Cell differentiation by mechanical stress

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ABSTRACT

Growth factors, hormones, and other regulatory molecules are traditionally required in tissue engineering studies to direct the differentiation of progenitor cells along specific lineages. We demonstrate that mechanical stimulation *in vitro*, without ligament-selective exogenous growth and differentiation factors, induces the differentiation of mesenchymal progenitor cells from the bone marrow into a ligament cell lineage in preference to alternative paths (i.e., bone or cartilage cell lineages). A bioreactor was designed to permit the controlled application of ligament-like multidimensional mechanical strains (translational and rotational strain) to the undifferentiated cells embedded in a collagen gel. The application of mechanical stress over a period of 21 days up-regulated ligament fibroblast markers, including collagen types I and III and tenascin-C, fostered statistically significant cell alignment and density and resulted in the formation of oriented collagen fibers, all features characteristic of ligament cells. At the same time, no up-regulation of bone or cartilage-specific cell markers was observed.

Key words: mechanical stimulation • bone marrow stem cells • ligament

Tissue engineering holds promise for providing an essentially unlimited pool of transplantable tissues (1). Tissue engineering can also provide a basis for quantitative *in vitro* studies of tissue development, by culturing cells on three-dimensional templates in the presence of specific biochemical or physical factors. Bioreactors can be designed to maintain physiological parameters at desired levels, enhance mass transport rates, and expose cultured tissues to specific stimuli (2). The requirements of functional tissue engineering include a) cellular components capable of differentiating into the appropriate lineages, b) a scaffold providing a structural template for tissue development, and c) a bioreactor providing the necessary biochemical and physical regulatory signals guiding cell differentiation and tissue development.

Mesenchymal progenitor cells isolated from bone marrow are becoming increasingly recognized for their potential to generate different cell types and thereby function effectively *in vitro* or *in vivo* in tissue repair (3). Even in older individuals, bone marrow stroma is relatively easily harvested, contains biosynthetically active precursors and multipotent cells, and thus can serve as a basis for tissue engineering of autologous implants without specific ethical issues or concerns about tissue rejection. Tissue engineering based on bone marrow-derived cells requires a judicious use of growth and differentiation factors to modulate cell commitment and differentiation and recapitulates the events occurring *in vivo* during tissue development (4). Preliminary studies have also shown that physical factors, including loading (5), electromagnetic fields (6), and ultrasound (7), play an important role in regulating the function of mesenchymal progenitor cells. We hypothesized that specific mechanical stimuli (multidimensional strain) can selectively differentiate mesenchymal progenitor cells into the ligament lineage. The results suggest a definitive role of mechanical stress in mesenchymal progenitor cell differentiation, thus opening new perspectives for ligament tissue engineering.

MATERIALS AND METHODS

Bioreactor

The bioreactor accommodated up to 12 individual tubes (2.5 cm in diameter × 4 cm long), each providing an environment for the growth of one vertically oriented ligament attached between two anchors positioned 2 cm apart. Tube dimensions and anchor placement were selected based on culture volumes previously used to grow ligaments (20 ml per ligament) (8), and the dimensions of anterior cruciate ligaments in goats (approximately 16 mm long) (9). The complete bioreactor system was placed in an incubator (37°C, humidified, 5% CO_2) and operated by using an external computer. Software used to control the device was written by using C programming language and Borland C++ Compiler Version 5.0 (Borland, Scotts Valley, CA). For both bovine and human cell studies, mechanical stimulation was applied after an initial period of 2 days allowed for gel hardening.

Anchors

Coral and cancellous bone anchors were used in the reactor tubes to mimic ligament-bone attachment *in vivo* and to support tissue ingrowth *in vitro*.

Collagen matrices

Type I collagen gel matrices were selected based on the ability of ligament fibroblasts to proliferate and organize in response to static tension (8). The final solution used to seed the cells was 1× Dulbecco's modified Eagle medium (DMEM) containing 450 mg glucose/L, 10 mg/L folic acid, 10 mM L-glutamine, 1 g/L $NaHCO_3$, 18.5% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/L streptomycin, 0.2 μg/ml Fungizone, 5×10^5 bone marrow stromal cells (BMSC)/mg collagen, 2 mg/ml collagen for bovine studies, and 2.25 mg/ml collagen for human studies; for human cells, medium was supplemented further with 100 μM/L L-ascorbic acid-2-phosphate. Medium was changed (100%) within the first 24 h and 50% twice per week thereafter.

Cells

Bovine bone marrow cells were obtained from fresh tibias and femurs of 2–3 week-old bovine calves (Research 87, Cambridge, MA). We obtained human unprocessed whole bone marrow aspirates from donors <25 years of age from Clonetics-Poietics (Walkersville, MD).

