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RESEARCH ARTICLE

ERK activation is required for hydrostatic pressure-induced tensile changes in engineered articular cartilage

G. D. DuRaine and K. A. Athanasiou*
Department of Biomedical Engineering, University of California, Davis, CA, USA

Abstract

The objective of this study was to identify ERK 1/2 involvement in the changes in compressive and tensile mechanical properties associated with hydrostatic pressure treatment of self-assembled cartilage constructs. In study 1, ERK 1/2 phosphorylation was detected by immunoblot, following application of hydrostatic pressure (1 h of static 10 MPa) applied at days 10–14 of self-assembly culture. In study 2, ERK 1/2 activation was blocked during hydrostatic pressure application on days 10–14. With pharmacological inhibition of the ERK pathway by the MEK1/ERK inhibitor U0126 during hydrostatic pressure application on days 10–14, the increase in Young's modulus induced by hydrostatic pressure was blocked. Furthermore, this reduction in Young's modulus with U0126 treatment during hydrostatic pressure application corresponded to a decrease in total collagen expression. However, U0126 did not inhibit the increase in aggregate modulus or GAG induced by hydrostatic pressure. These findings demonstrate a link between hydrostatic pressure application, ERK signalling and changes in the biomechanical properties of a tissue-engineered construct.

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Keywords **self-assembly; extracellular signal-regulated kinase 1/2 (ERK 1/2); hydrostatic pressure; chondrocyte; tissue engineering; cartilage**

1. Introduction

Within the body articular cartilage is found at the ends of long bones and functions as a load-dissipating, low friction-bearing surface. These functions are dependent on the composition of a dense extracellular matrix composed of 70–80% water and a collagen type II tensile network interlaced with compression-resisting proteoglycans. Cartilage is both avascular and aneural and has poor self-repair capabilities, with destruction of the tissue resulting in reduced mobility and pain. Tissue-engineered replacement cartilage is designed to fill this need, and our group has developed a scaffoldless self-assembly technology to produce cartilage constructs with clinically relevant properties approaching those of native articular cartilage (Hu and Athanasiou, 2006).

During normal motion articular cartilage encounters various forces, including shear, compression and hydrostatic pressure (Guilak *et al*., 2000). These forces are necessary to maintain tissue function, as immobilization results in matrix degradation (Buckwalter, 1995). Of these, hydrostatic pressure represents a major factor in modulating cartilage mechanobiology (Elder and Athanasiou, 2009b), with the physiological range in articular cartilage lying between 3 and 15 MPa (Afoke *et al*., 1987). Application of these mechanical stimuli has therefore been applied to improve matrix functional properties during cartilage tissue engineering (Elder and Athanasiou, 2009b). Previously, our group had identified a regimen of 10 MPa of hydrostatic pressure, statically applied for 1 h on days 10–14, that induced increases in both tensile and compressive mechanical properties in the self-assembled chondrocyte constructs (Elder and

*Correspondence to: K. A. Athanasiou, Department of Biomedical Engineering, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA. E-mail: athanasiou@ucdavis.edu

Athanasiou, 2009a). The addition of TGFβ1 prior to and during hydrostatic pressure application further improved construct properties in a synergistic manner (Elder and Athanasiou, 2008).

The mechanism by which hydrostatic pressure enhances self-assembled chondrocyte construct properties is not well understood. Multiple studies have produced conflicting reports on the signalling mechanisms involved during hydrostatic pressure application and the outcome on production of extracellular matrix components (Kraft *et al*., 2011). This is possibly a result of the wide range of hydrostatic pressure loading regimens, time scales and model systems used (Elder and Athanasiou, 2009b). In chondrocytes, results ranging from ion channel activation and intracellular Ca^{2+} release to modulation of extracellular signal-regulated kinase (ERK) activity have been detected, depending on the hydrostatic pressure regimen (Kopakkala-Tani *et al*., 2004; Mio *et al*., 2007). Of the mitogen-activated protein kinases (MAPKs), the ERK pathway has been observed to respond to mechanical signals in multiple cell types (Reusch *et al*., 1997; Jessop *et al*., 2002; Hatton *et al*., 2003; Laboureau *et al*., 2004; Bastow *et al*., 2005; Liu *et al*., 2006; Kook *et al*., 2009). MAPKs have been identified as signalling pathway components that transduce external signals into a plethora of cellular responses (Krishna and Narang, 2008). Downstream effects of ERK activation are context-specific (Schaeffer and Weber, 1999) and both stimulatory and inhibitory roles for ERK in cartilage have been reported (Nakamura *et al*., 1999; Hung *et al*., 2000; Murakami *et al*., 2000; Yoon *et al*., 2002; Bobick and Kulyk, 2004; Zakany *et al*., 2005; Ryan *et al*., 2009; Prasadam *et al*., 2010). For example, activation of the ERK pathway by FGF has been demonstrated to induce SOX9 expression in mouse primary chondrocytes (Murakami *et al*., 2000) and is required for TGFβ signalling during chondrogenesis (Li *et al*., 2010). Conversely, activation of ERK in primary chondrocytes or cartilage, following impact (Ryan *et al*., 2009; Ding *et al*., 2010) or TGFα (Appleton *et al*., 2010) application, has been linked to the down-regulation of matrix components.

