Static Compression of Single Chondrocytes Catabolically Modifies Single-Cell Gene Expression

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ABSTRACT Previous work has established that mechanical forces can lead to quantifiable alterations in cell function. However, how forces change gene expression in a single cell and the mechanisms of force transmission to the nucleus are poorly understood. Here we demonstrate that the gene expression of proteins related to the extracellular matrix in single articular chondrocytes is modified by compressive forces in a dosage-dependent manner. Increasing force exposure catabolically shifts single-cell mRNA levels of aggrecan, collagen IIa, and tissue inhibitor of metalloproteinase-1. Cytohistochemistry reveals that the majority of strain experienced by the cell is also experienced by the nucleus, resulting in considerable changes in nuclear volume and structure. Transforming growth factor-β1 and insulin-like growth factor-I offer mechanoprotection and recovery of gene expression of aggrecan and metalloproteinase-1. These results suggest that forces directly influence gene transcription and may do so by changing chromatin conformation.

INTRODUCTION

How cells receive and interpret their mechanical environment is of paramount importance to homeostasis and disease in many tissues, including cartilage, bone, muscle, vasculature, and skin. Processing mechanical signals is also extremely important for sensory functions such as hearing, touch, and proprioception (1). To date, studies have focused mainly on molecular mechanisms, populational cell responses, and tissue-level responses to mechanical forces. It is well established that mechanical stimuli can modify the gene expression in cells (2–4); however, an understanding of how an individual cell modifies its transcription in response to a defined mechanical environment is just beginning to be explored (5).

Explants and cell populations have been shown to respond to mechanical forces by regulating mRNA levels (2–4,6–10). The specific transcriptional changes largely depend on cell type and mechanical stimulus. A number of cell and tissue types within the body demonstrate sensitivity to their mechanical environment in terms of both transcriptional and translational responses. Articular chondrocytes offer a prime example of such force sensitivity. This is a direct reflection of the tissue from which these cells originate. Articular cartilage largely functions as a mechanical tissue and serves to absorb and evenly distribute compressive loads between long bones. Cartilage routinely experiences stress amplitudes of 10 to 20 MPa and compression up to 45% (11,12). Chondrocytes appear to be highly selective in their responses to mechanical stimuli. Dynamic hydrostatic and direct compression at physiologic loads and frequencies (1 Hz) appear to stimulate regenerative metabolism (6,7,13). Aberrant mechanical stimuli such as static compression, tensile stretch, and injurious compression can lead to catabolic changes and the disease osteoarthritis (6,8,10,14). Our group has begun to study the biomechanical responses of single chondrocytes to defined mechanical stimuli to better understand how single-cell responses contribute to tissue-level responses. Recently we reported that single chondrocytes modify their gene expression in response to compressive loading (5). This work reveals that dynamic compressive loading results in higher gene expression of collagen type II and aggrecan as compared with statically loaded cells. Static compression to forces of 50 and 100 nN suppressed type II collagen expression, whereas equivalent dynamic loads returned expression to control levels. This work validates and demonstrates the merits of the use of a single-cell approach (15) for studying the biological response of cells to mechanical stress.

The results of studies with optical tweezers, magnetic beads, and atomic force microscopy (AFM) have allowed for the construction of a nascent overview of a cell's local response to a small mechanical stimulus. These techniques have allowed researchers to observe specific biochemical changes in single cells and to understand the response of mechanically sensitive ion channels, cytoskeletal proteins, focal adhesions, and kinases (1,16). A recent study from our group has begun to reveal the mechanical stimuli required to elicit measurable gene expression changes in a single cell (5); however, distinct differences in single-cell gene expression among different static force levels were unclear, as was the mechanism of force sensing.

The objectives of this study are to determine whether different levels of static compression give distinct differences in single-chondrocyte gene expression, to determine how static force signals are conveyed to the nucleus to modify gene expression, and to use this information to attempt to provide single chondrocytes with treatments for reversing the effects of static compression. To accomplish this, we exposed single

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chondrocytes to varying magnitudes and durations of static force and utilized single-cell real-time reverse transcriptase polymerase chain reaction (RT-PCR) to quantify mRNA levels of extracellular matrix (ECM)-related genes: aggrecan, collagen IIa, tissue inhibitor of metalloproteinase-1 (TIMP-1), matrix metalloproteinase-1 (MMP-1), and cartilage oligomeric matrix protein (COMP). Cell and nuclear structure were studied with cytohistochemistry with or without static compression. The growth factors transforming growth factor-β1 (TGF-β1) and insulin-like growth factor-I (IGF-I) were used to attempt to modify the response of single chondrocytes to static loading. The hypothesis for this study was that static compression would have detrimental effects on single-cell gene expression and that TGF-β1 and IGF-I would alleviate the effects of compression.

MATERIALS AND METHODS

Cell culture

Middle-/deep-zone bovine articular chondrocytes were harvested aseptically from distal metatarsal cartilage of 18-mo-old heifers obtained from local abattoirs. Enzymatically digested (2 mg/ml collagenase type II and 3 mg/mL dispase in medium for 12 h or less in incubator) chondrocytes were seeded onto a tissue culture treated plastic 150 $\times$ 20 mm dish (plasma treated by Techno Plastic Products, Trasadingen, Switzerland) at an areal cell density of $\sim 3.3 \times 10^4$ cells/cm^2. The plates were incubated for 3 h at 37°C and 10% CO_2 before compression testing. TGF-β1 and IGF-I (PeproTech, Rocky Hill, NJ) were used at a single concentration each, 5 ng/ml and 100 ng/ml, respectively, as used previously (17).

Single-cell compression

To determine how a single chondrocyte responds both biomechanically and transcriptionally to a cell-wide mechanical stimulus, we have developed a device called the creep cytocompression apparatus (CCA), which can mechanically compress individual adherent cells (Fig. 1 *A*) (17–19). This device uses similar principles to AFM (beam theory and force control) to expose a single cell to a constant force while measuring the resulting creep behavior. A major difference from AFM is that instead of using a probe with a tip diameter of $\sim$10 nm, we are able to use a probe 50.8 μm in diameter, allowing us to perform macro-level unconfined compression on single cells.

After cell attachment, the medium was removed from the culture dish and replaced with supplemented medium containing 30 mM HEPES (Fisher Scientific, Pittsburgh, PA) warmed to 37°C. TGF-β1 or IGF-I was included in the fresh medium for the corresponding treatment group. The dish was then placed into the apparatus for creep testing. Unconfined compression was performed on attached chondrocytes. Compression of single cells began by ramping the probe down onto a cell, under closed-loop control. Depending on the treatment, a constant force of 25, 50, or 100 nN was applied to single cells for 30 or 60 s. Once the specified force was achieved, displacement data were collected. Cell height was determined by comparing probe contact with the cell to the measured distance to the dish. Each tested cell was recorded by noting its position on the dish, which was etched with crisscrossing lines before seeding. Compression testing lasted no longer than 45 min, after which time the medium was replaced with fresh supplemented DMEM warmed to 37°C. TGF-β1 or IGF-I was once again included in the fresh medium for the corresponding treatment group.