Cell culture

Bone marrow aspirates (human or bovine) were resuspended in DMEM supplemented with 10% FBS, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 mg/L streptomycin (P/S), and 1 ng/ml basic fibroblast growth factor (Life Technologies, Rockville, MD). We plated whole marrow aspirates at 5×10^3 mononuclear cells/cm^2 in tissue culture plastic, and bone marrow stomal cells were selected based on their ability to adhere to the tissue culture plastic; nonadherent hematopoietic cells were removed with the culture medium during medium replacement after 4 days in culture. Medium was changed twice per week thereafter. When primary bone marrow stromal cells became near confluent, after approximately 1–2 weeks, we detached them by using 0.25% trypsin/1 mM ethylenediaminetetraacetic acid and frozen in 8% dimethyl sulfoxide (DMSO)/10% FBS/DMEM for future use. First passage (P1) bovine BMSCs were replated at 5×10^3 cells/cm, trypsinized near confluency, and seeded directly into collagen gels. Human BMSCs were cultured to near confluency second passage (P2) and seeded directly into collagen gels.

Light microscopy

Tissue samples for histological evaluation were fixed in 10% neutral buffered formalin for 24 h at 4ºC and stored in 70% ethanol until embedded in paraffin, sectioned (5-µm thick), and stained with hematoxylin and eosin (H&E). Spatial cell distributions in the cross-sectional plane and cell alignment in longitudinal plane were assessed in six different ligaments (3 mechanical, 3 control) by videomicroscopy. For both cross-section cell density and longitudinal cell alignment, fields measuring 0.138 mm^2 at 10× magnification were chosen randomly (n=16 fields per sample in the cross-section; n=10 fields per sample in the longitudinal section). The number of cells in each field was determined by automated counting maintaining the minimum specimen counted be ≥10 pixels. We determined the percentage of aligned cells in longitudinal plane of the ligaments by taking the number of aligned cells over the total number of cells in each field; a cell with a length-to-width ratio of 3:1 within 30˚ of the long axis of the gel was considered aligned and counted in a blind study.

Transmission electron microscope

Longitudinal sections of the gels were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 h at 4ºC, rinsed in buffer, incubated in 1% osmium tetraoxide for 1 h on ice, and dehydrated though exposure to a gradient of alcohol. Specimens were infiltrated with propylene oxide, embedded in Epon resin, and cured overnight. Sections were cut along the longitudinal axis, stained with uranyl acetate and lead citrate, and examined by using a Philips (FEI Company, Hillsboro, OR) CM-10 electron microscope at 80 kV.

Antibodies

Longitudinal sections of mechanically stimulated and control ligaments were assessed for the expression of bovine and human collagen type I (rabbit antibovine, polyclonal, 1:200 dilution, Biodesign, Saco, ME; rabbit antihuman, polyclonal with <0.1% cross-reactivity to bovine, 1:40 dilution, Biodesign), collagen type III (rabbit antibovine, polyclonal with cross-reactivity to human, 1:50 dilution, Chemicon, Temecula, CA), and fibronectin (rabbit antihuman, polyclonal with cross-reactivity to bovine, 1:200 dilution, Dako, Carpinteria, CA). Secondary antibodies included FITC goat antirabbit IgG for bovine studies (1:160 dilution; Sigma, St. Louis, MO) and Cy-3 donkey antirabbit F(ab')$_2$ for human studies (1:200 dilution; Jackson Immunoresearch, West Grove, PA).

Immunohistochemistry

Immunohistochemical assessment of extracellular matrix (ECM) production and organization was performed on depariffinized sections from bovine ligaments and frozen sections from human ligaments.

Real-time quantitative reverse transciptase polymerase chain reaction (RT-PCR)

We extracted RNA by using Trizol according to the single-step acid-phenol guanidinium method (10). cDNA synthesis was performed by using Stratascript reverse transcriptase (Stratagene, Amsterdam, NL) in the presence of random hexamers (Catalys AG, Wallisellen, CH). We performed and monitored real-time PCR reactions by using an ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Biosystems, Foster City, CA), with settings as previously described (11). The level of expression of each target gene was normalized to the reference gene glyceraldehyde phosphate dehydrogenase (GAPDH) and then expressed as a fraction of the average expression levels measured in native ligaments. Primers and probes for GAPDH, collagen types I and II, osteopontin, and osteocalcin have been described previously (11). Primers and probes for collagen type III, tenascin-C, and bone sialoprotein were as follows. Collagen III: forward primer = 5'-ACACGTTTGGTTTGGAGAGTCC-3'; reverse primer = 5'-CTGCACATCAAGGACATCTTCAG-3'; probe = 5'-ATTGCCGTAGCTAAACTGAAAACCACCATCC-3'. Tenascin-C: forward primer = 5'-TCTCTGCACATAGTGAAAAACAATACC-3'; reverse primer = 5'-TCAAGGCAGTGGTGTCTGTGA-3'; probe = 5'-ACCACCACACGCTTGGATGCCC-3'. Bone sialoprotein: forward primer = 5'-TGCCTTGAGCCTGCTTCC-3'; reverse primer = 5'-GCAAAATTAAAGCAGTCTTCATTTTG-3'; probe = 5'-CTCCAGGACTGCCAGAGGAAGCAATCA-3'. We assessed a total of 24 gels based on human cells.