Therefore, in this study we sought to determine the activation state of ERK following hydrostatic pressure application and the role this plays in modulating the mechanical properties of self-assembled constructs. We hypothesized that: (a) ERK is activated by application of hydrostatic pressure; and (b) ERK is necessary for the enhanced self-assembled construct properties observed following the hydrostatic pressure treatment regimen.

2. Materials and methods

2.1. Chondrocyte isolation and construct seeding

Bovine stifle (knee) joints of 1 week old calves were obtained (Research 87, Boston, MA, USA) and opened under aseptic conditions to expose the femoral condyles. Articular cartilage was removed, minced and digested with 0.2% collagenase P (Worthington, Lakewood, NJ, USA) in culture medium containing 3% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA, USA) for 24 h, as described previously (Hu and Athanasiou, 2006); 5.5 million chondrocytes were seeded into 5 mm agarose wells and allowed to self-assemble, as previously described (Hu and Athanasiou, 2006; Elder and Athanasiou, 2009a). The constructs were cultured in a humidified incubator at 37°C and 10% CO_2 for a total of 4 weeks, then assayed. All cell culture medium components were purchased from Invitrogen (Carlsbad, CA, USA) or Sigma-Aldrich (St. Louis, MO, USA), unless otherwise noted.

2.2. Hydrostatic pressure

Hydrostatic pressure was applied to the constructs as described previously (Elder and Athanasiou, 2009a). Briefly, control and pressurized constructs were placed into sterile, heat-sealed bags containing medium. Specimens to be pressurized were transferred into the hydrostatic pressure chamber, which was maintained at 37°C during testing, while non-pressurized specimens (controls) were transferred into an adjacent container which was not pressurized. 10 MPa of static pressure was applied for 1 h during days 10–14. Daily, after hydrostatic pressure application, the constructs were transferred back to individual agarose-lined wells (Elder and Athanasiou, 2009a) and maintained in a humidified incubator at 37°C and 10% CO_2.

2.3. Study 1: identification of ERK activation

On day 10 post-seeding, self-assembled constructs for all groups were removed from the initial seeding wells and transferred to sterile, heat-sealed bags, as described above. Non-pressurized specimens were bagged and placed into an adjacent non-pressurized container. As previous studies in bovine cartilage (Ryan *et al*., 2009) have determined that ERK activation is maximal between 30 min and 2 h following mechanical loading, the constructs were collected 1 h after hydrostatic pressure treatment and flash-frozen in liquid nitrogen. To determine the initial change in signalling following hydrostatic pressure application, and whether this was still activated after multiple hydrostatic pressure applications, the samples were collected on days 10 (first day) and 14 (last day) of the hydrostatic pressure regimen, and were processed for immunoblot as described below.

2.4. Protein isolation and immunoblot

Protein was extracted using RIPA lysis buffer [50 mM sodium fluoride, 0.5% Igepal CA-630 (NP-40), 10 mM sodium phosphate, 150 mM sodium chloride, 25 mM Tris, pH 8.0, 1 mM phenylmethylsulphonyl fluoride, 2 mM ethylenediaminetetra-acetic acid (EDTA) and 1.2 mM sodium vanadate supplemented with protease inhibitor cocktail

(Sigma–Aldrich)]. Protein concentrations were measured using the Protein Assay Kit (Bio-Rad). Protein was electrophoresed on a 12% SDS–polyacrylamide gel and transferred to polyvinylidene difluoride membrane by electroblotting (Bio-Rad). The membranes were blocked with 5% dried, non-fat milk in TBST (25 mM Tris–HCl, 125 mM NaCl and 0.1% Tween 20) for 2 h, probed with primary antibody overnight and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. Antibodies were procured from Cell Signaling (Beverly, MA, USA). Immunoblot signal was visualized using goat anti-rabbit HRP (Pierce) and SuperSignal® West Pico Chemiluminescent Substrate (Pierce), according to the manufacturer's instructions.

