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The Chondrogenesis of PDLs by Dynamic Unconfined Compression Is Dependent on p42/44 and Not p38 or JNK

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UNIVERSITY OF MIAMI

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COMPRESSION IS DEPENDENT ON P42/44 AND NOT P38 OR JNK

By

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<u>The Chondrogenesis of PDLs by Dynamic</u>   (December 2009)
<u>Unconfined Compression is Dependent on</u>
<u>p42/44 and Not p38 or JNK.</u>

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Articular cartilage lines the surfaces of load bearing joints and has limited capabilities for self-repair due to its alymphatic and avascular structure. Attempts at making repairs to this tissue has resulted in substandard materials and/or causing further injury to the patient making this tissue a prime candidate for tissue engineering studies incorporating adult stem cells. These studies have given rise to some answers and many more questions including a search for alternative stem cell sources and what biochemical changes the cells undergo during the differentiation of these stem cells into chondrocytes, the cells which make up articular cartilage. Recently, periodontal dental ligament stem cells (PDLs) have come to the forefront as a practical alternative to other adult stem cells as well as the involvement of the mitogen-activated protein kinases (MAPKs) in stem cell differentiation via mechanical stimulation. During dynamic unconfined compression, levels of p42/44 MAPK increased by 50% ($p<0.05$). Additionally, the expression of the chondrogenic differentiation factor SRY (sex determining region Y)-box 9 (SOX-9) increased by 3-fold ($p<0.05$) as well as the chondrocyte marker aggrecan by over 2-fold after 4h of dynamic unconfined

compression. Addition of the p42/44 phosphorylation inhibitor PD98059, along with compression, yielded no change in SOX-9 or aggrecan expression levels from basal levels in uncompressed controls. Inhibition of p38 MAPK or JNK phosphorylation during unconfined compression had no effect on the elevated expression of SOX-9 and aggrecan as compared to compressed cells without the addition of an inhibitor. It is therefore the overall findings of this study that PDLs possess the ability to differentiate into chondrocytes by mechanical compression and this differentiation is mediated by the p42/44 MAPK cascade.

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Chapter 1 – Introduction

Articular cartilage is the tissue which lines the load bearing surfaces of bones in a joint to provide smooth, painless movement (1-5). Unfortunately, this tissue can become damaged due to two major factors: Trauma and disease (2;4;6). Due to its avascular and alymphatic architecture, articular cartilage has a very limited capacity for self-repair (1;2;5;7). Repairing the damage which has been done to articular cartilage has been the subject of study by many groups with many different strategies.

These strategies range from traditional surgical techniques to engineered tissue replacements. Unfortunately, these surgical approaches come with a long list of complications including damage done by surgical removal of healthy tissue or replacement of the missing cartilage with a substandard material (6). Engineering of replacement tissues seems to be a more viable answer to this problem. Early attempts at engineering replacements began with harvesting healthy chondrocytes and expanding them in vitro. However, this process also caused damage to healthy tissue and expansion of these harvested cells in monolayer culture led to the gradual loss of the cells phenotype over the course of passaging the cells (8;9).

The difficulties encountered with these attempts at articular cartilage repair led to a search for a different cell source. It was observed, if the injury to the cartilage penetrated the subchondral bone, a fibrin clot containing mesenchymal stem cells (MSCs) would migrate to the injury site from the bone marrow (1). However, as with all previous repair mechanisms, there were inherent

deficiencies. The cells would differentiate under biological and mechanical influences into a fibrocartilagous tissue (10-12), which is vastly inferior to native cartilage (1). It is because of these reasons the vast majority of tissue engineering in recent years has focused on the use of MSCs in the attempt to repair damage to articular cartilage (13).

Additionally, little is known about how these influences are translated into specific gene regulatory events (14;15). Multiple studies have been performed on human MSCs (hMSCs) where mechanical stimulation is utilized, but only chondrogenic markers are being investigated (15). Differing types of mechanical force have been applied including hydrostatic pressure (16), compressive loading (17;18), and tensile force (19) with very little investigation of the molecular cascade leading to chondrogenesis. It has been suggested the mitogen-activated protein kinase (MAPK) family of proteins are a major factor in this process (18-21).

The MAPKs are a family of proteins which are responsible for the transduction of extracellular stimuli into cellular responses, including differentiation, adaptation and responses to stress. The MAPK pathways are turned on by specific stimuli, including growth factors, and coordinate regulation of gene transcription and protein synthesis. There are multiple MAPK pathways, however, the three major pathways will be the subject of this study, and these are p42/44 MAPK, p38 MAPK, and c-Jun N-terminal kinase (JNK). These three pathways are activated by phosphorylation of a highly conserved tyrosine-X-threonine motif. Activation of these proteins can lead to translocation to the

nucleus, or the activated MAPK can bind to cytoplasmic substrates including various transcription factors.

It has recently been stated that very little work has been done to characterize the roles of the different MAPKs during chondrogenic differentiation (22). Studies on static compression of mouse MSCs (mMSCs) have shown evidence of differentiation to a chondroblast lineage by initiating the phosphorylation of p38 MAPK (23). Other studies have shown the inhibition of MEK, and therefore the phosphorylation of p42/44 MAPK, by addition of the inhibitor U0126, blocked upregulation of type II collagen transcripts due to treatment with transforming growth factor-β3 (TGF-β3) (24). Another study showed the involvement of JNK in the chondrogenesis of hMSCs (20). This study is in contrast to one done by Tuli et al. who show JNK is involved in chondrogenesis to a lesser extent than p42/44 or p38 MAPK (21). The differences in these observations could be attributed to selection of cell lines or differences in the conditions of the culturing of the cells (25).

The selection of a proper cell type is one of the central principles of tissue engineering. The proper cells can range from pluripotent stem cells to multipotent stem cells to unipotent stem cells. Pluripotent stem cells can become cells from any of the three germ layers: the endoderm, ectoderm, or most importantly in this case, mesoderm. Multipotent stem cells can also become multiple types of cells, but their range only encompasses a few different types of cells within the same germ layer, MSCs being a key example. Unipotent stem cells are the most differentiated of all stem cells, yet retain some of their stem-like properties. An

example of a unipotent stem cell is a reticulocyte which can only become erythrocytes.

The recent discovery of a new, easily attainable, viable adult dental stem cell, termed 'periodontal dental ligament stem cells' (PDLs), is an exciting alternative to MSCs. It has previously been shown addition of TGF-β3 has induced chondrogenesis of MSCs in a 3D culture by the elevation of expression of aggrecan and type II collagen messenger ribonucleic acid (mRNA) (24). These results are similar to our group's findings on the dynamic unconfined compression of hMSCs in a fibrin scaffold, without the addition of any exogenous cytokines (26). Also, a very common method for the induction of chondrogenesis in MSCs is by addition of TGF-β3 to a high density pellet culture (24). Our laboratory has induced chondrogenic differentiation of the PDLs by this same method, which leads us to believe mechanical stimulation of these cells will also direct them to a chondrogenic lineage.

The purpose of this study is to discover which MAPK is involved during chondrogenic induction of the PDLs by dynamic unconfined compression. Additionally, this study will determine if the PDLs behave similarly to other well-studied adult stem cell lines under the same dynamic compression conditions and other similar conditions. This will aid in determining if the use of this new, easily accessible adult stem cell line is a viable tool in further tissue engineering studies.

