametric Wilcoxon signed-ranks test was performed with significance set at $p \leq 0.02$ (Bonferroni adjustment).

Results

We performed loading experiments to determine the effect of intermittent hydrostatic pressure and cyclic tension on the expression of Cbfa1, Sox9 and their downstream

Table 2
Primer/probe sets for hypertrophic phenotype markers

Gene		5′ to 3′ sequence	Amplicon size	GenBank number	Efficiency
Cbfa1/Runx2	Forward	AGTGATTTAGGGCGCATTCCT	68	AF001447	0.980
	Probe	ATCCCAGTATGAGAGTAGGTGTCCCGCC			
	Reverse	GAGGGCCGTGGGTTCTG			
MMP-13	Forward	CAATGTTTTTCCTCGAACTCTCAA	94	AF072685	0.998
	Probe	AATTCACAATTCTGTAGGTTAA(A/G)TTCATTTTGGACCA			
	Reverse	TTCCACTTCAGAATGAGTCAGATCA			
Collagen 10	Forward	ACTTCTCTTACCACATACACGTGAAAG	108	X53556	0.941
	Probe	ACTTCTCTTACCACATACACGTGAAAG			
	Reverse	CCAGGTAGCCCTTGATGTACTCA			
TIMP-1	Forward	CACCCACAGACGGCCTTCT	90	AF144763	0.968
	Probe	CCACGAACTTGGCCCTGATGACG			
	Reverse	CTGGTATAAGGCAGTTTCATTGACTT			
TIMP-2	Forward	TGGGTCACGGAGAAGAACATC	71	AF144764	0.959
	Probe	ACGG(G/A)CACCAGGCCAAGTTCTTCG			
	Reverse	GAGCCGTCGCTTCTCTTGAT			
CTGF	Forward	CCTGGAGGAGAACATTAAGAAAGG	82	AF000137	0.923
	Probe	AAAGTGCATCCG(G/T)ACTCCCAAAATCTCC			
	Reverse	GCCAGAAAGCTGAAACTTGATAGG			
VEGF	Forward	GGCTGCTGTAATGACGAAAGTCT	127	M32976	0.935
	Probe	CACCATGCAGATTATGCGGATCAAACCT			
	Reverse	TGTTATGCTGTAGGAAGCTCATCTCT			
GAPDH	Forward	AAGCTCGTCATCAATGGAAAGG	74	U85042	0.925
	Probe	TCACCATCTTCCAGGAGCGAGATCC			
	Reverse	GCATCACCCCACTTGATGTTG			

targets. Three days of cyclic tension applied to primary bovine chondrocytes significantly increased the expression of cbfa1 (1.4 fold, $p = 0.02$) and its target MMP-13 (2.3 fold, $p = 0.01$, Table 4, Fig. 3). Three days of cyclic hydrostatic pressure resulted in a significant decrease in MMP-13 expression (0.7 of unloaded control, $p = 0.02$). Interestingly, both tension and hydrostatic pressure significantly upregulated expression of type X collagen, the effect being most pronounced for cyclic tension (1.5 fold over unloaded control, $p = 0.01$). Tissue inhibitors of matrix metalloproteinase, TIMP-1 and TIMP-2, showed an opposite response to each other with mechanical load. The expression of TIMP-1 was reduced by cyclic tension (0.6 of control levels, $p = 0.01$) and slightly, but significantly increased by cyclic hydrostatic pressure (1.14 time control, $p = 0.01$). Conversely, the expression of TIMP-2 was increased by cyclic tension (1.4 fold, $p = 0.01$). We also measured the expression of two angiogenic factors with