RESULTS

A novel bioreactor was developed to provide a combined tensile-compressive and torsional loading to mimic the unique combination of forces to which a ligament is exposed during physiological function and repair *in vivo* (Fig. 1) (12). Translational strain (10%, 2 mm) and rotational strain (25%, 90º) were applied concurrently at a frequency of 0.0167 Hz (one full cycle

of stress and relaxation per minute) to collagen gels seeded with bone marrow-derived cells; an otherwise identical bioreactor system without mechanical loading served as a control. Collagen gels, 2-cm long between anchors were seeded in a 2.54 cm tube with human or bovine bone marrow-derived cells and were cultured up to 21 days, with or without mechanical loading.

Helically organized collagen fibers formed in the direction of the applied load at the periphery of the mechanically stimulated ligaments, a feature absent in the controls (Fig. 2). When detached from the bone anchors for photographic documentation, fibers embedded within the ligaments form a ~17º angle with the horizontal plane (Fig. 2A); under translational and rotational strains at the extreme point of deformation, the fibers would have formed a ~70º pitch angle with the horizontal plane. Characteristic collagen banding patterns were clearly visible in the longitudinal transmission electron microscopy sections of mechanically stimulated gels, a pattern that was either absent or markedly less abundant in static controls. Collagen Type I fiber bundles (~20 μm diameter) formed in the mechanically challenged gels (13).

Mechanical stimulation of ligaments based on human or bovine bone marrow-derived cells induced elongated, ligament-like cell morphology, and cell alignment in the direction of loading, in contrast to the round and randomly distributed cells in static controls (Fig. 3). Cell alignment in the direction of mechanical loading was 2.5-fold higher (significant, $P<0.001$, paired Student's t-test between mechanically stimulated and static ligaments). Cross-sections through the gels confirmed that the cells in Figure 3b were not aligned perpendicular to the plane of the sections (data not shown). Compared with static controls, mechanically stimulated gels contracted by a significantly higher percentage (~58% versus 45%, respectively, for mechanical versus controls), and had significantly higher cross-sectional cell density after 21 days of culture ($P<0.001$, paired Student's t-test) (Fig. 3). Collagen Types I and III and fibronectin (markers of ligament extracellular matrix) (14) were all expressed in mechanically stimulated ligaments but not in controls, as evidenced by immunostaining (Fig. 4, bovine results shown, similar results for human stromal cells not shown). Collagen Types I and III could be detected in the form of fiber bundles oriented in the direction of loading.

The mRNA expression of collagen Types I and III and tenascin-C, typical markers of ligament cells (14, 15), was higher in the mechanically stimulated than in the control ligaments, as assessed by real-time quantitative RT-PCR (Fig. 5). After 14 days of culture, differences became statistically significant ($P<0.05$) and mRNA levels in the mechanically stimulated ligaments approached those quantified in native ligaments. These results are consistent with previous reports on the up-regulation of collagen types I and III and tenascin-C by fibroblasts isolated from anterior cruciate ligaments when exposed to mechanical stimulation (16, 17). The mRNA expression of bone sialoprotein and collagen type II, typical markers of bone and cartilage cells, respectively, was not detected in the engineered ligaments. Furthermore, the mRNA expression of osteocalcin and osteopontin, which characterize the progression of mesenchymal progenitor cells into the osteogenic lineage, was not up-regulated by mechanical stimulation and was comparable in engineered and native ligaments (data not shown). These data thus provide evidence that the specific mechanical regime induced a selective differentiation of the mesenchymal progenitor cells towards ligament cells.

DISCUSSION

Recently, several groups have developed apparati for the application of cyclic strain of two-dimensional cell-seeded constructs (16, 17). In other studies, cyclic mechanical strain promoted smooth muscle cell proliferation, organization, and extracellular matrix synthesis (18) and collagen Type I synthesis by anterior cruciate ligament fibroblasts (16). Compression improved the structure of cultured cartilage explants (19). The successful demonstration of bone marrow stromal cell differentiation into ligament-like cells with multidimensional strain in three-dimensional matrices suggests that mechanical forces can play an important role in the processes of cellular differentiation and not just in promoting specific tissue-types from differentiated cells. Because the culture medium was supplemented with serum capable of supporting the culture of any cell type, but not ligament-specific regulatory molecules, the specific mechanical regulatory signals appear to be responsible for supporting the selective differentiation toward ligament-like cells.