Chapter 2 – Background

The differentiation of different types of stem cells towards a chondrogenic lineage has been widely investigated; however the internal mechanism which occurs within the cells during the differentiation process is poorly understood.

The detection of chondrogenesis and chondrocytes is performed by only a limited number of methods. This procedure is generally performed by identification of increases in expression levels of either mRNA transcripts or protein expression levels of SRY (sex determining region Y)-box 9 (SOX-9), aggrecan, and type II collagen (25-28). It has been shown that during the differentiation process of MSCs to chondroblasts to proliferating chondrocytes, the expression levels of SOX-9 are at a maximum while type II collagen and aggrecan are both detectable (25). Additionally, the involvement of SOX9 during chondrogenesis has been extensively studied in mammalian developmental models and the protein was at first shown to have regulation over type II collagen during chondrogenesis (29). Furthermore, SOX-9 was proven to be the first transcription factor essential for chondrocyte differentiation (30) and is the master gene for chondrogenesis (31). Analysis of the changes in expression levels for this gene is important to prove chondrogenesis is taking place.

Aggrecan is a proteoglycan which forms large complexes with type II collagen and makes up the bulk of articular cartilage extracellular matrix (ECM). Also, aggrecan has not been shown to exist in any other structure of the mammalian anatomy. Aggrecan is expressed long before type II collagen (25),

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hence why this study will focus on the effects of dynamic unconfined compression on aggrecan and not type II collagen.

Monitoring changes in the expression of TGF-β3 gene and protein expression is necessary due to the chondroinductive effect the cytokine has on stem cells (24). Although the members of the TGF-β family of proteins share a similar sequence and structure, activation of TGF-β3 transcription is a very specific and unique process. The promoter region of the TGF-β3 gene contains both a cyclic adenosine monophosphate response element (CRE) and a binding site for activating protein-2 (AP-2) (32). The presence of a CRE begets the need to examine the changes in two early response genes, c-jun and c-fos. Primarily, c-jun can dimerize with activating transcription factor 2 (ATF2) and stimulate CRE-dependent transcription (33). The c-jun protein has been found to be bound directly to the CRE in the TGF-β3 promoter and shows some evidence of having a regulatory effect on the transcription of the gene. Additionally, overexpression of c-jun activates a portion of the c-fos gene, in which the newly transcribed c-fos contains only a CRE binding site (34). These three genes may have a pivotal role in the differentiation of PDLs into chondrocytes.

During the design process of any tissue engineering undertaking, cell selection is one of the key aspects of the design which must be well investigated. Part of this investigation is the search for adequate alternatives for the primary cell type selected. Work done in our laboratory has shown the ability of rabbit (35) and human (26) MSCs to be directed toward a chondrogenic lineage by dynamic unconfined compression alone with promising results. These

populations of MSCs used were enriched by recognition of the surface antigen Stro-1, a marker unique to this cell type. In lieu of these successes with MSCs, the identification of a satisfactory alternative is an ongoing process. As mentioned previously, PDLs were selected to be the alternative to hMSCs in this study.

This selection was made based on data from previous studies and observations. Primarily, the removal of the third molars, more commonly known as wisdom teeth, is a common process which a large portion of the population undergoes. Along with being a common procedure, it is beneficial to the patient and also much less invasive than the harvesting of bone marrow (27). Although highly advantageous to the patient, other properties of the cells are of greater consequence to their selection for tissue engineering applications; namely, the ability to differentiate into the target cells desired.

Along with PDLs, other dental stem cells which have gained recent popularity have also been analyzed to establish if they possess the ability to differentiate along a chondrogenic lineage. These alternative cell types include stem cells from human exfoliated deciduous teeth (SHED) and dental pulp stem cells (DPSC). As with MSCs, PDLs, SHED, and DPSC populations have also been purified by detection of the Stro-1 surface marker before any experiments were performed. In the attempt to determine if PDLs were an alternative to MSCs, both cell types were cultured under similar culture conditions for chondrogenic, adipogenic, and osteogenic differentiation by media supplementation. This set of experiments showed the differentiation of PDLs

coincided with that of MSCs, or was very comparable to it. Additionally, the proliferation rate of PDLs is much greater than that of MSCs. After 96 hours in culture, the number of PDLs in each culture was double that of the MSCs (27). These results were then independently verified demonstrating the multilineage potential of PDLs (36). For completeness, both SHED and DPSCs were tested for their ability to differentiation into mesodermal lineages. One such study compared PDLs and DPSCs which resulted in almost identical results for both cell types (37). Additionally, the similarities between SHED and DPSCs have also been assessed. Once again, the cells performed identically to each other during differentiation into chondrogenic, adipogenic, and osteogenic lineages (28).

Finally, it cannot be argued one of the most attractive characteristics of MSCs is their immunomodularity properties. More specifically, these cells, when implanted into an allogenic recipient, suppress the immune response of the recipient to the cells (38;39). Furthermore, this ability is retained when the MSCs are differentiated along a mesenchymal lineage (40). A recent study has established that PDLs also posses these immunomodularity properties (41). All these attributes taken together make PDLs the optimum and quite possibly superior, alternative to MSCs.

While the endpoint of any tissue engineering study is to attain a functional structure, it would behoove the investigator, and the scientific community as a whole, to understand the biochemical changes occurring in the cells during the differentiation to this endpoint. Previous experimental data collected both in our laboratory and by others point at the involvement of the different MAPK

pathways. Unfortunately, there is much discrepancy as to which MAPK pathway(s) is involved in the differentiation of stem cells toward a chondrogenic lineage. Many studies only report on the contribution or response of one or two of the MAPK pathways and the use of different cells and growth factors may also explain these inconsistencies (19). In one study performed on rat MSCs stimulated with TGF-β1 in both 2D and 3D configurations determined p38 MAPK was the key pathway involved in the differentiation of these cells, but failed to examine the effects of JNK (19). In contrast, induction of chondrogenesis of mMSCs by treatment with fibroblast growth factor 2 (FGF2), claims p42/44 MAPK is the major pathway involved the differentiation process. This claim is supported by the inhibition of SOX-9 expression by the addition of a p42/44 MAPK inhibitor, but refrained from reporting data for the other two MAPK pathways (31). Similarly, an investigation on hMSCs induced to a chondrogenic lineage by TGF-β3 only reported their findings on the phosphorylation of p42/44 MAPK and the effects of its inhibitor on the differentiation of the stem cells (24).

Fortunately, more complete studies have been performed on the differentiation of stem cells to chondrocytes which will increase our understanding of the biochemical changes occurring in the cells. One such investigation concentrated on the chondrogenesis of the mouse mesenchymal C3H10T1/2 cell line by the addition of bone morphogenetic protein-2 (BMP-2). This study noticed the addition of BMP-2 decreased the expression of the growth arrest-specific 6 (Gas6) during the differentiation of these cells. After addition of Gas6, the expression of phosphorylated p42/44 (p-p42/44) MAPK and

phosphorylated JNK (p-JNK) increased while the levels of aggrecan and type II collagen decreased suggesting these MAPKs are inhibitory to chondrogenesis (20). Additionally, experimentation on hMSCs without serum and with TGF-β1 exhibit the activation of p42/44 MAPK is the dominant pathway in chondrogenesis of these cells (21). However, it has also been shown p42/44 MAPK is the dominant pathway in the osteogenesis of hMSCs in the absence of exogenous growth factors (14).