Single chondrocyte isolation

Compressed and control chondrocytes were captured using a glass micropipette pulled and microforged to an inner diameter of $\sim$15 μm and a CellTram Vario hydraulic microaspirator (Eppendorf, Hamburg, Germany).

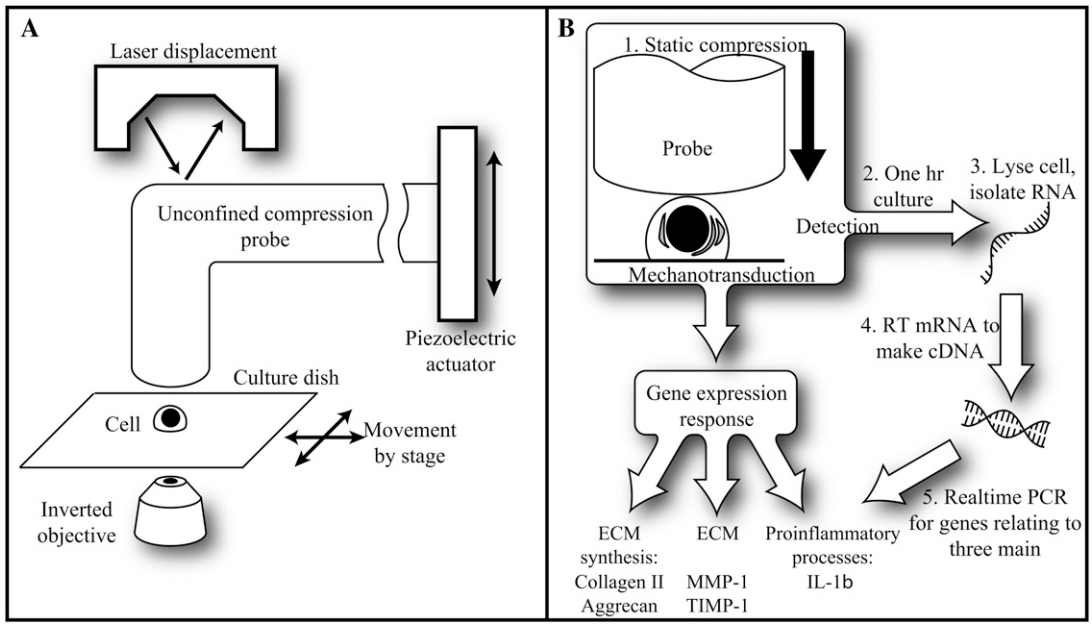

FIGURE 1 CCA for studying single-cell gene response to unconfined compression. (*A*) This device exposes single adherent cells to unconfined creep compression at forces ranging from 10 nN to 200 nN or greater. Beam theory is used to calculate the force while measuring the resulting cellular deformation. (*B*) Unconfined compression of single cells modifies cell gene expression by mechanotransduction. For chondrocytes, this modification could occur in genes related to major processes involved in cartilage homeostasis and disease. Gene expression is measured in compressed chondrocytes through single-cell RT-PCR.

Firmly attached single cells were collected by gentle suction pressure. Captured cells were then ejected into lysis buffer (Stratagene, La Jolla, CA) for RNA isolation. Specific compressed cells were identified for isolation by notes and drawings recorded during compression. Control cells were isolated at the same time from the dish between isolation of compressed cells.

RNA isolation and real-time RT-PCR (5′-nuclease assay)

Single-cell real-time RT-PCR was utilized to analyze transcriptional changes in these single chondrocytes. Assays were performed on each cell sample to determine the mRNA levels of seven genes relating to ECM production, collagen maintenance, and the proinflammatory process (Fig. 1 *B*). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for each sample. RT-PCR has been demonstrated to be sensitive enough to amplify mRNA isolated from a single cell (20) and has been successfully performed on smooth muscle cells after contractile events (21,22). Recent work from our group has shown that single cell real-time RT-PCR is a valuable tool for studying single chondrocyte gene expression response to growth factors and mechanical stimulus (5,23).

Total RNA was isolated from single chondrocytes using the Absolutely RNA Nanoprep protocol (Stratagene) with DNase I treatment. The purified total RNA for each chondrocyte was eluted into a volume of 8 μl for the RT reaction. Single-cell RNA was incubated with 1 mM dNTPs, 0.5 μM oligo$(dT)_{20}$ primers, and 0.5 μM random hexamers for 5 min at 65°C to anneal primers to the template RNA, followed by addition of buffer, 2.5 mM $MgCl_2$, 1 mM DTT, SuperScript III RT enzyme (Invitrogen, Carlsbad, CA), and RNase inhibitor for 10 min at 25°C for further primer annealing, 50 min at 50°C for reverse transcription, and 5 min at 85°C to terminate the reaction. Gene expression was assayed using multiplex real-time PCR performed on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) with HotStarTaq polymerase (Qiagen, Valencia, CA), 5 mM $MgCl_2$, and 2.5 mM dNTPs. The HotStarTaq polymerase was activated at 95°C for 15 min, followed by 55 cycles of 95°C for 15 s and 60°C for 30 s. Fluorescence measurements (on Cy5, FAM, and ROX) were taken every cycle at the end of the 60°C step to provide a quantitative real-time analysis of the genes analyzed. Primers were synthesized by Sigma-Genosys (Woodlands, TX), and gene-specific hydrolysis probes were synthesized by Biosearch Technologies (Novato, CA) and Eurogentec (Seraing, Belgium). The oligonucleotide sequences for aggrecan core protein, collagen IIa, COMP, GAPDH, IL-1β, MMP-1, and TIMP-1 were designed for real-time RT-PCR (5′-nuclease assay) from bovine mRNA sequences from the National Center for Biotechnology Information (Bethesda, MD). These sequences have been published previously (5,23) except for IL-1β: 5′ to 3′ forward primer (AAATGAACCGAGAAGTGGTG), reverse primer (CCAAGGCCACAGGAATC), and probe (CATGAGCTTTGTGCAAGGAGAGGAAAG). Three separate PCRs were performed on each single-cell cDNA sample: 1), aggrecan/collagen II/GAPDH, 2), MMP-1/TIMP-1/GAPDH, and 3), IL-1β/COMP/GAPDH.

Evaluation of gene expression

GAPDH abundance was determined quantitatively from real-time PCR. The threshold cycle (C_T) for GAPDH in each single-cell cDNA sample was determined at 20% of the maximum of the second derivative of fluorescence with respect to cycle number, as determined by comparative quantification analysis in the Rotor-Gene 6.0 program (Corbett Research, Concorde, NSW, Australia). Efficiency of the PCR was calculated by running a standard curve for serially diluted cDNA from large populations of bovine chondrocytes. In the case of IL-β1, synthesized product oligo was mixed in with bovine cDNA and serially diluted. Abundance values (A) were determined using a method adapted from Pfaffl (24). A is determined by the equation $A = 1/[(1 + E)\text{^}C_T]$, where E is primer efficiency. Higher abundance values indicate that the gene is expressed to a greater extent than genes with lower abundance values.