Mechanical stimulation enhanced cell differentiation for reasons that are not entirely clear. Presumably the influence of mechanical forces may be transduced via cell binding sites, leading to enhanced rates or extent of differentiation. It is reasonable to speculate that mechanical signals may trigger cell-surface stretch receptors and adhesion sites, resulting in cascades that involve activation of genes responsible for the synthesis and secretion of key ligament extracellular matrix components (16). It is conceivable that the mechanical forces induced differences in fluid flow in the gels relative to the controls, thus changing rates of mass transfer of nutrients, metabolites, and oxygen. Likely, combinations of mechanical signaling via cell membrane receptors and changes in transport play a role in the results observed in the present study. More detailed studies of cell receptors involved in transducing the mechanical signals and the associated cascades, as well as nutrient transport issues, will be needed in order to refine the sequence of events responsible for the enhanced differentiation and tissue formation. It should also be recognized that the influence of mechanical forces on the differentiation process could be transient and not permanent, and this is an issue to be addressed in continuing studies, including its dependence on sustained mechanical stimulation.

ACKNOWLEDGMENTS

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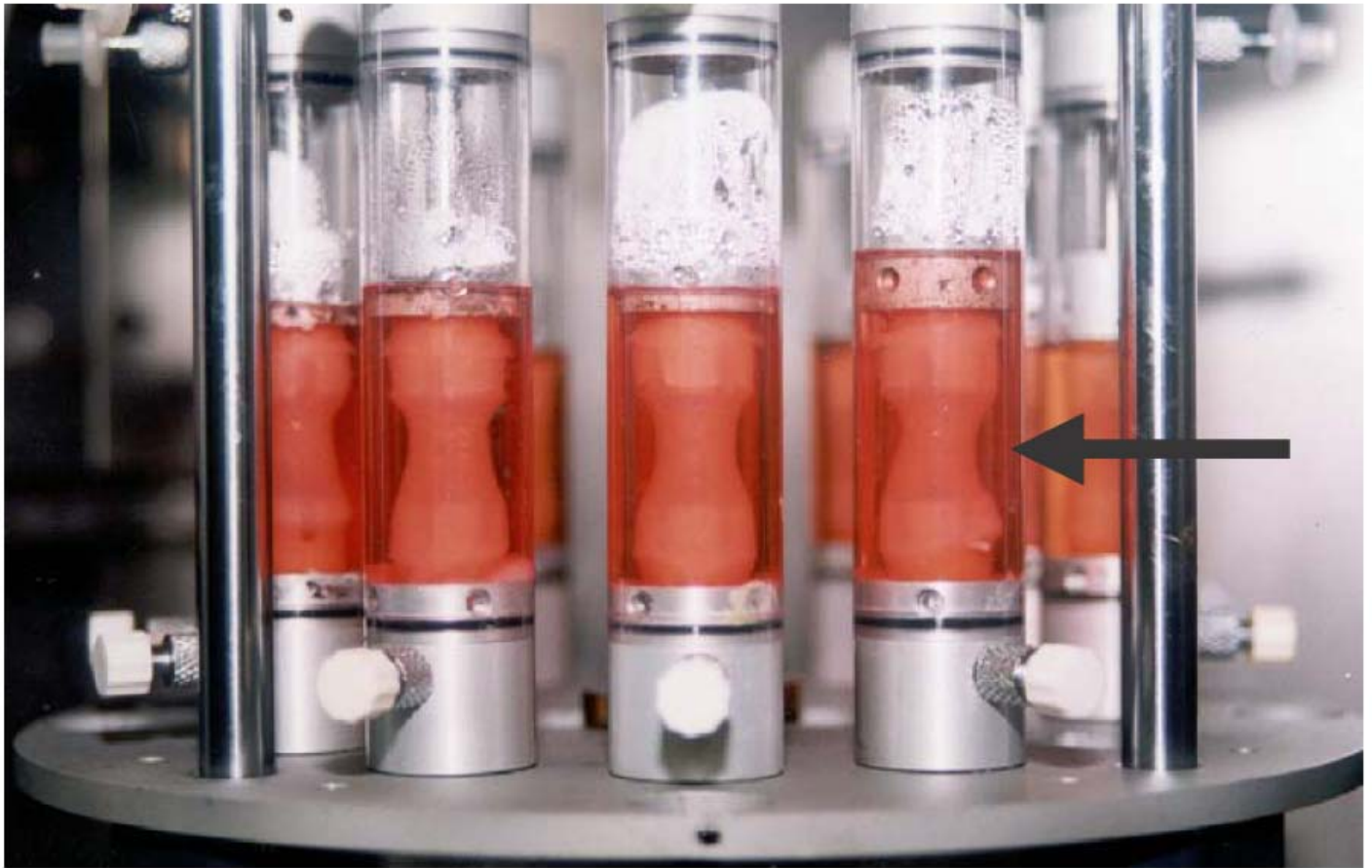
Fig. 1