After reviewing previous data and understanding of the differentiation mechanism of various types of stem cells, this study will examine the involvement of the three major MAPK pathways in the differentiation of PDLs along a chondrogenic lineage after dynamic unconfined compression without the addition of exogenous proteins or cytokines. It is another goal of this study to examine how these MAPK pathways individually affect the expression of chondrogenic genetic markers as a result of this compression.

Chapter 3 – Materials and Methods

Isolation of PDL cells

Human PDLs harvested from impacted wisdom teeth were collected from 3 patients (age 19-22 years) at the Clinic of the Nova Southeastern University College of Dental Medicine with their informed consent, according to approved institutional review board protocols. The PDLs from different teeth of the same donor were pooled and cells were released by overnight digestion at 37°C in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen Corp.), 1% antibiotic-antimycotic, 1 mg/ml collagenase (Worthington Biochemical Corp., Lakewood, NJ) and 0.6 mg/ml protease (Sigma-Aldrich Corp., St. Louis, MO). The resulting digestion was passed through a 70-µm cell strainer (BD Biosciences, Bedford, MA) in order to obtain single cell suspensions. The suspensions were plated on collagen-coated 6-well culture plates (at 1000 cells/well) in high glucose DMEM supplemented with 10% FBS and 1% antibiotics, and incubated at 37°C and 5% CO_2. Non-adherent cells were removed from the culture by changing the medium after 5 days. After two weeks, the cells of each well, referred to as a subpopulation, were passaged into separate 75cm^2 culture flask and deemed 'passage 1'.

The PDLs of each subpopulation were screened at passage 1 by examining the expression of Octamer-4 (Oct-4), NANOG, SOX-2, and Kruppel-like factor 4 (KLF4). The H9 human embryonic stem cell (ESC) line was used as

a positive control during the screening process. After screening, the ESC-marker-positive PDLs were expanded and examined at passages 3-5 for their capability of differentiating into derivatives of the three germ layers (ectoderm, endoderm, and mesoderm). All monolayer cultures were maintained at a subconfluent level to prevent spontaneous cell differentiation. (Huang et al., Manuscript in preparation).

<u>Cell Culture</u>

For subsequent passages, the cells were cultured in 10mL expansion medium on 75cm^2 tissue culture flasks (Nalgene Nunc, Naperville, IL) in a water-jacketed incubator at 37°C, 5.0% CO_2 and 100% humidity. Expansion medium consisted of high glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) plus 10% by volume fetal bovine serum (FBS) (Hyclone, Logan, UT) and 1% by volume Penicillin/Streptomycin mixture (Invitrogen, Carlsbad, CA). Cells were grown to 60% confluency to avoid spontaneous differentiation. Passaging was performed by lifting with 5mL 0.05% Trypsin-EDTA (Invitrogen, Carlsbad, CA) and incubation at 37°C, 5.0% CO_2 and 100% humidity for 5min. Trypsin was neutralized by adding 3mL of expansion media and the suspension was collected into a 50mL conical tube. The suspension was centrifuged at 1000rpm for 10min at 4°C. Supernatant was removed and the cells were resuspended in expansion medium and replated. For experiment, cells were counted prior to centrifugation by adding 0.1mL of the cell suspension to 0.9mL expansion medium in a counting vial and then diluted with 9mL Isoton II (Beckmann-Coulter, Hialeah, FL) and counted twice in a Z2 particle

counter (Beckmann-Coulter, Hialeah, FL). Cells were resuspended at a

concentration of 10x10^6 cells/mL in a solution of thrombin (Sigma-Aldrich, St.

Louis, MO) at 15 NIH units/mL and phosphate buffered saline (PBS) (Invitrogen,

Carlsbad, CA). Experiments were performed on passages 5-7.

<u>Fibrin Gels</u>

Gels were fabricated using a 1.5mm thick Teflon mold with six 8mm

diameter holes in it which was temporarily attached to a glass microscope slide

by use of six binder clips. Molds and slides were incubated in 100% ethanol prior

to the gel making process. Gels had a volume of 84μL composed of a 1:1 ratio of

80mg/mL bovine plasma fibrinogen (Sigma-Aldrich, St. Louis, MO) in high

glucose DMEM (Invitrogen, Carlsbad, CA) and thrombin at 15U/mL in PBS with a

cellular concentration of 10x10^{6}cells/mL for a final concentration of 5x10^6

cells/mL in the gels. Gels were made two-at-a-time by adding 84μL of thrombin

solution and 84μL of fibrinogen solution to a 1.5mL microcentrifuge tube and

84μL of the gel mixture was added to the mold. This procedure was repeated

three times to fill the six molds on the microscope slide. After all the gels were

poured, the entire assembly was incubated at 37°C, 5.0% CO_2 and 100%

humidity for two hours. After incubation, the molds were removed from the slides

and the gels were gently removed from the mold and stored in a 50mL conical

tube with 10mL of compression medium in an incubator at 37°C, 5.0% CO_2 and

100% humidity. Compression medium consisted of high glucose DMEM

(Invitrogen, Carlsbad, CA), 1% by volume Insulin-Transferrin-Selenium-A (ITS-A)

solution (Invitrogen, Carlsbad, CA), 1% by volume Penicillin/Streptomycin

mixture (Invitrogen, St. Louis, MO) and 0.1mg/mL aprotinin (Sigma-Aldrich, St. Louis, MO).

Dynamic Unconfined Compression

Gels were compressed in a custom built bioreactor (35). Gels were compressed at a rate of 1Hz and 10% strain for 4h in 12mL of compression medium with vehicle or inhibitors added to the media in the bioreactor. Compressions were carried out in water-jacketed incubator at 37°C, 5.0% CO_2 and 100% humidity. Control gels were maintained in the same medium as compressed gels in a 50mL conical tube in the same incubator for the same amount of time as the compressed gels. After compression, the bioreactor was disassembled and the gels were carefully removed. Control gels were also removed from their medium at this time. Proteins and mRNA were extracted from the gels as described in the following sections.

Inhibitors

To track the molecular interactions within the cells, selective inhibitors to halt the phosphorylation/activation of the MAPK signaling cascades were utilized. The p42/44 MAPK protein was inhibited by use of PD98059 at a concentration of 50µM, p38 MAPK was inhibited by use of SB202190 at a concentration of 10µM, and SP600125 was used to inhibit the JNK protein at a concentration of 10µM. All inhibitors were acquired from Invitrogen (Carlsbad, CA). Inhibitors and vehicles were added to the medium containing the fibrin gels at least 1h prior to unconfined dynamic compression and were also added to the medium in the

bioreactor to allow for the inhibitor to passively diffuse into the gels and penetrate the cells.