Relative abundance

Relative abundance for each gene of interest was normalized using GAPDH to account for variations in the overall transcriptional level in individual cells as well as for minor errors in pipetting and RNA isolation. Normalization was calculated only for cells that expressed GAPDH. Genes of interest were aggrecan, collagen IIa, COMP, IL-1β, MMP-1, and TIMP-1. The C_T for each gene of interest was calculated as described for GAPDH. Relative abundance (R) of each gene of interest (GOI) was calculated from E and C_T using the equation:

$$R_{GOI} = \frac{(1 + E_{GAPDH})^{C_{T,GAPDH}}}{(1 + E_{GOI})^{C_{T,GOI}}}. \quad (1)$$

Relative abundance values >1.0 indicate that the gene of interest is expressed to a greater extent than GAPDH.

Compression, fixation, and cytohistochemical staining of single chondrocytes

Chondrocytes were seeded using the protocol above, except they were cultured on glass 25 × 75 × 1 mm microscope slides (VWR, West Chester, PA) that were etched with crisscrossing lines. After 3 h of culture, slides were placed into the CCA for single-cell compression. A single chondrocyte was selected for compression and fixation. Before compression, the cell diameter and position of the cell were recorded. Cells were compressed to deformations of 0.7, 2.25, and 3.1 μm, corresponding to the mean deformations for control cells at 25 nN, 50 nN, and 100 nN, respectively. Before compression began, the probe was located above the cell so that it was just touching the top of the cell, and 3.7% paraformaldehyde in PBS, warmed to 37°C, was placed in the dish, and compression was begun immediately. Cells were fixed for 10 min and held under compression for the entire duration. Then the probe was raised off the, cell and the microscope slide was removed for staining. After compression and fixation, all samples were washed three times with PBS and then permeabilized in 0.1% Triton X-100 in PBS for 5 min. After three more PBS washes, the samples were incubated for 30 min with Alexa Fluor 647 phalloidin (2 U per coverslip; Molecular Probes, Eugene, OR), followed by three washes with PBS. The nucleus was stained with 10 μM Hoechst 33342 for 7 min, followed by three final washes with PBS. Samples were coverslipped with ProLong Gold antifade reagent (Molecular Probes).

Microscopy and image analysis

Cytohistochemistry samples were viewed with an Axioplan 2 microscope (Carl Zeiss, Oberkochen, Germany) and a $CoolSNAP_{HQ}$ charge-coupled device camera (Photometrics, Tucson, AZ). Images were acquired with a 100× objective with z-stacks (at 0.3 μm) through each cell using Metamorph 4.15 (Universal Imaging, Downingtown, PA). All images were acquired in grayscale, processed with two-dimensional deconvolution (nearest neighbors), and colorized for presentation using Metamorph. Orthogonal yz planes and three-dimensional reconstructions were also performed with Metamorph. Measurements of cell heights and widths were made digitally using images of cellular F-actin stained with Alexa Fluor 647 phalloidin. Nuclear heights and widths were made using Hoechst 3342 images.

Strain calculations

Mechanical testing data (creep curves and cell heights) were collected for all cells that were compressed for single-cell RT-PCR. Cells selected for single-cell RT-PCR exhibited a viscoelastic creep response, as observed previously (17,25). The mean final deformation was calculated from each set of creep data by averaging the deformation over the final 10 s. Dividing this number by the cell height yielded axial cell strain.

Lateral and axial strains were additionally calculated from cells that were fixed in compression. Cell axial and lateral strains were calculated by com-

paring diameter and height measurements taken before compression with diameters and heights calculated from three-dimensional reconstructions of the cortical F-actin using Metamorph 4.15. Nuclear lateral and axial strains were calculated by comparing heights and diameters of compressed cells to the mean height and diameter of 25 control chondrocytes, selected from the same slide as compressed cells. The mean nuclear diameter and height were $5.52 \pm 0.51\ \mu$m and $6.33 \pm 0.50\ \mu$m for control chondrocytes. Cell apparent Poisson's ratio (υ_A) was calculated from the equation $\upsilon_A = -\varepsilon_l/\varepsilon_a$ where ε_l is the lateral strain and ε_a is the axial strain (26).

Statistics and data analysis

All results are reported as mean ± SD. Statistical analysis of the data was performed using JMP IN 5.1 (SAS Institute, Cary, NC). The effects of force times, duration, and growth factor treatment were tested with two-factor ANOVA ($\alpha = 0.05$). Where ANOVA revealed differences, a Tukey's honestly significant difference post hoc test was performed to make pairwise comparisons among means. To test whether the gene expression of single chondrocytes followed normal distribution, data were examined using the Shapiro-Wilk W test for normality, where $p < 0.05$ indicated a significant departure from normality. Where data failed the Shapiro-Wilk W test, a Box-Cox normality plot was performed to determine the proper transformation for normalization. Multiple linear regression analyses were used to compare growth factor treatment on axial cell strain versus gene relative abundance data. The following second-order equation was used:

$$Y_i = \beta_0 + \beta_1 X_{i1} + \beta_2 X_{i2} + \beta_3 X_{i3} + \beta_{12} X_{i1} X_{i2} + \beta_{13} X_{i1} X_{i3}, \quad (2)$$

where Y_i is the gene abundance data, $\beta_0, \beta_1, \beta_2, \beta_3, \beta_{12}, \beta_{13}$ are constants, X_{i1} is the axial cell strain, $X_{i2} = 1$ if TGF-β1 treated and $X_{i2} = 0$ if not, and $X_{i3} = 1$ if IGF-I treated and $X_{i3} = 0$ if not. These analyses provided statistical comparisons of slope coefficients to each other.

RESULTS

Single-cell transcriptional response to static compression

Initial analysis of single cell RT-PCR data showed a non-Gaussian distribution for all genes that were analyzed, as we have observed previously in single chondrocytes (5,23). GAPDH abundance data and aggrecan, collagen IIa, TIMP-1, and COMP relative abundance data all failed the Shapiro-Wilk W test for normality ($p < 0.05$). Box-Cox transformations provided lambda values of 0 for all data suggesting a logarithmic data transformation. Therefore, GAPDH abundance data were $\log_{10}$ transformed to a Gaussian scatter. All relative abundance data were transformed by adding 1 to all nonzero data and calculating the $\log_{10}$ of this sum. The resulting transformed data passed the Shapiro-Wilk W test ($p > 0.05$) and were statistically analyzed with linear regression (ANOVA) in JMP. Fig. 2 shows the distribution for GAPDH abundance before and after $\log_{10}$ transformation.

The transformed gene expression data demonstrated that, overall, static compression elicited a catabolic shift in single chondrocyte gene expression (Fig. 3, *A–C*). For each force level, we were able to calculate the axial cellular strain that single cells experienced from data collected during stimulation with the CCA. One-factor ANOVA revealed that the average axial strain significantly increased for each force level ($p < 0.0001$). Increasing duration from 30 to 60 s at 100 nN did not yield a significant change in average strain levels. Increasing force exposure to single cells decreased the mRNA levels of two of the principal ECM proteins in cartilage, aggrecan core protein (Fig. 3 *A*, $p < 0.05$) and collagen IIa (Fig. 3 *B*, $p < 0.05$), while maintaining constant GAPDH mRNA expression. Concomitantly, expression of TIMP-1 increased with greater force in a dose-dependent manner (Fig. 3 *C*, $p < 0.05$). Extending the duration of 100 nN exposure time to 60 s did not significantly modify gene expression responses compared with 30 s. MMP-1 expression in single cells was recorded in only 5 of the 149 cells that were analyzed in the first part of experimentation. The proinflammatory mediator IL-1β was not detected in any single chondrocytes.