Table 3
Primer/probe sequences for sox9 and ECM genes

Gene		5′ to 3′ sequence	Amplicon size	GenBank number	Efficiency
SOX9	Forward	ACGCCGAGCTCAGCAAGA	71	AF278703	0.997
	Probe	CGTTCAG(T/G)AGTCTCCAGAGCTTGCCCA			
	Reverse	CACGAACGGCCGCTTCT			
Collagen 2	Forward	AGCAGGTTCACATATACCGTTCTG	73	X02420	0.919
	Probe	CCGGT(A/G)TGTTTCGTGCAGCCATCCT			
	Reverse	CGATCATAGTCTTGCCCCACTT			
Aggrecan	Forward	CAGCCAGGCCACCCTAGAG	74	BTU76615	0.927
	Probe	ATGGAACACGATGCCTTT(C/T)ACCACGA			
	Reverse	GGGTGTAGCGCGTGGAGAT			
COMP	Forward	CCAGAAGAACGACGACCAGAA	128	X74326	0.955
	Probe	ACGGCGACCGGATCCGCAA			
	Reverse	TCTGATCTGAGTTGGGCACCTT			
Collagen 1	Forward	ACATGCCGAGACTTGAGACTCA	86	AB008683	0.975
	Probe	CCACCCAGAATGGAGCAG(T/C)GGTTACTACTG			
	Reverse	GCATCCATAGTACATCCTTGGTTAGG			
Lubricin (Superficial zone protein)	Forward	TTATTTCTGGATGCTGACTCCATTTA	73	AF056218	0.965
	Probe	TCCACCACCTCCACCTCGGAGAATTA			
	Reverse	GGGAATGCCCCAAACTTCA			