2.5. Study 2: inhibition of ERK activation

U0126 functions to block ERK activity by inhibiting MEK1, the immediate upstream activator of ERK. Previous studies (Bobick and Kulyk, 2004; Elluru *et al*., 2009) have indicated that 20 μM U0126 is sufficient to block ERK activation, therefore 20 μM U0126 was added to constructs 1 h prior to hydrostatic pressure application. All other constructs were treated with the vehicle control (3 μl/ml ethanol). Inhibitor or vehicle control was added at days 10–14 at the same time as hydrostatic pressure treatment.

2.6. Quantitative biochemistry and histology

Briefly, construct pieces were weighed and lyophilized for 96 h. After lyophilization, the specimens were reweighed and then digested to completion, using sequential pepsin–elastase digestion. This digest was used for both collagen and glycosaminoglycan (GAG) content assays. Collagen content was assayed using the chloramine-T hydroxyproline assay (Woessner, 1961). GAG content was assayed using the Biglycan GAG Assay Kit (Biocolor, UK), according to the manufacturer's instructions (Hu and Athanasiou, 2006). For histology, specimens were frozen in OCT cutting medium and cut into 14 μm-thick sections on a cryotome. All sections were adhered to Superfrost Plus slides and fixed in formalin prior to staining. Sections were stained for safranin-O/fast green and picosirius red, as previously described (Hu and Athanasiou, 2006).

2.7. Creep indentation and tensile mechanical testing

Compressive and tensile mechanical properties were determined as previously described (Elder and Athanasiou, 2008). Briefly, the compressive aggregate modulus was determined using a creep indentation apparatus (Athanasiou *et al*., 1994), using a 0.8 mm flat porous indenter tip, applying a tare weight of 0.2 g and a test load of 0.7 g (Elder and Athanasiou, 2009a). The discs were bathed in PBS during the duration of testing. Data were modelled using the linear biphasic theory (Mow *et al*., 1989).

Tensile tests consisted of an uniaxial pull-apart test until failure, as previously described (Aufderheide and Athanasiou, 2007). Specimens were cut into dogbone-shaped pieces and both ends glued to paper test strips. Gauge length represented the distance between the paper test strips. Sample thickness and gauge length were measured using digital calipers (Hu and Athanasiou, 2006). The rate of displacement was 1% of the gauge length/s and achieved using an Instron 5565 materials-testing system (Instron, Norwood, MA, USA). Using the cross-sectional area, stress–strain curves were calculated from the load–displacement curves. Young's modulus was determined from the linear region of the stress–strain curve.

2.8. Statistical analysis

For experimental groups in study 1, three samples/group were used for immunoblotting. For study 2, five or six samples/group were used for both biomechanical and biochemical analysis. Groups were analysed by one-way ANOVA and the Tukey–Kramer *post hoc* test was conducted when appropriate, using the statistical analysis software package JMP (SAS, Cary, NC, USA), with $p < 0.05$ being defined as being statistically significant. All data are reported as mean ± standard deviation (SD).

3. Results

3.1. Study 1

3.1.1. Hydrostatic pressure activates ERK

Activation of ERK was detected by immunoblot following initial application of hydrostatic pressure at day 10 of culture (first application of hydrostatic pressure) through to day 14 of culture (last application of hydrostatic pressure) (Figure 1). Activation at day 14 of culture by hydrostatic pressure was less than that at day 10. Minimal ERK activation was detected in control constructs at day 10 or 14, indicating the basal activity of this pathway in control constructs remains low or non-existent during this time. As ERK was activated by hydrostatic pressure, the relevance of inhibiting ERK activation during hydrostatic pressure treatment was chosen to carry forward into study 2.