Cytohistochemistry of single chondrocytes

Single cells were compressed at deformations equal to the mean deformations seen at 25, 50, and 100 nN during mechanical stimulation of cells or 0.7, 2.25, and 3.1 μm, respectively. Fixation, labeling, and fluorescent microscopy of single chondrocytes allowed us to observe visible alterations

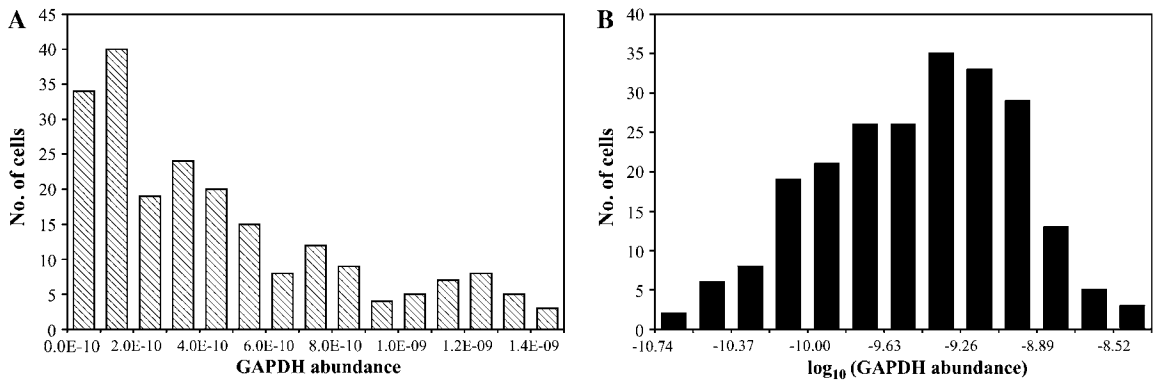

FIGURE 2 Distributions of GAPDH mRNA levels for all cells analyzed. (*A*) The distribution of GAPDH was found to be non-Gaussian. (*B*) Log_{10} transformation of the data creates a Gaussian distribution, allowing linear statistics to be performed.

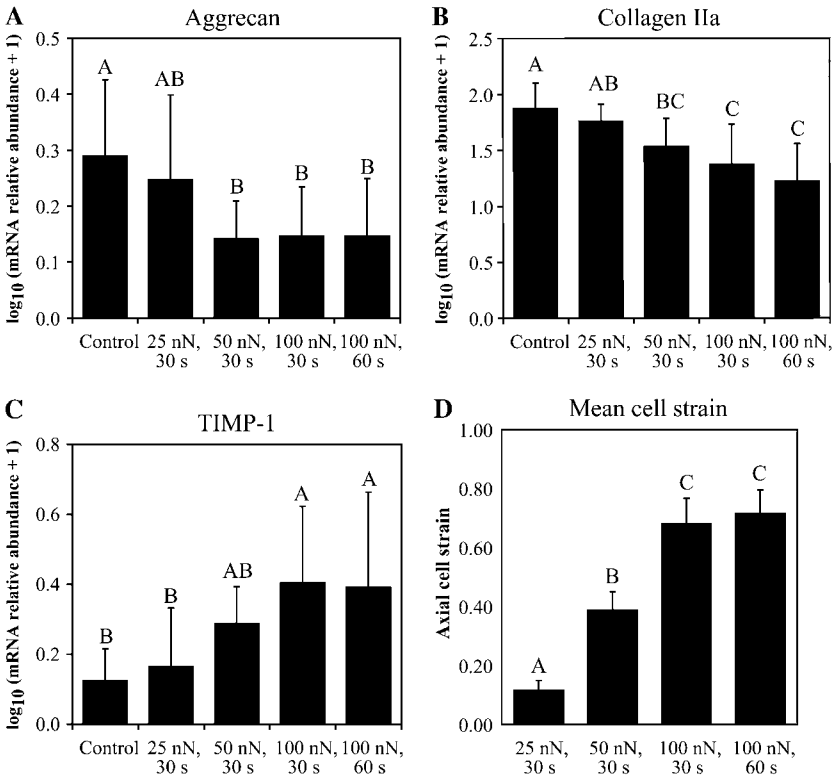

FIGURE 3 Abundance of mRNA in single chondrocytes normalized to GAPDH as determined by single-cell real-time RT-PCR. All values + 1, transformed with $\log_{10}$ to obtain a Gaussian distribution. Only cells that had detectable GAPDH were analyzed. Sample sizes ranged from 31 to 11. All results presented as mean ± SD. Letters indicate significance determined by ANOVA with post hoc analysis ($p < 0.05$). (*A*) Aggrecan core protein and (*B*) collagen IIa mRNA levels normalized to GAPDH decreased with increasing force. Aggrecan expression experienced a sharp decrease after compression with 50 nN of force, whereas collagen IIa showed a dosage decrease from control cells to those exposed to 100 nN of force. (*C*) TIMP-1 mRNA levels increased in a dosage-dependent manner from control cells to those exposed to 100 nN of force. (*D*) Calculation of cell axial strain shows significant increases at each force level ($p < 0.0001$).

to the F-actin and the nuclei of compressed cells as compared with controlled chondrocytes (Fig. 4). Generally, increasing deformation resulted in cells as well as nuclei that were increasingly flattened. Increased deformation also resulted in noticeable changes to chromatin organization within the nucleus (Fig. 4). Three-dimensional image reconstructions of single chondrocytes allowed us to estimate the axial and lateral strains experienced in both the cell and nucleus (Fig. 5). Analysis revealed that increasing compression led to increased strain for both the cell and the nucleus (Fig. 5, *A* and *B*). Plotting the nuclear strain versus the cellular strain yielded a slope of 0.95 for the axial results (Fig. 5 *A*) and a slope of 1.10 for the lateral results (Fig. 5 *B*). Using the lateral and axial strain measurements, we were next able to calculate nuclear and cellular apparent Poisson's ratios (Fig. 5, *C* and *D*). One-factor ANOVA with post hoc analysis revealed that the cellular apparent Poisson's ratio was not significantly different after compression to 0.7 and 2.25 μm (0.45 ± 0.07 and 0.49± 0.04) but showed a significant increase after 3.1 μm of compression (0.96 ± 0.05) (Fig. 5 *C*, $p < 0.0001$). The nuclear apparent Poisson's ratio showed significant increases from 0.42 ± 0.02 at 0.7 μm to 0.67 ± 0.03 at 2.25 μm to 1.07 ± 0.14 at 3.1 μm (Fig. 5 *D*, $p < 0.0001$).