Figure 1. Individual bioreactor vessels housed in the mechanical device. The arrow indicates a collagen gel suspended between two bone anchors as it is stimulated translationally and torsionally.
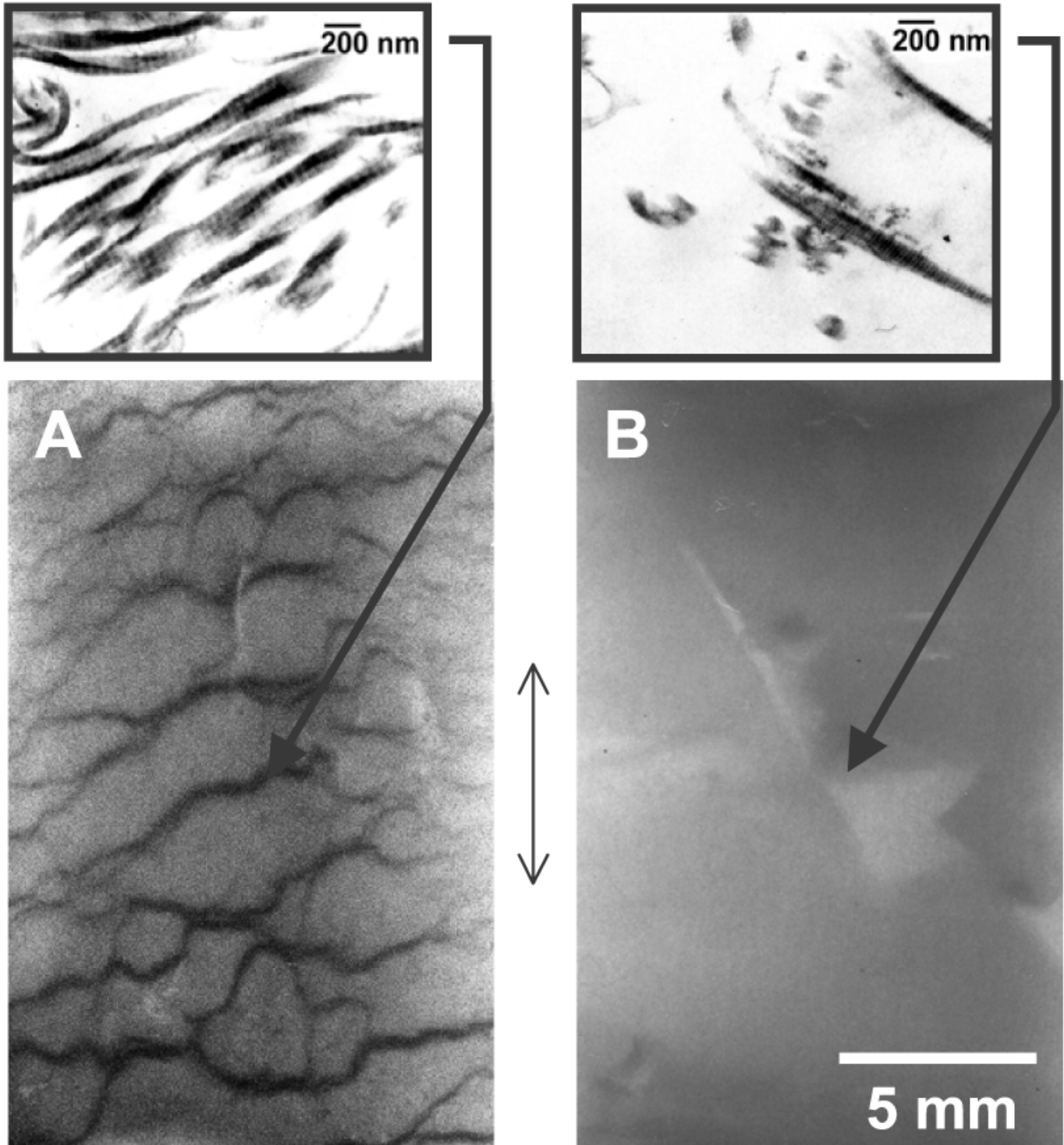
Fig. 2