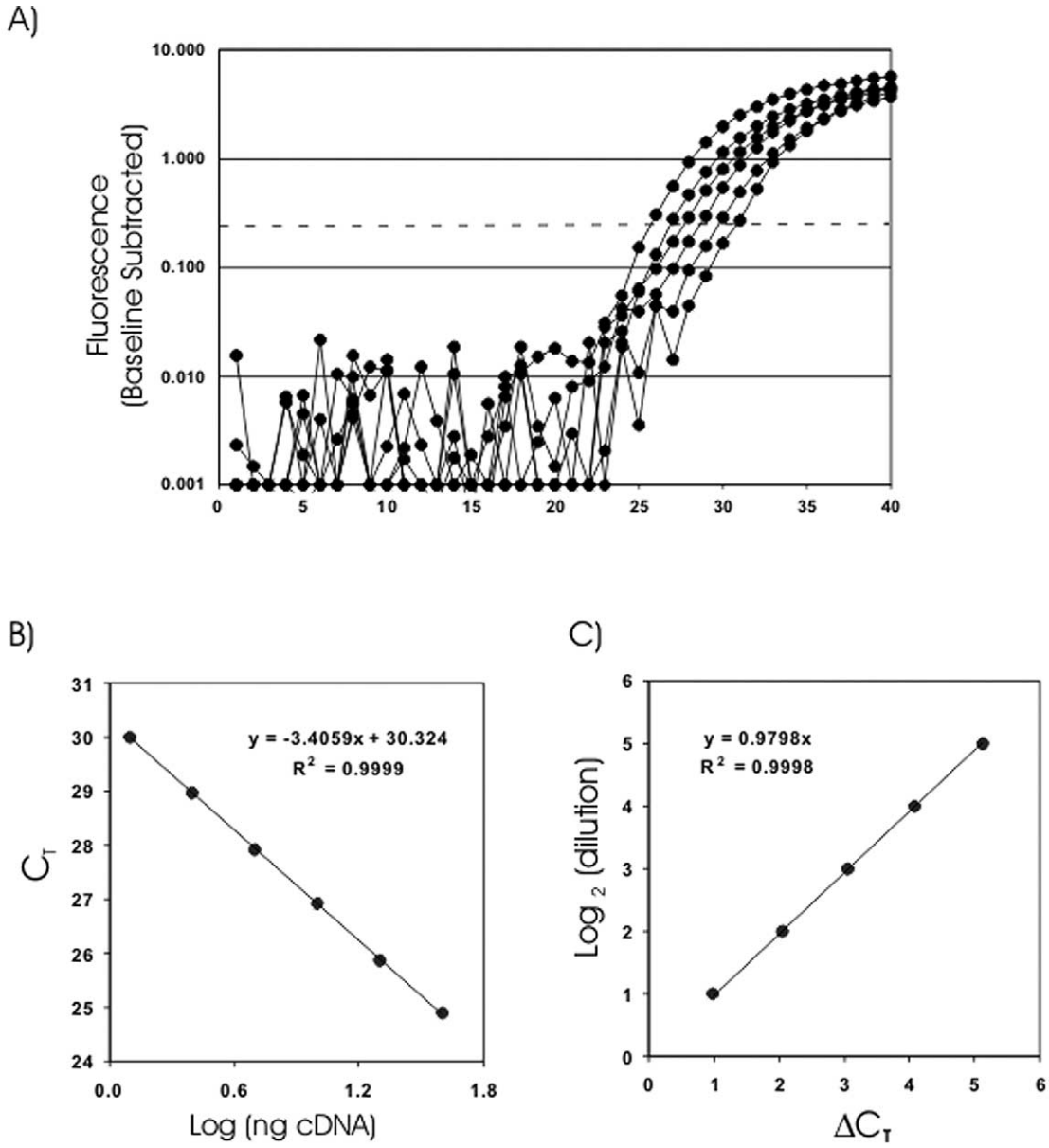


Fig. 2. Standard curves for real-time PCR of cbfa1 transcript. (A) The accumulation of PCR product for the cbfa1 probe/primer set is detected by a measurement of the emitted fluorescence. The plots of fluorescence versus cycle number for serial dilutions of 40, 20, 10, 5, 2.5, and 1.25 ng of cDNA are given. C_T was defined as the cycle number at which a fluorescence threshold of 0.15 was attained. All negative fluorescence values were set to 0.001 for the sake of graphing. (B) The C_T value for Cbfa1 transcript plotted versus the log of the starting amount of cDNA. (C) The measured ΔC_T versus the expected change given a 1:2 dilution [log2(dilution factor)]. The efficiency of the reaction is given by the slope of this curve, in this case 0.98 or 98%. See [38] for further details.

important roles in endochondral ossification. The expression of CTGF was increased by cyclic tension 1.6 fold ($p = 0.01$). VEGF was significantly upregulated by both cyclic tension and hydrostatic pressure ($p = 0.01$ and $p = 0.01$).

We hypothesized that the expression of Sox9 and its extracellular matrix targets would be differentially regulated by the two types of loading. There was, however, no significant effect of loading on sox9 expression (Table 5, Fig. 4). Expression of type 2 collagen was increased with cyclic tension (1.3 fold, $p = 0.01$), but the expression of aggrecan was not affected by either loading mode. Cyclic tension also significantly increased the expression of COMP (1.8 fold), lubricin (superficial zone protein) (1.5 fold), and type I collagen (1.1 fold) compared to unloaded control specimens. Hydrostatic pressure on the other hand, did not significantly alter the expression of any gene in Table 5, with the exception of reducing the expression of type I collagen (0.73 of control, $p = 0.01$).

Discussion

Gradients of shear and hydrostatic pressure are created throughout the cartilaginous skeleton during muscle con-

Table 4
Expression of hypertrophic markers genes relative to the GAPDH control (a value of one equals the same expression level as GAPDH). Expression is calculated as $2^{\Delta C_T}$, where C_T is the cycle number at which the fluorescence level reaches a threshold value of 0.151 and $\Delta C_T = C_{T(\mathrm{GAPDH})} - C_{T(\mathrm{Target})}$. p values were determined using the Wilcoxon signed-ranks test with significance set at $p \leq 0.02$.