<u>mRNA Extraction and Quantification</u>

Gels for mRNA extraction were immediately placed into 0.5mL of TRIzol LS (Invitrogen, Carlsbad, CA) in glass douncers and promptly pulverized. After pulverization in TRIzol, the samples were transferred into a clean, 1.7mL microcentrifuge tube. The samples were incubated at room temperature for 5min and then 100µL of chloroform (Sigma-Aldrich, St. Louis, MO) was added. The tube was briefly vortexed and incubated at room temperature for 15min. Samples were then centrifuged at 12,000 x g for 15min at 4°C. After centrifugation, the upper colorless phase containing the mRNA was decanted to a fresh tube. The lower red phase was retained for protein isolation. To precipitate the mRNA, 250µL of isopropanol (Sigma-Aldrich, St. Louis, MO) was added to the colorless phase and then incubated at room temperature for 15min. Samples were then centrifuged at 12,000 x g for 10min at 4°C and the isopropanol was removed carefully as to not disturb the pellet. The mRNA pellet is washed by adding 0.5mL of 75% ethanol (Sigma-Aldrich, St. Louis, MO) and mixed by vortexing. The samples were then centrifuged at 7,500 x g for 15min at 4°C. The ethanol was removed and the pellet was air-dried for 5min. The mRNA was resuspended in 12µL of DEPC water and heated in a water bath at 55°C for 10min. Samples were quantified using a NanoDrop (ThermoFisher Scientific, Waltham, MA) by placing 2µL of each sample onto the pedestal, connected to a computer equipped with the ND-1000 software and using the built-in protocol "Nucleic

Acids" with the "RNA-40" option selected. The sample was recollected from the

pedestal and was then wiped clean before quantifying the next sample.

Complimentary DNA synthesis

Complimentary deoxyribonucleic acid (cDNA) synthesis was performed

utilizing the ThermoScript PCR Kit (Invitrogen, Carlsbad, CA). Reactions were

carried out according the manufacturer's protocol for 1μg or less of mRNA

template. Messenger RNA samples were normalized to 1.0μg in 9μL of PCR-

grade water in a 200μL PCR reaction tube. The first master mix was made by

adding 1μL of Oligo(dT)$_{20}$ and 2μL 10mM deoxynucleotide triphosphate (dNTP)

for each reaction in a clean 1.5mL microcentrifuge tube, always making extra to

account for pipetting, and adding 3μL of this master mix to each sample. The

samples were then placed in a GeneAmp 2700 ThermoCycler (Applied

BioSystems, Foster City, CA) and incubated at 55°C for 5min to denature the

mRNA. The samples were then removed from the thermocycler and a second

master mix was prepared containing: 4μL 5X cDNA Buffer, 1μL 0.1M DTT, 1μL

RNaseOUT, 1μL PCR-grade water and 1μL ThermoScript RT for each sample.

From this master mix, 8μL were added to each sample to attain a final volume of

20μL. The samples were then returned to the thermocycler and incubated at

45°C for 50min then 85°C for 5min to denature the enzyme, and then 4°C forever

to preserve the newly created cDNA. The samples were removed from the

thermocycler and stored at -20°C until needed. This protocol yielded samples

containing 50ng/μL of original mRNA template.

Polymerase Chain Reaction (PCR) Primer/Probes

Primer/probe mixtures were purchased from Applied Biosystems (Foster City, CA) at a 20X concentration. Primer/probes were selected from the catalog for c-Fos (Hs00182371_m1), c-Jun (Hs99999141_s1), TGF-β3 (Hs00234245_m1), SOX-9 (Hs00165814_m1), aggrecan (Hs00153936_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) using the criteria that the probe spans an exon/exon boundary so genomic DNA was not amplified.

Real-time PCR

Real-time PCR was carried out with the LightCycler TaqMan Master kit (Roche Applied Sciences, Indianapolis, IN). The Master Mix (5X) was prepared by adding 10µL of "Enzyme" to the "Reaction Mix" and mixed by pipetting up and down and kept on ice. The PCR Mix was prepared by mixing 13µL of PCR-grade water, 1µL of 20X primer mix, and 4µL of 5X Master Mix for each sample in a clean 1.5mL microcentrifuge tube. LightCycler Capillaries (20µL) were placed into a MagNA Pure LC Cooling Block with LC Centrifuge Adapters (Roche Applied Sciences, Indianapolis, IN) and 18µL of the PCR Mix was pipetted into each Capillary. Then, 2µL of each cDNA sample was added to the Capillaries and the tubes were capped. The Capillaries in the LC Adapters were placed into a table-top microcentrifuge (Eppendorf USA, Westbury, NY) and centrifuged at 700 x g for 5s and then the Capillaries were transferred to the LightCycler Sample Carousel. Reactions were performed in a LightCycler 2.0 Instrument (Roche Applied Science, Indianapolis, IN) connected to a computer with the

LightCycler 4.05 Software installed under the conditions recommended by the manufacturer and are enumerated in Table 3.1.

Table 3.1: Conditions for Roche LightCycler 2.0

Analysis Mode	Cycles	Segment	Target Temperature	Hold Time	Acquisition Mode
Pre-Incubation					
None	1		95°C	10min	None
Quantification					
Quantification	40	Denaturation	95°C	10s	None
		Annealing	60°C	20s	None
		Extension	72°C	1s	Single
Cooling					
None	1		40°C	30s	None

Standard curves for each gene of interest were generated by diluting the calibrator sample at a ratio of 1:10 four times to generate a logarithmic standard curve. These samples were run through the thermocycler. Once the thermocycler protocol was completed, the calculations of the crossing points (Cp's) was adjusted to obtain the best standard curve which includes an efficiency near 100%, signified by a value of 2.0, and a minimal error. This was performed in the LightCycler 4.05 software by the following procedure. First, the method of calculation was changed in the Analysis window from 'Automated' to 'Fit Points'.

Then, three tabs labeled Step 1, Step 2 and Step 3 appear. The 'Step 1: Background' tab remains unchanged. In the 'Step 2: Noise Band' tab, the

horizontal red line is adjusted so it is just slightly above the fluorescence level of the sample containing only water.

Next, in the 'Step 3: Analysis' tab, the horizontal red line, called the threshold, is adjusted to minimize the error or bring the Efficiency as close to 2.0 as possible. The 'Minimize Error' button was used to find a starting point for further adjustments.

Finally, the Cp values were read from the far left panel of the Analysis window and used for further calculations in the ΔΔCt method for calculating fold changes. The Noise Band and Threshold numbers were input into each Real-time PCR performed for each gene of interest to conserve inter-assay comparability.

<u>Protein Isolation</u>

Protein isolation was carried out immediately after compressions were completed. Gels were removed from the bioreactor and placed into a pre-cooled ceramic mortar. Liquid nitrogen was then poured onto the gels and the ceramic pestle was placed on top of the gels. Once the liquid nitrogen had evaporated, the gels were ground into a powder. The powder was placed into a 1.7mL microcentrifuge tube and the lysis buffer was added. Once all the gels had been ground, cell lysis was carried out by mechanical means. Each tube was vortexed for 1min every 10min for 40min and kept on ice between each vortexing. After the 40min protocol, the tubes were centrifuged at 14,000rpm at 4°C for 30min to pellet the cell membranes, cytoskeleton and the bulk of the fibrin. The

supernatant was transferred to a clean 1.7mL microcentrifuge tube for Western blotting and enzyme-linked immuosorbent assay (ELISA).

<u>Lysis Buffer</u>

The lysis/protein storage buffer used consisted of 20mM Tris Base, 140mM sodium chloride, 10mM sodium pyrophosphate, 10mM sodium fluoride, 2mM sodium orthovanadate, 3mM ethylenediaminetetraacetic acid (EDTA) and 10% v/v anhydrous glycerol. The pH of the solution was then adjusted to 7.4 using 10N hydrochloric acid (HCl). One tablet of complete Mini protease inhibitor cocktail (Roche, Indianapolis, IN) was added to each 10mL of the solution and vortexed until dissolved immediately before use. Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich, St. Louis, MO) was also added just before use at 10μL/mL.