3.2. Study 2

3.2.1. Gross appearance and histology

ERK inhibition did not alter gross morphology. Constructs from all groups reached a diameter of approximately 5 mm and a thickness of approximately 0.66 (0.66 ± 0.08,

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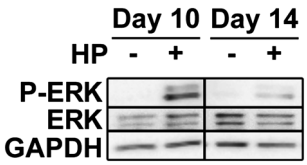

Figure 1. Immunoblot of phosphorylated ERK (P-ERK) with hydrostatic pressure (HP) treatment. Assembled from a single larger gel, equal amounts of protein were loaded in each lane. Hydrostatic pressure induced ERK activation in self-assembled constructs at day 10 and (weakly) at day 14

0.69 ± 0.08, 0.65 ± 0.05, 0.67 ± 0.06, for control, U0126, hydrostatic pressure and U0126 + hydrostatic pressure groups, respectively; $p > 0.05$). Addition of U0126 did not grossly alter morphology or matrix accumulation. Representative constructs for diameter, thickness and histological staining are displayed in Figure 2.

3.2.2. Quantitative biochemistry

Inhibition of ERK during hydrostatic pressure reduced total collagen accumulation, but did not reduce GAG accumulation. Hydrostatic pressure resulted in a significant increase in total collagen content (Figure 3) from control levels [4.91 ± 0.24 to 5.79 ± 0.62 percentage wet weight (ww %)]. Constructs treated with U0126 blocked the increase in collagen induced by hydrostatic pressure treatment (4.96 ± 0.63 ww%) and were not significantly different from control or U0126 alone (4.53 ± 0.33 ww%). GAG accumulation in U0126-treated constructs was significantly more than control (Figure 3). However, the addition of U0126 did not significantly affect the accumulation of GAGs in hydrostatic pressure-treated constructs (Figure 3) compared to hydrostatic pressure treatment alone.

3.2.3. Mechanical evaluation

Inhibition of ERK during hydrostatic pressure application inhibited the increase in tensile properties induced by hydrostatic pressure, but did not inhibit changes in compressive properties. Hydrostatic pressure treated

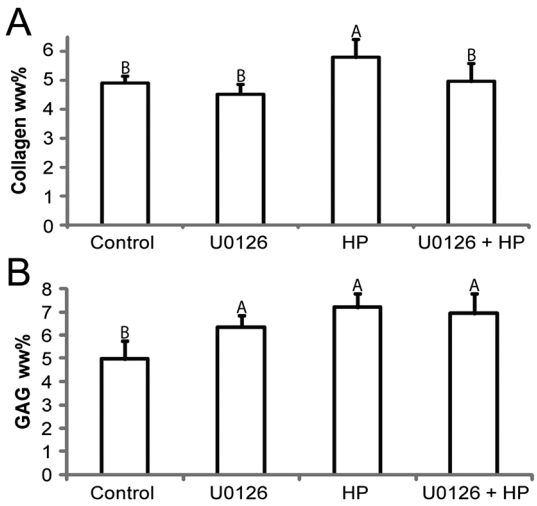

Figure 3. Biochemistry for total collagen wet weight percentage (wwt%) (A) and total GAG wwt% (B). Application of hydrostatic pressure increased total collagen; this was inhibited by application of U0126 during hydrostatic pressure treatment. (B) GAG was increased by application of hydrostatic pressure or U0126 alone. Application of U0126 during hydrostatic pressure treatment did not inhibit GAG accumulation. Groups with different letters are significantly different ($p < 0.05$)

constructs had a significantly increased tensile modulus (791 ± 204 kPa) over control (no treatment) constructs (461 ± 108 kPa). The tensile modulus of constructs treated with U0126 during hydrostatic pressure application (438 ± 149 kPa) was not significantly different from control constructs. Addition of U0126 by itself did not significantly alter tensile modulus compared to control constructs. Compressive properties did not significantly change with addition of U0126 in any group, although a trend for increased compressive properties (aggregate modulus) was observed (Figure 4).