Figure 2. Intact gels seeded with bovine bone marrow derived cells after removal from the bioreactor following 21 days of culture. Images were acquired with a 35 mm Nikon camera on a dissection scope at 10× magnification. Double-sided arrow indicates the longitudinal axis of the ligaments. Mechanical stimulation (tension and torsion) induced collagen fiber bundle formation and organization in the periphery and in a helical formation that was absent in statically grown controls (**B**). Inserts are TEM images showing organized collagen fibrils present in the gel that had been triggered mechanically (above **A**) versus the static control (above **B**).
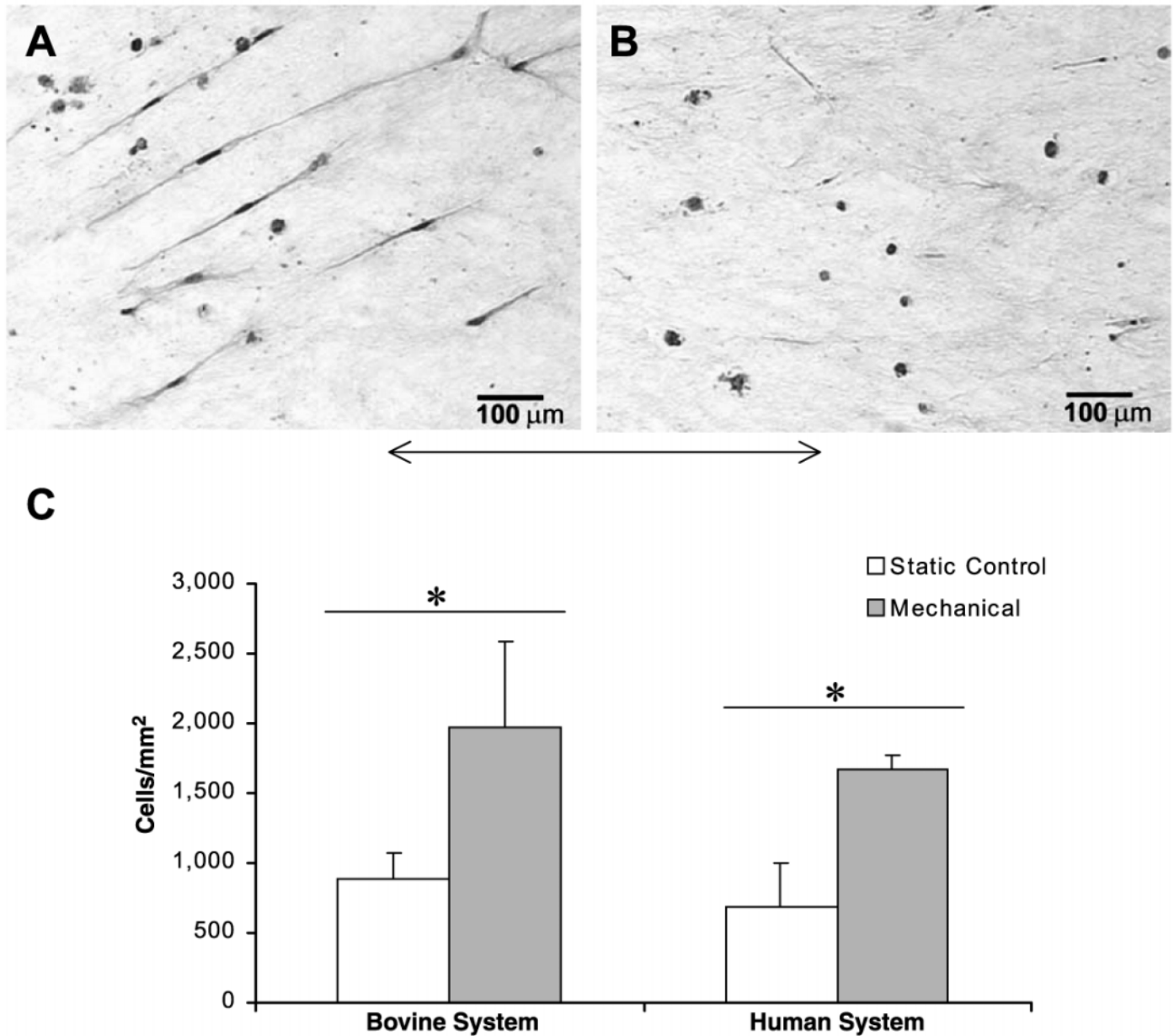
Fig. 3