<u>Western blot</u>

Western (Immuno) blotting was performed in a Bio-Rad Mini-PROTEAN Tetra Electrophoresis System (Hercules, CA). Sodium dodecyl sulfate (SDS)-Polyacrylamide Gels were cast in a Mini-PROTEAN casting frame (Bio-Rad, Hercules, CA) and stand two-at-a-time. Stand, frame, and gel cassettes were assembled according to the diagrams furnished by the manufacturer. Resolving gels were cast at 12% and consisted of 3.4mL distilled-deionized (DDI) water, 4mL 30% acrylamide/bis-acrylamide solution, 37.5:1 (Bio-Rad, Hercules, CA), 2.5mL 1.5M Tris-HCl, pH 8.8, 0.1mL 10% w/v SDS, 50μL 10% ammonium persulfate (APS), and finally 5μL N,N,N',N'-Tetramethylethylenediamine (TEMED) to begin polymerization. A volume of 3.2mL of the gel mixture was added to each cassette, gently topped with 0.5mL 100% ethanol, and allowed to

stand for 45min to 60min. The gels were then washed with distilled water and dried with filter paper, being careful not to touch the gel. The stacking gel was then prepared with 3ml DDI water, 0.65mL 30% acrylamide/bis-acrylamide solution, 37.5:1, 1.25mL 0.5M Tris-HCl, pH 6.8, 50µL 10% w/v SDS, 25µL 10% APS, and finally 5µL TEMED to begin polymerization. The gel mixture was then added to the top of the resolving gel and filled to the top of the short plate. The gel combs were inserted to the gel cassette and allowed to stand for 30min to 45min.

Gel cassettes were removed from the casting frame and stand and placed immediately into the electrode assembly of the Mini-PROTEAN System according to the diagrams supplied by the manufacturer and placed into the Mini-PROTEAN Tetra cell tank. Running buffer was then added to the inner chamber of the electrode assembly until the level of the buffer was just below the top of the chamber and the remainder of the buffer was added to the tank to assist with heat distribution. Running buffer was made by combining 3.03g Tris-base, 14.4g glycine and 1g SDS in a volumetric flask in 900mL of DDI water and raising the volume to 1L after the powders had dissolved. Equal volumes of protein samples were combined with the Pierce 5X Lane Marker reducing sample buffer (Rockford, IL) in a fresh 1.7mL microcentrifuge tube, briefly vortexed and immersed in boiling water for no more than 5min. A total of 30µg of total protein was loaded into each of the wells of the SDS gel. Volumes loaded were calculated based on the results of the protein assay. Pierce 3-Color Prestained Molecular Weight Marker Mix was resuspended in 10µL DDI water and loaded

onto each gel. After all samples and the marker were loaded, the lid of the assembly was placed onto the tank and connected to a Bio-Rad PowerPac Basic Power Supply set to a constant voltage of 150V and allowed to run until the sample buffer ran out of the bottom of the resolving gel.

Transfer of the separated proteins from the resolving gel to a polyvinylidene difluoride (PVDF) membrane was done in a Bio-Rad Mini Trans-Blot Transfer Cell. Prior to electrophoretic transfer, two fiber pads and two filter papers were incubated in transfer buffer at 4°C during gel electrophoresis. The transfer buffer was prepared by adding 3.03g Tris-base, 14.4g glycine, and 200mL 100% methanol to 700mL DDI water. After the powders had dissolved, the mixture was raised to 1L in a volumetric flask. When gel electrophoresis was completed, the apparatus was disassembled and the gel cassette was gently opened. The stacking gel was gently separated from the resolving gel using a razor blade and discarded. The resolving gel was then separated from the glass plate and placed into the pre-chilled transfer buffer and allowed to equilibrate for 15min. During this, the PVDF membrane was wetted in 100% methanol for 3-5s and then placed into the pre-chilled transfer buffer. Now the gel sandwich was prepared. First, the gel holder cassette was opened and one of the pre-wetted fiber pads was placed on the black side. Next, one of the filter papers was placed on the fiber pad, followed by the gel and the PVDF membrane and the second filter paper. After this, a glass test tube was used as a "rolling-pin" to ensure complete contact between the gel and the membrane. Finally the second filter pad was added and the gel holder cassette was closed and locked and

placed into the electrode module in the Tetra Cell tank. The remainder of the pre-chilled transfer buffer was added to the tank and the Bio-Ice cooling unit which had been frozen at -20°C was placed into the tank. The lid was placed onto the tank, connected to the PowerPac, and set to run for 90min at a constant voltage of 100V.

After the transfer was completed, the apparatus was again disassembled, and the gel sandwich was removed. The membrane with the proteins on it was removed, placed into a new smooth glass container and incubated for 1h at room temperature under constant agitation with a solution of Tris-buffered saline (50 mM Tris-HCl and 150 mM sodium chloride) plus 0.05% Tween 20 (TBS-T) with 1% non-fat dry milk and 1% bovine serum albumin added to block the non-specific binding sites on the membrane. Proteins of interest were p38, p42/44, JNK1/2, and β-Actin. Primary antibodies to specific proteins were diluted in the blocking buffer at a ratio of 1:500 and incubated overnight at 4°C under constant agitation. The antibody to β-Actin was diluted 1:5000 and only allowed to incubate for 30min at room temperature under constant agitation. All primary antibodies were obtained from Cell Signaling Technology (Danvers, MA), except β-Actin which was purchased from Sigma-Aldrich (St. Louis, MO).

When incubation with the primary antibody was complete, the antibody solution was removed and frozen at -20°C for repeated use. The membrane was then briefly rinsed with 2 changes of TBS-T and then washed for 15min in TBS-T followed by three 5min washes. Wash buffer was changed between each wash period. After the final wash, the glass container was sufficiently drained and the

secondary antibody was added and incubated at room temperature for 1h under constant agitation. Secondary, horseradish peroxidase (HRP)-conjugated, antibodies were obtained from Invitrogen (Carlsbad, CA) and diluted in the blocking buffer at a ratio of either 1:5000 or 1:10000. Next, the same wash protocol used for the primary antibody was repeated.

Subsequent to the washing, the membrane was removed from the container, gently blotted on a lab wipe, and placed on a piece of clean glass. The membrane was then covered with 1mL of the chemilumenescent reagent. One of two reagents was used, either the Amersham ECL Plus Western Blotting Detection reagent (Piscataway, NJ) mixed at a ratio of 40:1 or the Pierce SuperSignal West Pico Substrate (Rockford, IL) mixed at a ratio of 1:1. The membrane was allowed to stand for 5min, then blotted dry on a lab wipe and placed between two plastic sheets. The plastic sheets with the membrane were temporarily fixed in an autoradiography cassette. A sheet of Amersham Hyperfilm ECL autoradiography film (Piscataway, NJ) was placed into the cassette under dark room conditions and exposed for times varying from 30s to 1h and processed in an automatic film processor. The processed film was scanned into a computer and the image was analyzed with ImageJ (NIH, Bethesda, MD).