4. Discussion

This study identified ERK-mediated changes in the mechanical properties of tissue-engineered articular cartilage

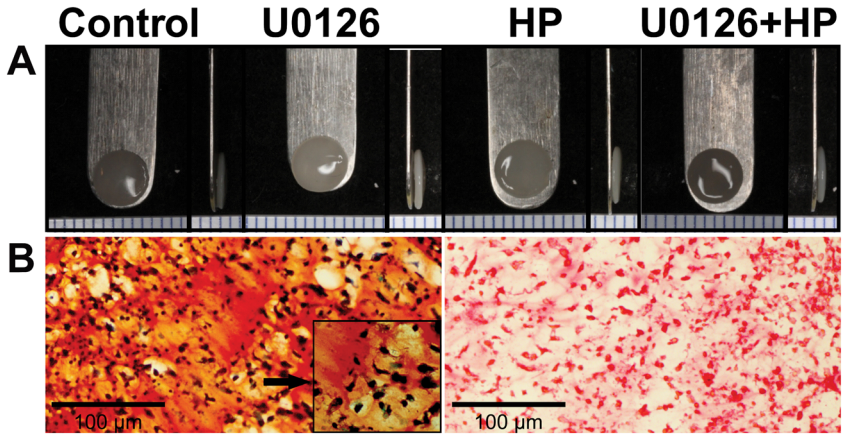

Figure 2. Gross morphology and representative histological staining of constructs. (A) Gross morphology and thickness appeared similar between all groups. (B) Histological images are representative of all groups: (left) safranin-O, fast green; (right) picrosirius red; magnification = ×20; left safranin-O inset magnification = ×40

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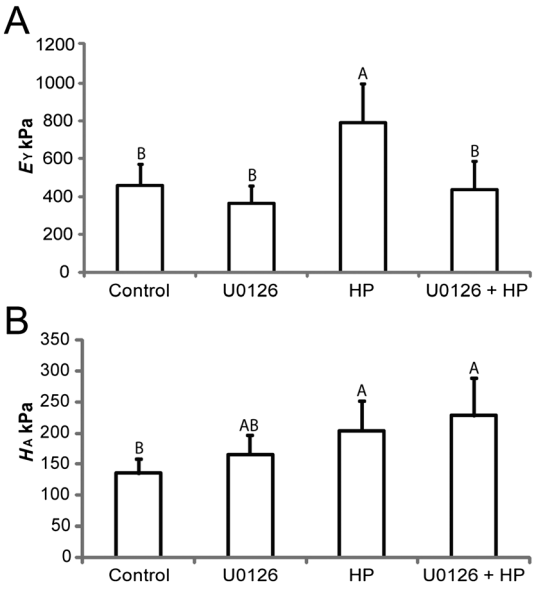

Figure 4. Hydrostatic pressure induces increases in Young's modulus (E_Y) of self-assembled constructs; this was blocked by ERK inhibitor U0126 (A) but does not inhibit the aggregate modulus (H_A) (B) induced by hydrostatic pressure. Groups with different letters are significantly different ($p < 0.05$)

constructs following hydrostatic pressure treatment. This study was designed to: (a) identify the signalling state of ERK activation during initial hydrostatic pressure application; and (b) quantify the effect of ERK inhibition during hydrostatic pressure application on the mechanical and biochemical properties of self-assembled constructs. Application of 1 h of static 10 MPa hydrostatic pressure on day 10 was sufficient to activate ERK, although activation of ERK at day 14 was much less than at day 10. Given the low level of basal ERK activation seen in the control, this is likely due to an ERK negative-feedback loop following multiple applications of stimuli (Shin *et al*., 2009).

The hypothesis that ERK activation is required for tensile property enhancements due to hydrostatic pressure application was confirmed. Changes in tensile properties with hydrostatic pressure application were inhibited by the addition of the ERK activation inhibitor U0126. Treatment with U0126 during hydrostatic pressure application resulted in decreased collagen ww% compared to hydrostatic pressure application alone; this decrease in collagen may have resulted in the decreased tensile properties of this group.

However, ERK signalling does not explain the change in compressive properties induced by hydrostatic pressure in self-assembled cartilage, as inhibition of ERK did not inhibit changes in compressive properties. The increase in compressive properties corresponds to the increase in GAG content seen in both the U0126 and hydrostatic pressure groups. As the changes in compressive properties by hydrostatic pressure did not appear to be negatively altered by inhibiting ERK, it can be concluded that these may be mediated by a different pathway. As GAG content increased with U0126 addition, this may indicate that ERK activation is counterproductive to increased GAG content.

Changes in GAG content with U0126 application may be due to multiple scenarios. It is possible that U0126 may inhibit low basal levels of ERK activation in the non-hydrostatic pressure-treated constructs. Another possibility is that U0126 may increase GAG content through unknown secondary effects. Although U0126 has extremely high specificity, other protein kinases can be weakly inhibited (Davies *et al*., 2000). Interestingly, this U0126-induced increase in GAG content appears to be overshadowed when hydrostatic pressure is applied, as the combination of hydrostatic pressure and U0126 did not significantly increase GAG content over hydrostatic pressure alone.