Growth factor stimulation of single-cell transcription after static compression

Single-cell RT-PCR analysis demonstrated that TGF-β1 and IGF-I significantly and differentially reduced the catabolic effects of static compression on single chondrocytes (Fig. 6). Two-factor ANOVA with post hoc analysis revealed that treatment with TGF-β1 prevented the decreased aggrecan core protein mRNA levels seen in untreated chondrocytes exposed to increasing static compression ($p < 0.001$) and offered a stimulatory effect at 25 nN and 30 s of compression (Fig. 6 *A*, $p < 0.05$). IGF-I treatment prevented TIMP-1 mRNA levels from significantly increasing (Fig. 6 *C*, $p < 0.001$). ANOVA revealed that TGF-β1 and IGF-I treatment significantly reduced collagen IIa RNA levels in single chondrocytes as compared with controls (Fig. 6 *B*, $p < 0.01$). Analysis of cell axial strain revealed that both TGF-β1 and IGF-I significantly reduced the cellular strain experienced by single cells as compared with untreated chondrocytes (Fig. 6 *D*, $p < 0.0001$).

COMP, a protein that has been previously associated with osteoarthritic changes in articular cartilage (27) as well as matrix assembly (28), did not significantly increase its gene expression but showed an increasing trend for cells exposed to 100 nN of force (Fig. 7). TGF-β1 and IGF-I significantly decreased mRNA levels of COMP in single cells as compared with controls, and IGF-I showed a significant decrease over both controls and TGF-β1 (Fig. 7, $p < 0.05$).

To further analyze the effects of strain on single chondrocytes, the mRNA levels of aggrecan, collagen IIa, TIMP-1, and COMP were correlated to the axial strain each chondrocyte experienced, and multiple linear regression analyses were performed (Fig. 8, *A–D*). For aggrecan, this correlation showed that TGF-β1 significantly increased the strain/

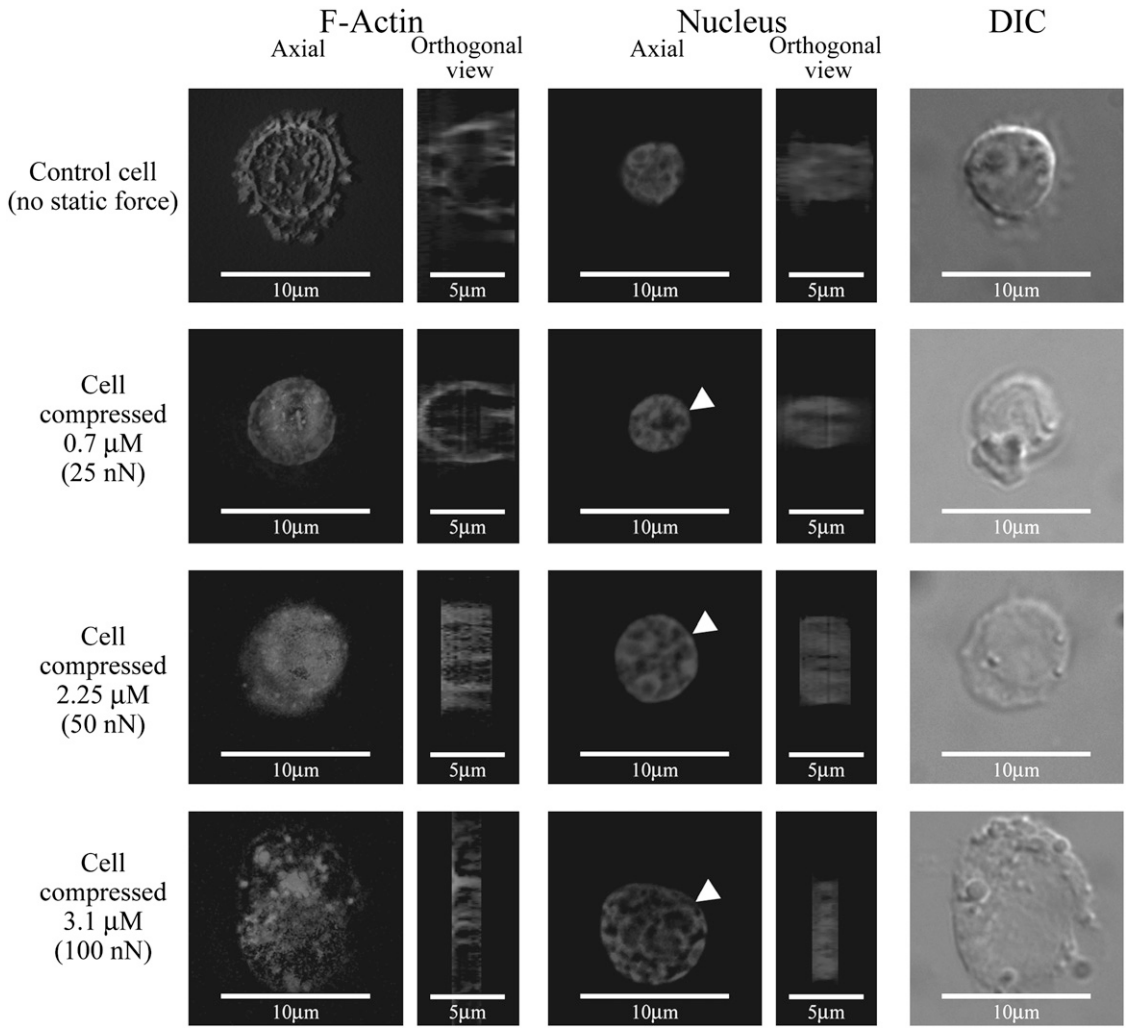

FIGURE 4 Microscopy of compressed chondrocytes. Single cells were compressed and fixed with paraformaldehyde at three levels of deformation (0.7, 2.25, and 3.1 μm) equal to mean cell deformations during single-cell gene expression analysis. F-actin was stained with Alexa Fluor 647 phalloidin, and the nucleus/chromatin with Hoechst 33342. For each cell, image z-stacks were acquired at 0.3-μm spacing. Two-dimensional deconvolution was performed for all fluorescent images, and three-dimensional reconstruction was performed for the F-actin axial view. The nuclear axial view is a single plane imaged near the center. Orthogonal yz planes from the center of the cell were created from z-stacks of F-actin and the nucleus. In the nuclear axial view, nuclei and openings in nucleus are larger with increasing deformation (*arrows*).

mRNA abundance slope parameter as compared with controls (Fig. 8 *A*, $p < 0.001$). Similarly, IGF-I significantly decreased the strain/mRNA abundance slope parameter for collagen IIa and TIMP-1 (Fig. 8, *B* and *C*, $p < 0.001$). No significant differences were observed for the strain/mRNA abundance slope parameters for COMP (Fig. 8 *D*).

MMP-1 was difficult to detect and was recorded in only 10 of the 380 cells that were analyzed. MMP-1 mRNA was detected only in uncompressed cells and cells compressed at 100 nN. The frequency of detection of MMP-1 went from 3.8% in uncompressed cells to 5.4% in cells compressed at 100 nN. In cells where MMP-1 was detected, TIMP-1 mRNA levels were 32-fold higher than MMP-1 in uncompressed cells and 54-fold higher in cells compressed at 100 nN. IL-1β was not detected in any single cells in this study.