Figure 3. Cell morphology, alignment and cross-sectional cell density as assessed by hematoxylin and eosin (H&E) staining and image analysis. A) H&E of longitudinal sections of gels seeded with bovine bone marrow-derived cells and mechanically challenged gels for 21 days in culture. **B)** H&E of longitudinal sections of statically grown control gels indicated little to no cell alignment in the direction of stimulation. Mechanical stimulation induced a 2.5-fold (*P*<0.001, paired Student's *t*-test) increase in cellular alignment along the longitudinal axis of the ligaments (double-sided arrow) compared with controls. Similar results were observed in gels seeded with human bone marrow-derived cells, mechanically and statically grown gels after 14 days of culture (data not shown). **C)** Cross-sectional cell density of gels seeded with bovine bone marrow-derived cells after 21 days of culture and gels seeded with human cells after 14 days of culture. Image analysis showed a statistically significant (* indicates *P*<0.001, paired Student's *t*-test) increase in cells density of ~twofold for mechanical versus control gels in both systems (*N*=3 ligaments for each group, *N*=16 fields from each ligament).
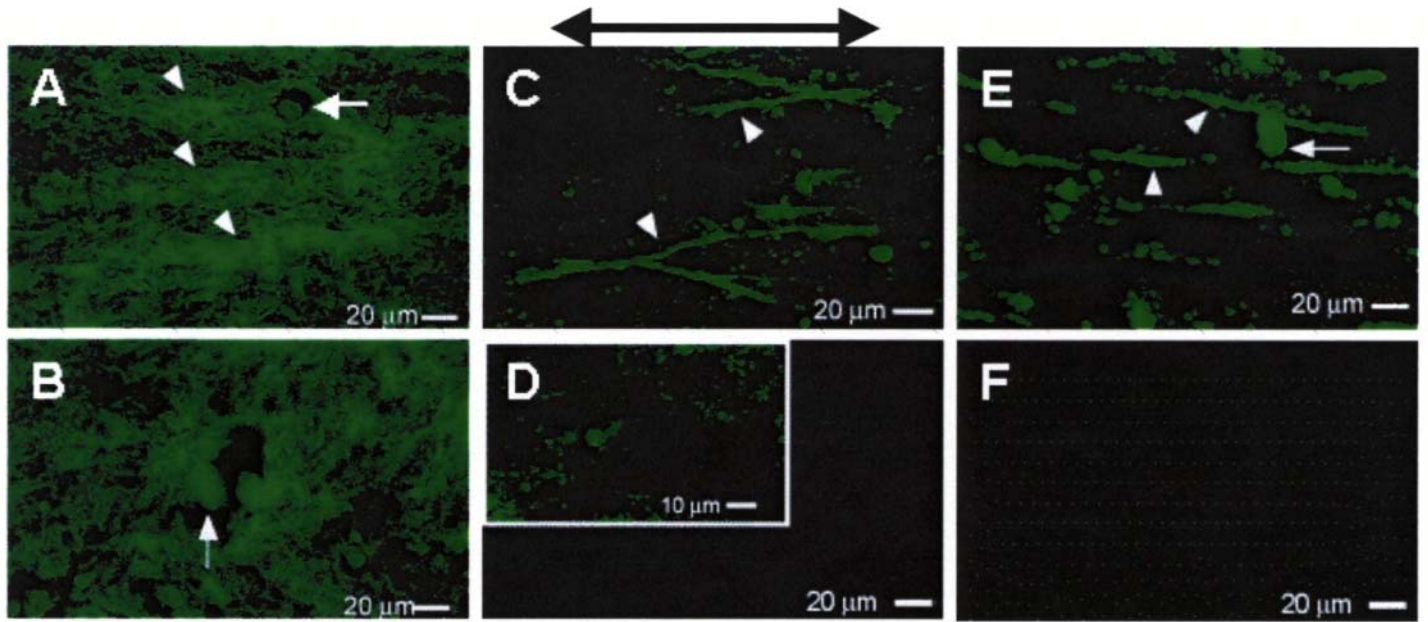
Fig. 4