Enzyme-linked Immunosorbent Assay (ELISA)

Due to low levels of protein expression, ELISA's were utilized for the detection of the activated/phosphorylated forms of the MAPK proteins. The kits for phosphorylated p38 (p-p38) MAPK, p-p42/44 MAPK and p-JNK1/2 were obtained from Endogen (Rockford, IL) and all followed a similar protocol which

will be summarized here. The samples used in this protocol are the ones isolated from the pulverized fibrin gels discussed in the previous sections. All incubations were carried out at room temperature. First, 100µL of standards, sample, and blanks were added to the 96-well plate which was provided with each kit and allowed to incubate for 1h. The bottom of each well was coated with an antibody to the particular protein for which the kit was designed. Now, the wells were emptied and washed with 400µL of wash buffer four times. Next, 100µL of the detection antibody was added to each well and allowed to incubate for 1h. The same wash protocol was repeated. Following this, 100µL of the conjugate was added to each well and allowed to incubate for one hour and then washed again with the same wash protocol. Finally, the 100µL of the tetramethylbenzidine (TMB) substrate was added and allowed to incubate for 30min at room temperature followed by 100µL of the stop solution. The plate was read in a MRX Revelation densitometer (Dynex Technologies, Chantilly, VA) using a 450nm filter. Expression levels were normalized with the β-actin western blot densitometry. This method was employed due to the high amounts of fibrin dissolved in the cell lysates which would skew any type of protein assay.

<u>Statisitcal Analysis</u>

All statistical analysis, data organization and figure design was done with the Prism 5.0 statistical package except for the Grubb's Test for outliers, which was performed with an online calculator available at http://www.graphpad.com.

A two-way ANOVA will be utilized for comparisons of experimental data. A two-way analysis of variance (ANOVA) analyzes two variables both

independently and then the interaction of the two variables on the data. In this case, the independent variables are the MAPK pathway being inhibited and the application of compression. For all tests, the α-value will be set at 0.05 which calls for a more stringent statistical analysis. The p-value returned has the same interpretation as a one-way ANOVA or a standard t-test. Finally, the F-value returned is a measure of the impact of each independent variable or their interaction on the results. The further the departure from 1 of the F-value, the more impact that condition has on the results. For example, a F-value of 1.6 has little impact while a value of 6.2 has a great impact on the results.

<u>Chondrogenic differentiation of PDLs by treatment with TGF-β3</u>

To determine if PDLs have the ability to differentiate into chondrocytes,

the cells were grown in pellet culture and treated with TGF-β3 (10ng/mL) for 14

days. RT-PCR was performed for aggrecan and type II collagen to assess

chondrogenic differentiation. Cells treated with TGF-β3 express higher levels of

aggrecan than cells that did not receive TGF-β3 treatment (Figure 4.1). Most

importantly, cells treated with TGF-β3 express type II collagen while the

untreated cells do not.

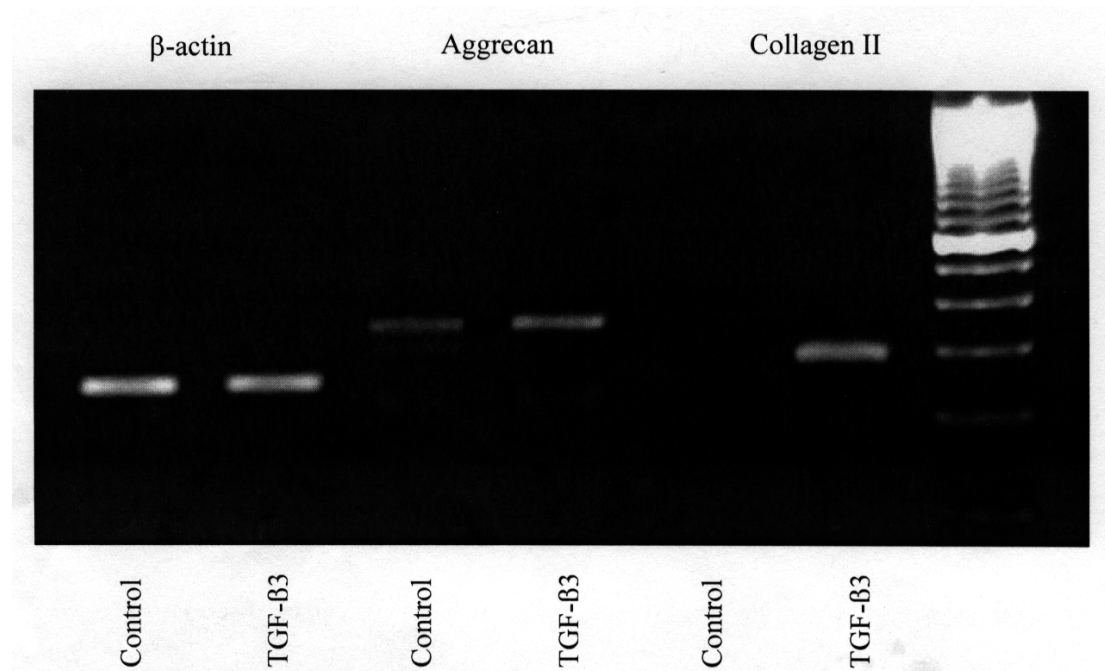<u>Figure 4.1</u> - PDL's grown in pellet culture for 14 days express chondrogenic markers when stimulated with TGF-β3 (10ng/mL). RT-PCR was performed to assess chondrogenic differentiation and β-actin was used as a loading control. Right-most lane is molecular weight marker. Cells treated with TGF-β3 express type II collagen and higher levels of aggrecan mRNA than non-treated cells.

<u>Expression of phosphorylated-p42/44 MAPK protein expression levels are</u>

<u>increased by dynamic unconfined compression</u>

Measurement of the p-p42/44 MAPK was performed by sandwich ELISA specific to the phosphorylated form of the protein and then normalized by β-actin expression as measured by Western blot as described in Chapter 3. Expression of phosphorylated-p42/44 MAPK activated protein normalized to cellular β-actin increased as a result of dynamic unconfined compression by approximately 50% (Figure 4.2A, $p<0.05$). The inhibitor SB202190, which specifically blocks the phosphorylation of p38 MAPK, when added to the bioreactor failed to attenuate the increase in protein expression of p-p42/44 MAPK after compression. Similarly, the inhibitor SP600125, which specifically blocks the activation of JNK, had no effect upon p-p42/44 MAPK expression under the conditions of these experiments (Figure 4.2B). With and without the inhibitors for p38 MAPK and JNK, protein expression of p-p42/44 increased by 50-75% ($p<0.005$ for p-p38 MAPK inhibitor as compared to uncompressed control cells).