DISCUSSION

The results of this study provide what we believe are new insights into the role of mechanical forces in regulating cell transcriptional responses. The CCA allowed a precise loading regiment to be applied to single cells and for measurement of the resulting strains. Real-time RT-PCR analysis proved to be a powerful tool for gauging single chondrocyte modification of gene expression in response to static compression. Initial RT-PCR results demonstrated the negative action of static compression on single chondrocytes (Fig. 3). These data suggest that static compression is initiating a catabolic transcriptional profile as compared with control single chondrocytes. Increasing force exposure to single cells decreased the expression of two of the primary ECM proteins

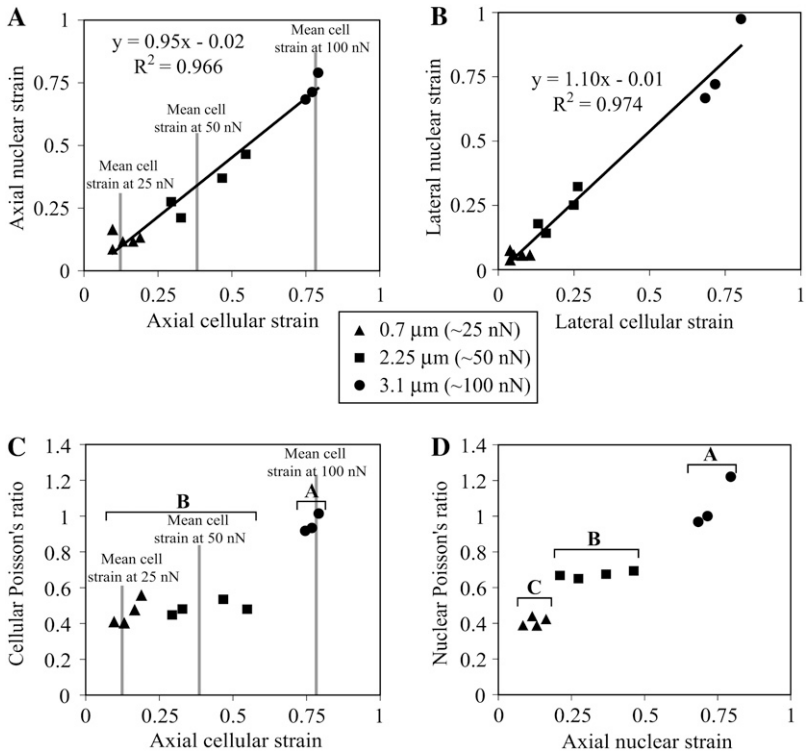

FIGURE 5 Strain analysis of cells and nuclei during compression. Letters indicate significance determined by ANOVA with post hoc analysis. (*A*) Axial nuclear strain versus axial cellular strain reveals a nearly one-to-one correlation. Mean cell strain values from Fig. 3 *D* are included for comparison. (*B*) Lateral nuclear strain versus lateral cellular strain shows that the nucleus experiences more lateral strain than the cell. (*C*) The cellular apparent Poisson's ratio suggests incompressibility at 0.7 and 2.25 μm. It also shows a significant increase and deviation from isotropy at 3.1 μm ($p < 0.0001$). (*D*) The nuclear apparent Poisson's ratio increased in each deformation group ($p < 0.0001$), also indicating deviation from isotropy at higher strains.

in cartilage, aggrecan (Fig. 3 *A*) and collagen II (Fig. 3 *B*). Our group has previously observed similar decreases in single chondrocytes after static compression with the same device using similar methods (5). In this study, we additionally observed that increasing force exposure led to increased TIMP-1 expression in a dosage-dependent manner (Fig. 3 *C*). The catabolic-type response of single chondrocytes to static compression suggests that cells may be downregulating new ECM synthesis in addition to protecting existing ECM, as indicated by decreased aggrecan core protein and collagen IIa and increased TIMP-1 mRNA levels (Fig. 3, *A–C*). Additionally, statically compressed single cells

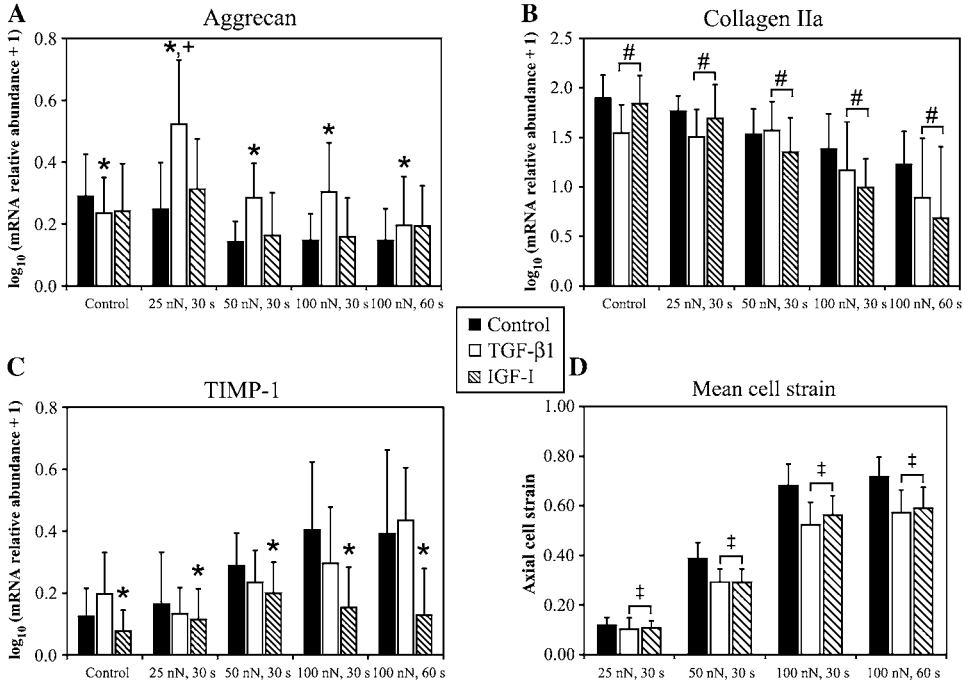

FIGURE 6 Gene expression and strain analysis of compressed single chondrocytes exposed to the growth factors TGF-β1 (5 ng/ml) and IGF-I (100 ng/ml). All results presented mean ± SD. Sample sizes ranged from 31 to 7. (*A*) With TGF-β1, aggrecan mRNA levels do not decrease significantly in response to static loads ($^{*}p < 0.001$), are stimulated at 25 nN, and are significantly different from all control groups ($^{+}p < 0.05$). (*B*) Post hoc analysis shows that TGF-β1 and IGF-I treatment resulted in collagen IIa mRNA levels that are less than those in controls ($^{\#}p < 0.01$). (*C*) TIMP-1 mRNA increases from static compression are prevented with IGF-I ($^{*}p < 0.001$). (*D*) Calculation of the axial strains of all chondrocytes tested with RT-PCR shows that both TGF-β1 and IGF-I significantly reduced the amount of axial strain experienced by cells ($^{\ddagger}p < 0.0001$).