Figure 4. Immunohistochemical assessment of gels seeded with bovine (A–F) bone marrow- derived progenitor cells. Mechanically stimulated bovine gels **(A, C, E)** compared with static controls **(B, D, F)** cultured for 21 days showed **(A)** ordered collagen type I fiber bundles (arrow heads) in the direction of load; that is, along the longitudinal axis of the ligaments (double-arrow), compared with static controls **(B)**, where the absence of fiber bundle organization at 400× magnification is observed. Arrows indicate nonspecific FITC-stained cell nuclei around which ECM is found. Collagen type III was produced in the direction of load in mechanically stimulated gels **(C)** (arrow heads) compared with static controls **(D)** at 400× magnification and at the same exposure times and conditions. **D)** Inset box: static control gel at 1,000× and longer exposure time showing nonspecific binding of the secondary antibody as in the negative controls. Mechanical stimulation up-regulated fibronectin production along the axis of load in mechanically stimulated gels at 400× magnification **(D)**; single arrow indicates a cell nucleus and surrounding cytoplasm (not seen) involved in the production and secretion of fibronectin. At the same magnification and exposure times, fibronectin was absent in static controls **(E)**; only a nonspecific signal was observed at 1,000×.
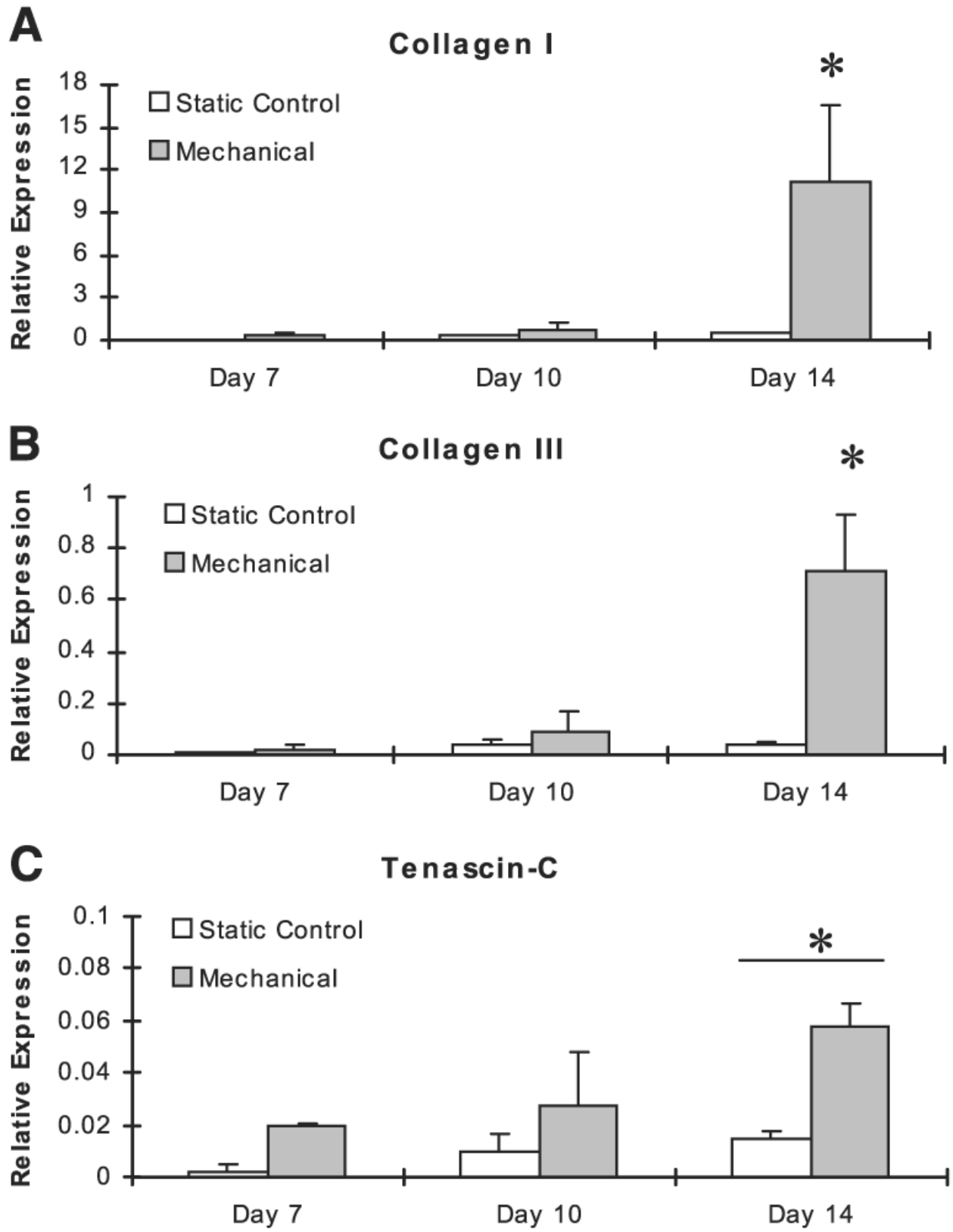
Fig. 5

Figure 5. Real-time RT-PCR results illustrating data for days 7, 10, and 14 for mechanical versus static control gels. Statistically significant differences are shown with an asterisk based on $P<0.05$ for the collagen results (**A** and **B**), and for Tenascin-C (**C**) using 2-way ANOVA, Tukey-Kramer test.