Mechanosensitive ion channels have been documented in multiple cell systems (Martinac, 2004), with multiple ion channels responding to hydrostatic pressure stimulation (Browning *et al*., 1999; Hall, 1999; Olsen *et al*., 2011). In chondrocytes, ion flow has been proposed to be a signal transducer of mechanical loading (Mow *et al*., 1999). Application to chondrocytes of hydrostatic pressure in the 10 MPa range induces inhibition of the Na–K ion channel and activation of the Na–H pump (Browning *et al*., 1999; Hall, 1999). Natoli *et al*. (2010) have shown that Ca^{+2} or Na/K ion channel modulation by ionomycin and ouabain in self-assembled cartilage constructs resulted in mechanical changes tantamount to those resulting from the application of hydrostatic pressure. In multiple systems, activation of ERK by ion channel activity has been described (Rane, 1999) and both ionomycin and ouabain have been reported to modulate ERK activity in other systems (Hanson and Ziegler, 2002; Soltoff and Hedden, 2008; Sweadner, 2008) and to increase intracellular Ca^{+2} levels. Synthesizing these data, a model can be proposed that hydrostatic pressure may act to modulate ion channel activity and therefore intracellular ion flux (mainly Ca^{+2}), with a portion of this signal transducing through the ERK pathway, resulting in upregulation of extracellular matrix molecules (Elder and Athanasiou, 2009b).

The changes in mechanical and biochemical properties of tissue-engineered cartilage seen with ERK activation are likely not limited to hydrostatic pressure application. The ERK pathway is known to be regulated by multiple external stimuli, especially growth factors, such as TGFβ1 and FGF2, and may thus play a role in the beneficial effects of these stimuli on tissue-engineered constructs (Elder and Athanasiou, 2008, 2009c). Further studies should be undertaken to identify the mechanism of upstream ERK activation and downstream gene activation (e.g. *SOX9* in chondrocytes) during hydrostatic pressure application, within the context of ion channel activity. This mechanism also likely functions in native tissues that are exposed to hydrostatic pressure, and may have relevance in other tissue-engineered materials.

Modulation of mechanical properties in tissue engineering remains an important objective, as the need for functional tissue replacements requires that engineered tissues exhibit biomechanical properties on a par with native tissue. Identification of signalling pathways that regulate mechanical properties will further this goal.

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Conflict of interest

The authors have declared that there is no conflict of interest.

References

Afoke NY, Byers PD, Hutton WC. 1987; Contact pressures in the human hip joint. *J Bone Joint Surg Br* **69**(4): 536–541.

Appleton CT, Usmani SE, Mort JS, Beier F. 2010; Rho/ROCK and MEK/ERK activation by transforming growth factor-α induces articular cartilage degradation. *Lab Invest* **90**(1): 20–30.

Athanasiou KA, Agarwal A, Dzida FJ. 1994; Comparative study of the intrinsic mechanical properties of the human acetabular and femoral head cartilage. *J Orthop Res* **12**(3): 340–349.

Aufderheide AC, Athanasiou KA. 2007; Assessment of a bovine co-culture, scaffold-free method for growing meniscus-shaped constructs. *Tissue Eng* **13**(9): 2195–2205.

Bastow ER, Lamb KJ, Lewthwaite JC *et al*. 2005; Selective activation of the MEK–ERK pathway is regulated by mechanical stimuli in forming joints and promotes pericellular matrix formation. *J Biol Chem* **280**(12): 11749–11758.

Bobick BE, Kulyk WM. 2004; The MEK–ERK signaling pathway is a negative regulator of cartilage-specific gene expression in embryonic limb mesenchyme. *J Biol Chem* **279**(6): 4588–4595.

Browning JA, Walker RE, Hall AC *et al*. 1999; Modulation of $Na^+ \times H^+$ exchange by hydrostatic pressure in isolated bovine articular chondrocytes. *Acta Physiol Scand* **166**(1): 39–45.

Buckwalter JA. 1995; Osteoarthritis and articular cartilage use, disuse, and abuse: experimental studies. *J Rheumatol Suppl* **43**: 13–15.

Davies SP, Reddy H, Caivano M *et al*. 2000; Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* **351**(1): 95–105.