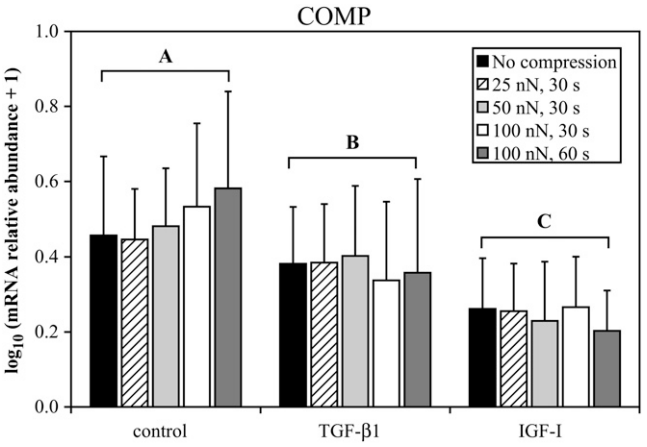

FIGURE 7 COMP gene expression of compressed single chondrocytes exposed to the growth factors TGF-β1 (5 ng/ml) and IGF-I (100 ng/ml). The mRNA abundance in single chondrocytes normalized to GAPDH as determined by single-cell real-time RT-PCR. COMP mRNA levels were not significantly affected by static compression. However, TGF-β1 and IGF-I significantly decreased the expression of COMP in single cells. Letters indicate significance determined by ANOVA with post hoc analysis ($p < 0.05$).

had detectable TIMP-1 mRNA levels in the majority of cells, whereas MMP-1 was not detected. In native articular cartilage, TIMP-1 and MMP-1 are responsible for tissue maintenance and remodeling. MMP-1 is an important component in collagen degradation and remodeling, whereas TIMP-1 acts to keep the effects of MMP-1 in check (29). In this study, it appears that compressed chondrocytes have up-regulated TIMP-1 expression to counter potential MMP-1 activity and matrix destruction. This is especially true for chondrocytes compressed to 100 nN, which had the highest TIMP-1 and MMP-1 mRNA levels.

Aside from the recent work from our group (5), the closest comparisons to this study are made to previous studies detailing the response of cartilage explants to compression. These comparisons are difficult because mechanical stimulation and measurement of the response occur on different time scales. Additionally, cells in explants experience a complex mechanical environment made up of compression, hydrostatic pressure, and shear. The advantage of our single-cell compression approach is that we are able to finely tune the mechanical environment experienced by cells and, in this case, limit them to a purely compressive force. The results of static single-cell compression can be compared with a study by Ragan et al. (8), which saw similar down-regulation of collagen IIa and aggrecan mRNA levels after compression of cartilage explants to 50% of their original thickness for 2 h. Injurious compression of cartilage explants to 50% strain in $<$0.5 s did not result in significant changes in collagen II and aggrecan gene expression; however, increases in TIMP-1 along with other catabolic genes were observed after mechanical insult (10). Neither study detected gene expression

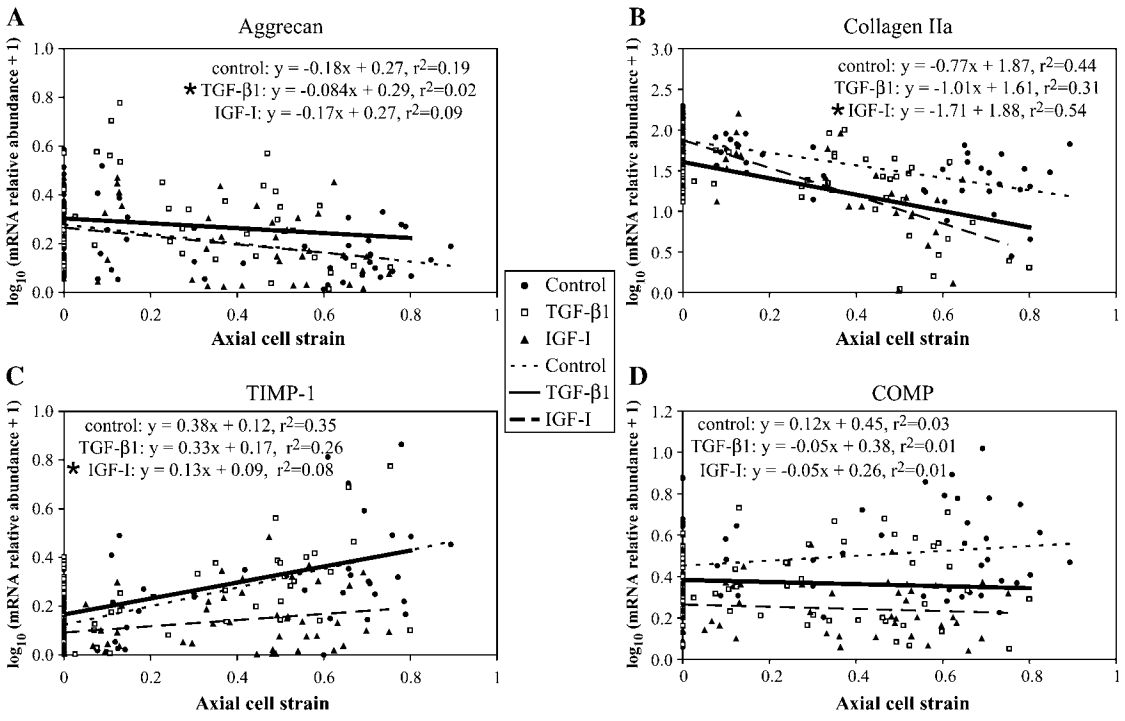

FIGURE 8 Correlation of gene relative abundance to axial cell strain. Multiple linear regression analyses show that (*A*) TGF-β1 significantly increases the slope parameter for aggrecan ($*p < 0.001$), and (*B* and *C*) IGF-I significantly decreases the slope parameter for collagen IIa and TIMP-1 ($*p < 0.001$). (*D*) Correlation of COMP relative abundance to axial cell strain showed no significant effect on abundance/axial cell strain slope parameter.

changes until hours after mechanical stimulation. Comparisons of single-cell compression results with previous work with cartilage explants (6–8,10,13,14) demonstrate that the study of short durations of mechanical stimulation (<1 min) holds promise, especially toward modifying transcriptional events. Future work should study not only short durations but the lasting effects of a short mechanical stimulus on gene expression and protein synthesis. Work has already demonstrated that proteoglycan synthesis in cartilage explants can be altered by short durations of direct compression (30) and that a single cycle of dynamic compression can initiate sustained calcium signaling (31).

To further understand how compression affects single chondrocytes, we carried out experiments in which single chondrocytes were fixed with formaldehyde during compression with the CCA (Fig. 4). These images illustrate that static compression leads to drastic changes to both the cell and the nucleus as compared with uncompressed control cells. Changes to F-actin, the nucleus, and chromatin structure are visible at deformations of 2.25 and 3.1 μm or ~50 and 100 nN of compressive force. Using digital video capture, we have previously demonstrated the mechanical recovery of live chondrocytes exposed to a wide range of axial strains (6–63%) and determined that permanent cellular deformation occurs above 30% strain (26). Comparing the results of this work to our strain calculations from the CCA (Fig. 3 *D*), we know that cells exposed to 25 nN of force should fully recover, whereas cells experiencing 50 and 100 nN of compressive force sustain lasting changes to their morphology after compression because axial strain exceeds 30%. Although these cells experience considerable deformation, they remain viable 1 h later and provide measurable mRNA levels, as determined by single-cell RT-PCR.