Analysis of the p-p42/44 MAPK data as a whole was performed by two-way ANOVA with $\alpha = 0.05$. The ANOVA was tuned to compare compressed versus non-compressed results across the four inhibition conditions and then the four inhibition conditions against compression status. There was no significant effect due to pathway inhibited alone or to the interaction of the pathway inhibited and compression. There was only significant effect due to compression: $F(1,10)=8.661$, $p=0.01$.
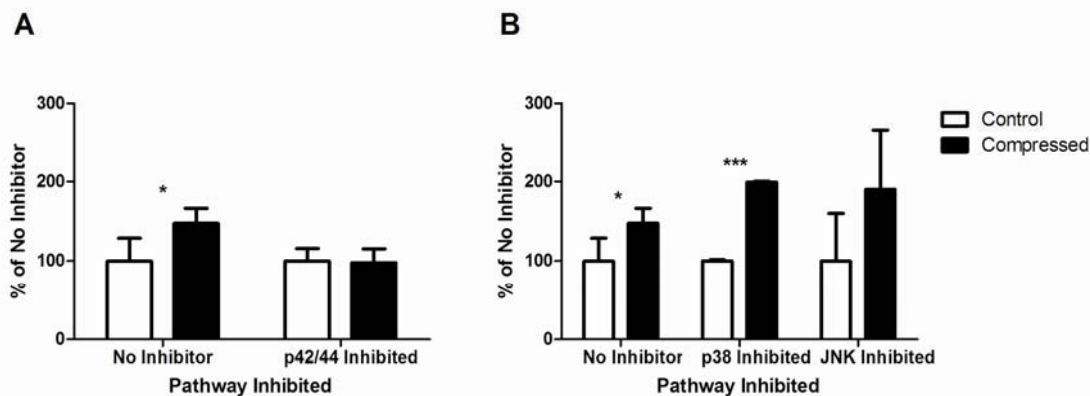
Figure 4.2 - Levels of p-p42/44 MAPK increase due to 4h of dynamic compression, except when the cells are pretreated with the selective p-42/44 MAPK phosphorylation inhibitor PD98059 (50µM) and is added to the media in the bioreactor during compressions. Data is expressed as a percent of the control (non-compressed) expression of p-p42/44 MAPK ± SD and the X-axis represents which MAPK cascade was inhibited. Protein levels were measured by ELISA and normalized based on β-actin expression. The data was analyzed by two way ANOVA with α=0.05, there was no significant effect on protein expression by the Pathway inhibited: $F_{(3,10)}=1.284$; $p=0.33$. There was also no significant effect by the interaction of the Pathway inhibited and Compression: $F_{(3,10)}=1.284$; $p=0.33$. However, there was a significant effect due to Compression: $F_{(1,10)}=8.661$; $p=0.01$. * indicates $p<0.05$; *** indicates $p<0.001$ as compared to its own control.

Expression of phosphorylated-p38 and phosphorylated-JNK proteins remain unchanged

The level of expression of p-p38 MAPK remains unchanged due to compression without the addition of an inhibitor. It also remains unchanged with the addition of the inhibitors for p38 MAPK and JNK phosphorylation. However, there is an increase in the expression of the p-p38 MAPK protein when the inhibitor for p42/44 MAPK phosphorylation is added. As these pathways are very closely linked, and this phenomena has been observed before in human cells (42), this result is not surprising and was more or less expected (Figure 4.3).
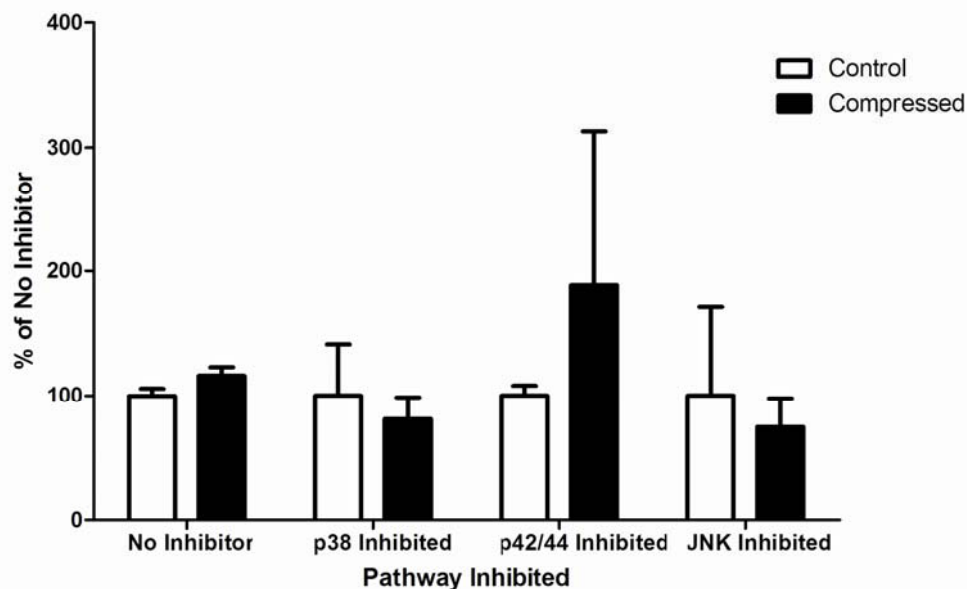
Figure 4.3 - Levels of p-p38 MAPK remain virtually unchanged due to 4h of dynamic compression. Data is expressed as a percent of the control (non-compressed) expression of p-p38 MAPK ± SD and the X-axis represents which MAPK cascade was inhibited. Protein levels were measured by ELISA and normalized based on β-actin expression. The data was analyzed by two way ANOVA with α=0.05, there was no significant effect on protein expression by the Pathway inhibited, the effect due to Compression, or the interaction of the Pathway inhibited and Compression: $F_{(3,10)}=1.36$, $p=0.31$; $F_{(1,10)}=2.316$, $p=0.16$; $F_{(3,10)}=1.36$, $p=0.31$; respectively.

Due to the high standard deviation in the p42/44 MAPK phosphorylation inhibited data, and virtually unchanged expression in the other treatment groups, there is no statistical significance to the data. Albeit, this is still a favorable result and is in agreement with our hypothesis.

Additionally, there was a complete lack of change of expression of p-JNK due to dynamic unconfined compression removing the need to test the effect of the inhibitors on its expression (Figure 4.4).

Figure 4.4 – JNK phosphorylation does not change due to 4h of dynamic compression.

Expression of early response genes increases with application of dynamic unconfined compression

The two early response genes c-fos (Figure 4.5A) and c-jun (Figure 4.5B) were analyzed by Real-time PCR in duplicate in PDLs subjected to mechanical force. Increases of expression of these response genes on the order of 4-fold ($p<0.005$ for c-fos) and 3-fold ($p<0.05$ for c-jun), were observed following the 4 hour compression process. The elevation of the early response genes were evaluated for potential induction of TGFβ-3 gene transcription.
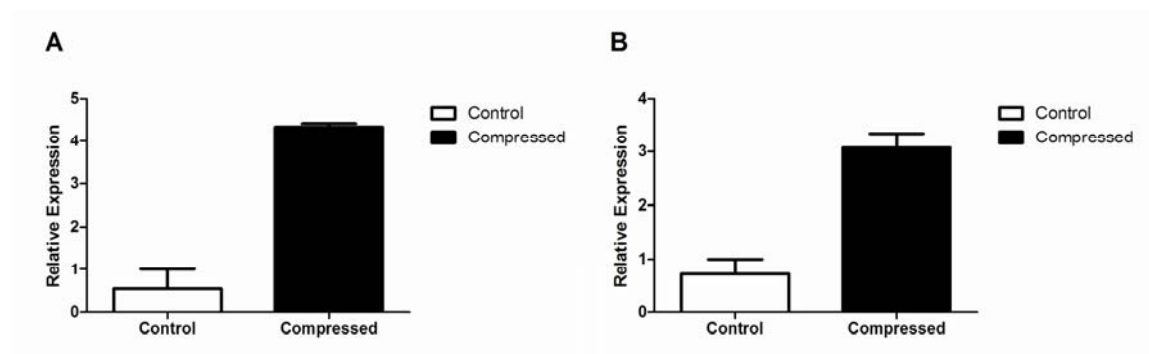

Figure 4.5 – Expression of early response genes as a result of mechanical stimulation. c-Fos (A) and c-Jun (B) message increase as a result of dynamic unconfined compression (*=$p<0.05$, **=$p<0.005$).