Gene	Loading type	Unloaded mean (sd)	Loaded mean (sd)	P value
Cbfa1/Runx2	Hydrostatic pressure	0.0108 (0.0020)	0.0098 (0.0013)	0.04
	Tension	0.0072 (0.0037)	0.0099 (0.0051)	0.02
MMP13	Hydrostatic pressure	0.0025 (0.0028)	0.0019 (0.0025)	0.02
	Tension	0.0007 (0.0004)	0.0017 (0.0011)	0.01
Type X collagen	Hydrostatic pressure	0.0063 (0.0052)	0.0077 (0.0061)	0.01
	Tension	0.0052 (0.0034)	0.0076 (0.0040)	0.01
TIMP-1	Hydrostatic pressure	0.305 (0.087)	0.349 (0.082)	0.01
	Tension	0.662 (0.187)	0.387 (0.109)	0.01
TIMP-2	Hydrostatic pressure	0.188 (0.085)	0.167 (0.069)	0.04
	Tension	0.095 (0.025)	0.134 (0.028)	0.01
CTGF	Hydrostatic pressure	0.402 (0.288)	0.351 (0.291)	0.26
	Tension	0.382 (0.137)	0.602 (0.194)	0.01
VEGF	Hydrostatic pressure	0.033 (0.025)	0.042 (0.030)	0.01
	Tension	0.041 (0.027)	0.057 (0.033)	0.01

tractions and movement and may exert importance influences on cartilage biology. The results of this study demonstrate that the pattern of chondrocyte gene expression is differentially responsive to varying mechanical signals. The two loading environments used in this study were selected because they each incorporate certain features of how articular cartilage functions and is loaded in vivo. Five megapascals of hydrostatic pressure is within the range of fluid pressure created in the middle and deep zones of articular cartilage during normal activity. Tensile strains, on the other hand, are created tangential to the joint surface as the joint articulates and at the interface region between cartilage and bone [12].

We report in this study on the unique mechanical regulation of MMP-13 and its inhibitors by cyclic tension and hydrostatic pressure. MMP-13 expression was significantly upregulated in chondrocytes in alginate subjected to cyclic tension and down regulated in cells exposed to hydrostatic pressure. This effect was enhanced by the upregulation of TIMP-1 by hydrostatic pressure and its down-regulation by cyclic tension. These findings have important implications for endochondral ossification because MMP-13 is the dominant proteolytic enzyme in hypertrophic cartilage [13,14] and is required for vascularization of the calcified cartilage. MMP-13 efficiently hydrolyzes type II collagen [15] and aggrecan [16] preparing the way for invasion by endothelial