Ding L, Heying E, Nicholson N *et al*. 2010; Mechanical impact induces cartilage degradation via mitogen activated protein kinases. *Osteoarthr Cartilage* **18**(11): 1509–1517.

Elder BD, Athanasiou KA. 2008; Synergistic and additive effects of hydrostatic pressure and growth factors on tissue formation. *PLoS One* **3**(6): e2341.

Elder BD, Athanasiou KA. 2009a; Effects of temporal hydrostatic pressure on tissue-engineered bovine articular cartilage constructs. *Tissue Eng Part A* **15**(5): 1151–1158.

Elder BD, Athanasiou KA. 2009b; Hydrostatic pressure in articular cartilage tissue engineering: from chondrocytes to tissue regeneration. *Tissue Eng Part B Rev* **15**(1): 43–53.

Elder BD, Athanasiou KA. 2009c; Systematic assessment of growth factor treatment on biochemical and biomechanical properties of engineered articular cartilage constructs. *Osteoarthr Cartilage* **17**(1): 114–123.

Elluru RG, Thompson F, Reece A. 2009; Fibroblast growth factor 18 gives growth and directional cues to airway cartilage. *Laryngoscope* **119**(6): 1153–1165.

Guilak F, Setton LA, Kraus VB. 2000; Structure and function of articular cartilage. In Principles and Practice of Orthopaedic Sports Medicine, Garrett WE, Speer KP, Kirkendall DT *et al*. (eds). Lippincott Williams & Wilkins: Philadelphia, PA.

Hall AC. 1999; Differential effects of hydrostatic pressure on cation transport pathways of isolated articular chondrocytes. *J Cell Physiol* **178**(2): 197–204.

Hanson DA, Ziegler SF. 2002; Regulation of ionomycin-mediated granule release from rat basophil leukemia cells. *Mol Immunol* **38**(16–18): 1329–1335.

Hatton JP, Pooran M, Li CF *et al*. 2003; A short pulse of mechanical force induces gene expression and growth in MC3T3-E1 osteoblasts via an ERK 1/2 pathway. *J Bone Miner Res* **18**(1): 58–66.

Hu JC, Athanasiou KA. 2006; A self-assembling process in articular cartilage tissue engineering. *Tissue Eng* **12**(4): 969–979.

Hung CT, Henshaw DR, Wang CC *et al*. 2000; Mitogen-activated protein kinase signaling in bovine articular chondrocytes in response to fluid flow does not require calcium mobilization. *J Biomech* **33**(1): 73–80.

Jessop HL, Rawlinson SC, Pitsillides AA *et al*. 2002; Mechanical strain and fluid movement both activate extracellular regulated kinase (ERK) in osteoblast-like cells but via different signaling pathways. *Bone* **31**(1): 186–194.

Kook SH, Hwang JM, Park JS *et al*. 2009; Mechanical force induces type I collagen expression in human periodontal ligament fibroblasts through activation of ERK/JNK and AP-1. *J Cell Biochem* **106**(6): 1060–1067.

Kopakkala-Tani M, Elo MA, Sironen RK, *et al*. 2004; High hydrostatic pressure induces ERK and PI3 kinase phosphorylation in human HCS-2/8 chondrosarcoma cells. *Cell Mol Biol* **50**(4): 485–490.

Kraft JJ, Jeong C, Novotny JE *et al*. 2011; Effects of hydrostatic loading on a self-aggregating, suspension culture-derived cartilage tissue analog. *Cartilage* **2**(3): 254–264.

Krishna M, Narang H. 2008; The complexity of mitogen-activated protein kinases (MAPKs) made simple. *Cell Mol Life Sci* **65**(22): 3525–3544.

Laboureau J, Dubertret L, Lebreton-De Coster C *et al*. 2004; ERK activation by mechanical strain is regulated by the small G proteins rac-1 and rhoA. *Exp Dermatol* **13**(2): 70–77.

Li J, Zhao Z, Liu J *et al*. 2010; MEK/ERK and p38 MAPK regulate chondrogenesis of rat bone marrow mesenchymal stem cells through delicate interaction with TGF-β1/Smads pathway. *Cell Prolif* **43**(4): 333–343.

Liu J, Liu T, Zheng Y *et al*. 2006; Early responses of osteoblast-like cells to different mechanical signals through various signaling pathways. *Biochem Biophys Res Commun* **348**(3): 1167–1173.