The results of our image analysis of compressed single chondrocytes (Fig. 5) allowed us to explore how cellular deformation relates to nuclear deformation. By comparing the axial (Fig. 5 *A*) and lateral strains (Fig. 5 *B*) of compressed cells, we are able to show that axially, in the direction of force exposure, nearly all of the strain experienced by the cell is experienced by the nucleus, as evidenced by the nearly one-to-one relation of axial nuclear strain to axial cell strain (Fig. 5 *A*). Laterally, the nucleus experiences slightly more strain than the cell (Fig. 5 *B*). In situ, chondrocytes have been shown to experience slightly larger cell axial strains (14.7 ± 6.4%) than nuclear axial strains (8.8 ± 6.2%) from a 15% surface-to-surface tissue compression (32). For comparison, cells compressed 0.7 μm in this study experienced 14.0 ± 0.04% axial strain, whereas the nucleus experienced 12.0 ± 0.03% axial strain. The results of the axial strain correlation demonstrate that, in these compressed chondrocytes, the cytoskeleton and cytoplasm are doing very little to buffer the nucleus from an application of compressive force to the cell. Closer study of images of nuclei make it evident that compression not only deforms the nucleus but also changes the arrangement of the chromatin (Fig. 4). Our calculations of nuclear apparent Poisson's ratio (Fig. 5 *D*) demonstrate an anisotropic response and indicate increasing nuclear volume. This begins to explain the larger spaces that were observed within the nucleus with increased deformation (Fig. 4). Connecting these results to the gene expression results make it apparent that compressive force may be directly influencing transcriptional events by initiating force-mediated conformational changes in the chromatin. Previous work (33) has demonstrated that tensile forces applied to the cell membrane are transferred through the cytoskeleton to the nucleus. Our work suggests that for relatively large cellular strains, the cytoskeleton functions less as a mechanosensory network and more as a system for shielding the cell from the full extent of the compressive force, thereby aiding in morphological recovery after strain.

With this in mind, we attempted to provide chondrocytes with a means of mechanoprotection as well as a way to recover gene expression after compression. Previous research has demonstrated that the growth factors TGF-β1 and IGF-I can lead to integrin activation (34,35) and result in stiffening of the actin cytoskeleton in chondrocytes (17). Additionally, these growth factors have a well-documented ability to stimulate matrix synthesis and maintain phenotype in chondrocytes (36–38).

Exposing chondrocytes to TGF-β1 or IGF-I for the entire process of attachment, cell compression, and postcompression incubation significantly reduced the strain levels chondrocytes experienced at all force levels (Fig. 6 *D*). The combination of growth factor treatment and reduced strain allowed selective recovery of gene expression (Fig. 6, *A–C*). TGF-β1 treatment reversed the negative (decreasing) levels of aggrecan core protein initiated by static compression and was stimulatory at 25 nN (Fig. 6 *A*). IGF-I exposure had a different influence on single-chondrocyte gene expression and prevented increased TIMP-1 with increased static force (Fig. 6 *C*). Neither growth factor reversed the decreased levels of collagen IIa mRNA elicited by static compression (Fig. 6 *B*), suggesting that collagen IIa transcription may be more, and perhaps even permanently, sensitive to static compression compared with the other genes studied. Statistical analysis also revealed that chondrocytes exposed to TGF-β1 and IGF-I have significantly lower levels of collagen IIa mRNA than control cells.

COMP mRNA levels were not significantly regulated by static compression; however, TGF-β1 and IGF-I significantly lowered single-cell expression of COMP as compared with controls (Fig. 7). These results, together with the observed decrease in collagen IIa expression, merit further study because COMP and collagen II have been connected in ECM assembly (28). These data may suggest a common transcriptional factor for both of these genes that is influenced by TGF-β1 and IGF-I signaling.

Analysis of cell axial strain revealed that both TGF-β1 and IGF-I significantly reduced the cellular strain experienced by single cells, especially at 50 and 100 nN of force (Fig. 6 *D*). To further analyze the influence of strain on transcription, we

correlated mRNA levels to the axial strain each chondrocyte experienced (Fig. 8). This correlation confirmed that TGF-β1 lessened the impact of static compression on aggrecan expression and strain levels as compared with untreated cells (Fig. 8 *A*). Similarly, IGF-I exposure reduced TIMP-1 expression and strain levels (Fig. 8 *C*).

Our single-cell approach had several limitations that may have contributed to results we have reported in this study. An obvious limitation is that chondrocytes are removed from the three-dimensional matrix of cartilage and subjected to two-dimensional culture. The removal of cell-matrix interactions likely affects chondrocyte mechanotransduction. Another limitation is the fact that gene expression measurements were taken at a single time point. One hour after compression may not be the appropriate time to look at gene expression of proteins such as IL-1β or MMP-1, which we had difficulty detecting. For MMP-1, expression was observed only in controls and cells compressed to 100 nN. This result might be caused by the single time point we analyzed or because the sample size for the 25- and 50-nN treatments was not as large as that of the control (uncompressed) group. Instead of getting a glimpse of the transcriptional changes occurring at a single time point, it would be beneficial to track changes occurring in a single cell over time after mechanical insult. Because RT-PCR is a destructive assay, other techniques would need to be utilized such as a gene reporter system.

The results of the growth factor studies provide evidence that stiffening the cytoskeleton with bioactive agents is not adequate to resist all of the deleterious effects of high levels of static compression and the resulting strains. Exposing a cell to a longer duration of force does not appear to be a significant factor, whereas the amount of strain that is imposed on the nucleus does so appear. A combination treatment of TGF-β1 and IGF-I could offer better recovery of gene expression to static compression, especially for aggrecan and TIMP-1. However, at force levels of 50 and 100 nN, the cell and nucleus will most likely still receive a sufficient amount of strain to initiate harmful transcriptional changes. From our analysis of cellular response to strain, it appears that a threshold of cellular axial strain exists between 14% and 41%, at which the injurious effects of static compression are initiated (Fig. 5 *A*). In terms of nuclear axial strain, this correlates to between 12% and 33% strain (Fig. 5 *A*). We propose that within this range of axial strain lies a point where forces across the cell become large enough to deform the nucleus such that conformational changes to chromatin occur. These force-mediated changes in turn directly influence a cell's transcriptional state. In terms of chondrocytes, these changes appear to shift chondrocytes into a matrix-protective state, as demonstrated by decreased mRNA levels of the primary cartilage ECM proteins aggrecan and collagen II and increased mRNA levels of TIMP-1. Growth factors reduce the amount of strain the nucleus experiences and seem to alter a chondrocyte's specific transcriptional response to compression in terms of the genes we analyzed. This knowledge is directly applicable to how cells respond to their mechanical environment and demonstrates that cells respond to static compressive force by modifying gene expression related to ECM synthesis and maintenance. Further, it appears that this response is directly related to the amount of nuclear deformation that occurs. The next step in understanding the process of force-mediated transcription would be to observe the changes occurring to the chromatin in real time. With such information, the forces required for conformational chromatin changes and the exact thresholds for transcriptional changes could be determined.

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