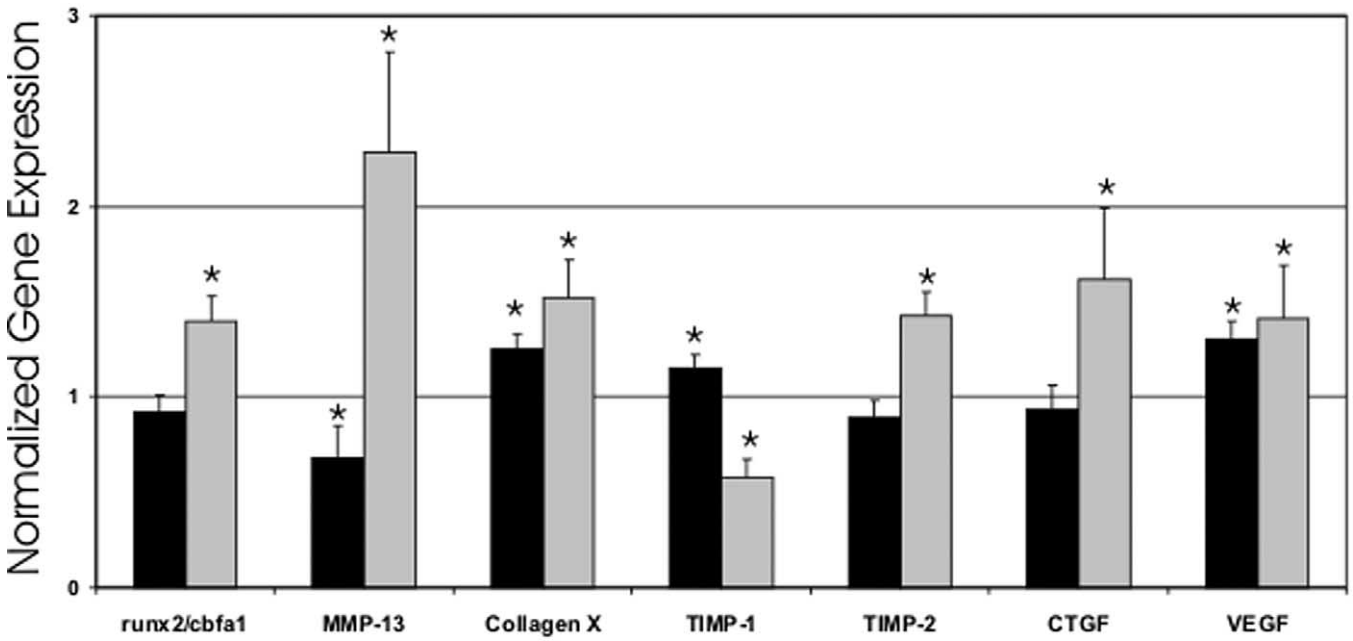


Fig. 3. The gene expression of hypertrophic markers by intermittent hydrostatic pressure (■) and tension (□). Data has been normalized to unloaded controls, where a 1.0 represents no effect of loading. *Refers to a significant effect of loading with respect to unloaded control ($p \leq 0.02$).

Table 5
Expression of sox9 and ECM genes relative to the GAPDH control (a value of one equals the same expression level as GAPDH). Expression is calculated as $2^{\Delta C_T}$, where C_T is the cycle number at which the fluorescence level reaches a threshold value of 0.151 and $\Delta C_T = C_{T(\mathrm{GAPDH})} - C_{T(\mathrm{Target})}$. p values were determined using the Wilcoxon signed-ranks test with significance set at $p \leq 0.02$.

Gene	Loading type	Unloaded	Loaded	p value
Sox9	Hydrostatic pressure	0.280 (0.242)	0.305 (0.257)	0.07
	Tension	0.165 (0.077)	0.167 (0.055)	0.33
Type 2 collagen	Hydrostatic pressure	67.22 (27.10)	64.27 (23.57)	0.26
	Tension	26.92 (4.13)	35.81 (4.92)	0.01
Aggrecan	Hydrostatic pressure	1.285 (0.863)	1.325 (0.768)	0.21
	Tension	0.999 (0.313)	0.994 (0.316)	0.89
COMP	Hydrostatic pressure	1.597 (1.010)	1.677 (1.054)	0.09
	Tension	0.925 (0.330)	1.705 (0.683)	0.01
Type 1 collagen	Hydrostatic pressure	0.0023 (0.0009)	0.0015 (0.0010)	0.01
	Tension	0.0015 (0.0003)	0.0017 (0.0004)	0.02
Lubricin	Hydrostatic pressure	0.089 (0.060)	0.078 (0.052)	0.05
	Tension	0.065 (0.039)	0.095 (0.055)	0.01

and mesenchymal cells. The degradation of the matrix by MMP-13 may also activate MMP-9 (gelatinase B) and release sequestered stores of angiogenic and growth factors. Chemical inhibition of MMP-13 activity suppresses degradation of type II collagen, type X collagen expression, and calcium uptake [17]. In animal models, a lack of MMP13 expression in the hypertrophic cartilage is associated with the absence of vascular invasion and diminished or absent endochondral bone formation [18,19]. The effects of mechanical loading on MMP-13 expression seen in this study are consistent with the hypothesis that endochondral ossification is accelerated in regions of cyclic tension. In fact, the tension/shear created at the cartilage/bone interface may provide an important regulatory signal which restricts the expression of MMP-13 to the hypertrophic zone.