Martinac B. 2004; Mechanosensitive ion channels: molecules of mechanotransduction. *J Cell Sci* **117**(12): 2449–2460.

Mio K, Kirkham J, Bonass WA. 2007; Possible role of extracellular signal-regulated kinase pathway in regulation of *Sox9* mRNA expression in chondrocytes under hydrostatic pressure. *J Biosci Bioeng* **104**(6): 506–509.

Mow VC, Gibbs MC, Lai WM *et al*. 1989; Biphasic indentation of articular cartilage – II. A numerical algorithm and an experimental study. *J Biomech* **22**(8–9): 853–861.

Mow VC, Wang CC, Hung CT. 1999; The extracellular matrix, interstitial fluid and ions as a mechanical signal transducer in articular cartilage. *Osteoarthr Cartilage* **7**(1): 41–58.

Murakami S, Kan M, McKeehan WL *et al*. 2000; Upregulation of the chondrogenic *Sox9* gene by fibroblast growth factors is mediated by the mitogen-activated protein kinase pathway. *Proc Natl Acad Sci USA* **97**(3): 1113–1118.

Nakamura K, Shirai T, Morishita S *et al*. 1999; p38 mitogen-activated protein kinase functionally contributes to chondrogenesis induced by growth/differentiation factor-5 in ATDC5 cells. *Exp Cell Res* **250**(2): 351–363.

Natoli RM, Skaalure S, Bijlani S *et al*. 2010; Intracellular Na^+ and Ca^{2+} modulation increases the tensile properties of developing engineered articular cartilage. *Arthritis Rheum* **62**(4): 1097–1107.

Olsen SM, Stover JD, Nagatomi J. 2011; Examining the role of mechanosensitive ion channels in pressure mechanotransduction in rat bladder urothelial cells. *Ann Biomed Eng* **39**(2): 688–697.

Prasadam I, van Gennip S, Friis T *et al*. 2010; ERK-1/2 and p38 in the regulation of hypertrophic changes of normal articular cartilage chondrocytes induced by osteoarthritic subchondral osteoblasts. *Arthritis Rheum* **62**(5): 1349–1360.

Rane SG. 1999; Ion channels as physiological effectors for growth factor receptor and Ras/ERK signaling pathways. *Adv Second Messenger Phosphoprotein Res* **33**: 107–127.

Reusch HP, Chan G, Ives HE *et al*. 1997; Activation of JNK/SAPK and ERK by mechanical strain in vascular smooth muscle cells depends on extracellular matrix composition. *Biochem Biophys Res Commun* **237**(2): 239–244.

Ryan JA, Eisner EA, Duraine G *et al*. 2009; Mechanical compression of articular cartilage induces chondrocyte proliferation and inhibits proteoglycan synthesis by activation of the ERK pathway: implications for tissue engineering and regenerative medicine. *J Tissue Eng Regen Med* **3**(2): 107–116.

Schaeffer HJ, Weber MJ. 1999; Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol Cell Biol* **19**(4): 2435–2444.

Shin SY, Rath O, Choo SM *et al*. 2009; Positive- and negative-feedback regulations coordinate the dynamic behavior of the Ras–Raf–MEK–ERK signal transduction pathway. *J Cell Sci* **122**(3): 425–435.

Soltoff SP, Hedden L. 2008; Regulation of ERK1/2 by ouabain and Na–K–ATPase-dependent energy utilization and AMPK activation in parotid acinar cells. *Am J Physiol Cell Physiol* **295**(3): C590–599.

Sweadner KJ. 2008; A third mode of ouabain signaling. Focus on regulation of ERK1/2 by ouabain and Na–K–ATPase-dependent energy utilization and AMPK activation in parotid acinar cells. *Am J Physiol Cell Physiol* **295**(3): C588–589.

Woessner JF Jr. 1961; The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys* **93**: 440–447.

Yoon YM, Kim SJ, Oh CD *et al*. 2002; Maintenance of differentiated phenotype of articular chondrocytes by protein kinase C and extracellular signal-regulated protein kinase. *J Biol Chem* **277**(10): 8412–8420.

Zakany R, Szijgyarto Z, Matta C *et al*. 2005; Hydrogen peroxide inhibits formation of cartilage in chicken micromass cultures and decreases the activity of calcineurin: implication of ERK1/2 and Sox9 pathways. *Exp Cell Res* **305**(1): 190–199.