Our data also suggest that Cbfa1/Runx2 signaling could provide the molecular link between mechanical loading and the observed effect on chondrocyte maturation. Cbfa1/Runx2 is a transcription factor with a fundamental role in bone formation. Its targets include MMP-13, alkaline phosphastase, osteocalcin, and osteonectin, all highly expressed by hypertrophic chondrocytes as well as osteoblasts. Cbfa1 −/− mice show an absence of MMP-13 expression, cartilage calcification and vascular invasion [20], whereas overexpression of Cbfa1 accelerates calcium uptake and alkaline phosphatase activity [21]. In our study, Cbfa1 expression was upregulated 40% by cyclic tension. Other studies have shown that mechanical deformation of osteoblastic cells increases expression and DNA binding of Cbfa1 [6]. These data suggest the intriguing hypothesis that load-induced alterations in bone remodeling and endochondral ossification could be regulated by Cbfa1.

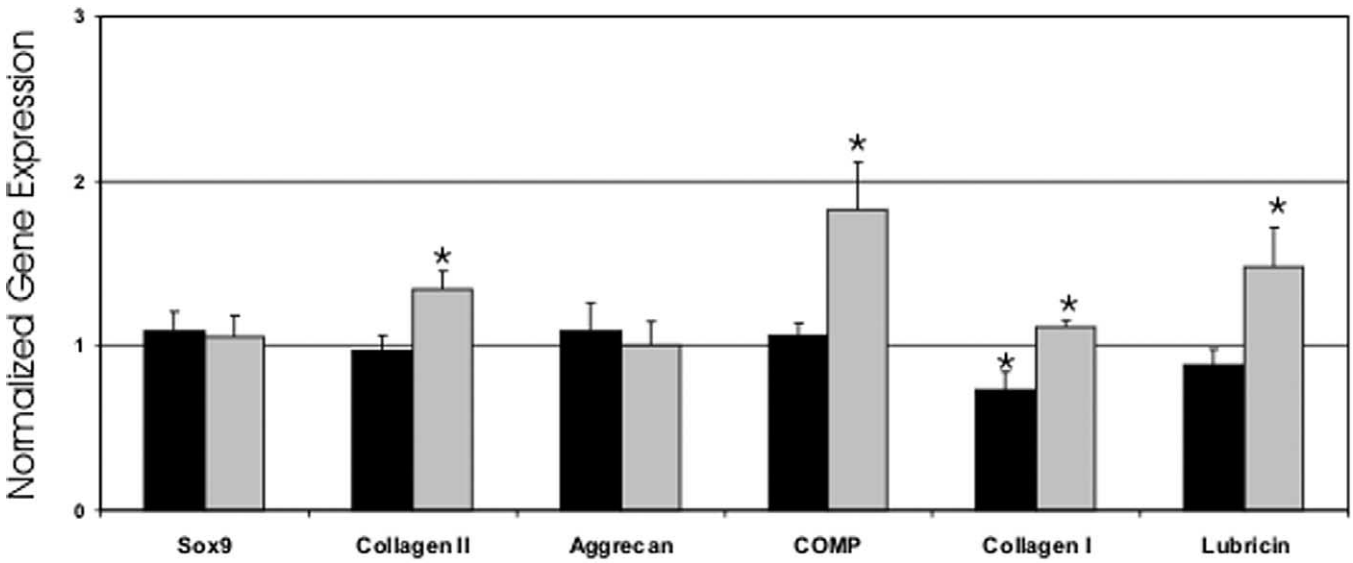


Fig. 4. The gene expression of Sox9 and cartilage matrix proteins by intermittent hydrostatic pressure (■) and tension (□). Data has been normalized to unloaded controls, where a 1.0 represents no effect of loading. *Refers to a significant effect of loading with respect to unloaded control ($p \leq 0.02$).

Type X collagen is a third hypertrophic marker upregulated by tension. Although not a direct target of Cbfa1, the expression of type X collagen is linked to the expression of MMP-13. When MMP-13 activity was chemically inhibited, the expression of hypertrophic markers (type x collagen, Cbfa1, and MMP-13) was also inhibited [17]. Type X collagen expression has also been shown to be upregulated by cyclic deformation in explants (data not shown) and collagen gels [22] which suggests that this phenomenon is a generalizable one with relevance to in vivo physiology. Although the function of type X collagen is under debate, it is interesting to note that the severity of type X collagen-associated lesions correlate with severity of loading. Cartilage lesions in human disease (Schmid's chondroplasia) and knockout mice are most severe in situations of high mechanical loading and are mild in situations of low load (i.e, immobilization and bedrest) (23–25). One explanation is that the upregulation of type X collagen in sites of high shear/tensile stress increases the mechanical resilience of the matrix, possibly by influencing the efficiency of calcification and mineralization.

Angiogenic factors were of particular interest in this study, because it is the shift in the balance of pro- and anti-angiogenic factors which may allow a previously avascular tissue to become vascularized. The upregulation of CTGF and VEGF by tensile strain may contribute in an additive fashion to the pro-angiogenic effect of increased MMP-13 expression. Both CTGF and the related CYR61 are found in hypertrophic cartilage and have a putative role in its neovascularization [26,27]. CTGF is a secretable signalling protein which induces proliferation, adhesion and migration of endothelial cells in in vitro and in vivo angiogenesis assays. Furthermore, the expression of CTGF and CYR61 are both sensitive to mechanical loading [4,28]. VEGF is a mitogen for endothelial cells, causes vessels to become more permeable and also stimulates migration of osteoblasts [29,30]. In other cell systems, VEGF expression has also been shown to be responsive to mechanical signals [3,31].

Sox9, together with Sox5 and Sox6, are crucial for the induction of the cartilage phenotype during skeletal development. Most of the major cartilage matrix proteins (type II, IX and XI collagen, aggrecan, COMP) contain binding sites for this transcription factor in their promoters and cartilage fails to form in Sox9 $-/-$ mutant mice. We hypothesized that the expression of Sox9 would be upregulated by hydrostatic pressure, a hypothesis which was not confirmed by our findings. Other studies, however, have shown that physiologic levels of hydrostatic pressure result in an upregulation of type II collagen and aggrecan and are chondroprotective [11,32–35]. Differences in culture conditions, scaffold properties, cell density, and loading magnitude, interval and frequency could all conceivably have an effect on biosynthesis. In our study, the primary effect of hydrostatic pressure was to inhibit type 1 collagen dedifferentiation and MMP- 13 activity, rather than increase expression of ECM associated structural proteins.

The regulation of endochondral ossification is a complex process. Each stage of the differentiation is under control of a number of growth factors, regulatory molecules, angiogenic factors, and proteases and their inhibitors. Although it is not possible to judge how mechanical factors modulate this process by looking at the expression of a single gene, we believe that the general picture provided by the results of this study supports the notion that cyclic tension accelerates expression of hypertrophic markers in articular chondrocytes. The magnitude of the changes we saw in our loading experiments were relatively small. However, the changes were reproducible across eight independent experiments, and the statistical significance was found using a conservative p level of 0.02. Mechanical regulation of cartilage biology may consist of accumulated effects of small magnitude changes over relatively long periods of time, instead of an off/on effect. It must also be emphasized that these changes have been found at the transcriptional level only and further studies must be performed to quantify protein levels by western blotting and immunohistochemistry. Evidence suggests, at least for COMP, that mechanically induced increases in transcript are accompanied by an equally great increase in protein [36,37].

The unique response of chondrocytes to different types of mechanical loading suggests that mechanical stresses can modulate the patterns of ossification and distribution of extracellular matrix proteins, ultimately yielding a skeleton which is uniquely adapted to the physical forces it experiences.

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