TGF-β3 expression is not increased by elevations in early response genes or compression

In quantitative real-time PCR expression of TGF-β3 mRNA remained unchanged, relative expression was 0.8 ± 0.2 in unstressed PDL cells in comparison to stressed stem cells 1.14 ± 0.01. The application of mechanical force demonstrated no functional impact on TGF-β3 transcription levels (Figure 4.6) despite the observed increases in c-fos and c-jun.
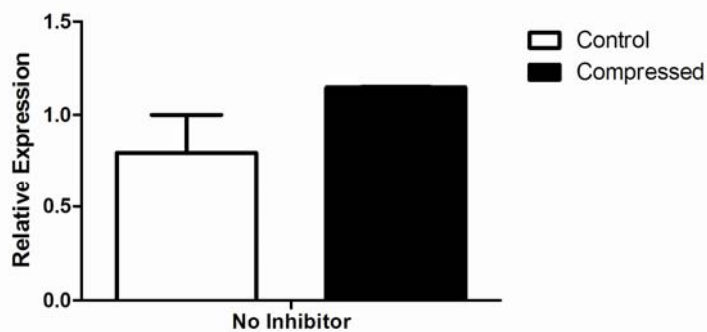

Figure 4.6 – Expression of TGF-β3 does not change due to 4h of dynamic compression

SOX-9 and aggrecan mRNA expression is elevated following dynamic unconfined compression

SOX-9, a gene that is elevated in cells undergoing the transition from stem cell to chondrocyte, increased by nearly 3-fold after the cells were subjected to compression (Figure 4.7A, p<0.05). This increase in SOX-9 gene expression remained constant in the compressed PDLs in fibrin gels after pre-treatment with the p38 MAPK and JNK activation inhibitors (Figure 4.7B). Conversely, the expression of SOX-9 is completely suppressed to non-compressed levels with the addition of the p42/44 MAPK pathway inhibitor (Figure 4.7A). Two-way ANOVA analysis of the SOX-9 results comparing the affect of compression

across the four inhibition conditions indicates there is a significant affect due to compression of the cells: $F_{(1,14)}=10.30$, $p=0.06$.
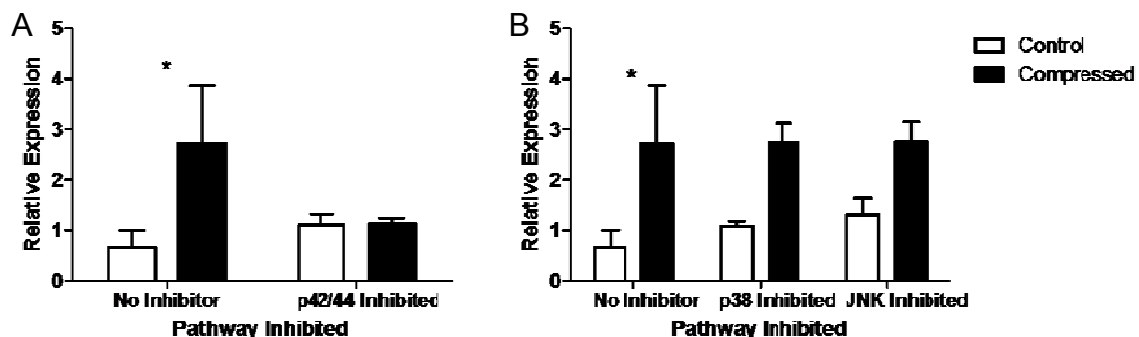
Figure 4.7 - Expression of the differentiation factor SOX-9 increases after 4h of compression, except when the cells are pretreated with the selective p42/44 MAPK phosphorylation inhibitor PD98059 (50µM). Data is expressed as relative expression of the control (non-compressed) ± SD and the X-axis represents which MAPK cascade was inhibited. Real-time PCR was used to quantify the amount of mRNA and normalized to GAPDH expression. The data was analyzed by two way ANOVA with α=0.05, there was no significant effect on protein expression by the Pathway inhibited: $F_{(3,14)}=1.06$; $p=0.40$. There was also no significant effect by the interaction of the Pathway inhibited and Compression: $F_{(3,14)}=1.259$; $p=0.33$. However, there was a significant effect due to Compression: $F_{(1,14)}=10.30$; $p=0.006$. * indicates $p<0.05$ as compared to its own control.

Similarly to SOX-9, the chondrogenic marker aggrecan increased under the conditions of dynamic compression (Figure 4.8). Increase of expression in this case was only 2-fold, but two-way ANOVA states a significance of the effect of compression ($F_{(1,10)}=6.059$, $p=0.034$). Maintenance of the increased expression levels of the aggrecan gene after compression was not affected by p38 MAPK ($p<0.05$ versus uncompressed cells) and JNK phosphorylation inhibitors. Complete suppression of the compression induced aggrecan gene expression elevation was observed in the presence of the p42/44 MAPK pathway inhibitor.
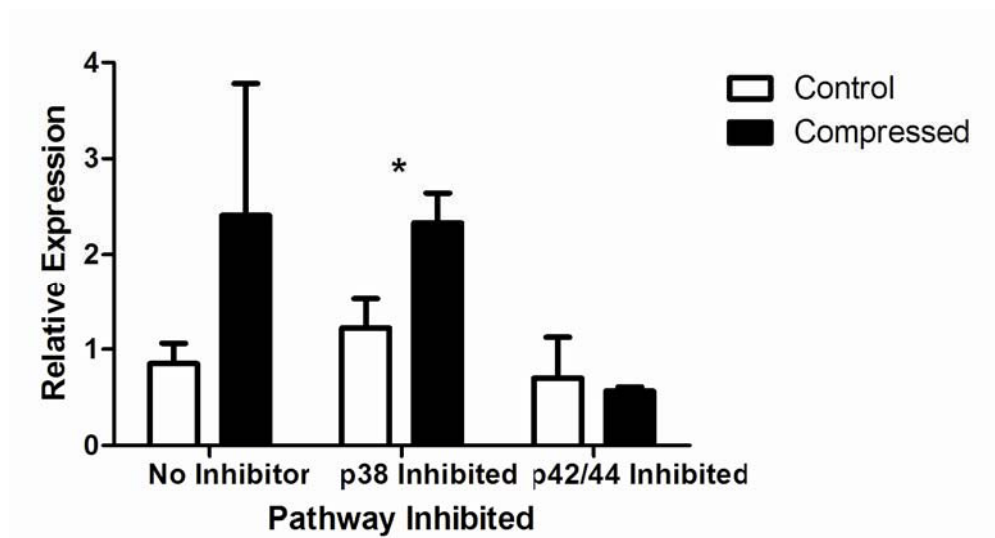
Figure 4.8 - Levels of aggrecan mRNA increase due to 4h of compression, except when the cells are pretreated with the selective p42/44 phosphorylation inhibitor PD98059 (50μM). Data is expressed as relative expression to the control (non-compressed) expression and the X-axis represents which MAPK cascade was inhibited. Messenger RNA levels were measured by Real-time PCR and normalized to GAPDH expression levels. The data was analyzed by two way ANOVA with α=0.05, there was significant effect on protein expression by the Pathway inhibited: $F_{(2,10)}=4.33$; $p=0.44$. There was also no significant effect by the interaction of the Pathway inhibited and Compression: $F_{(2,10)}=2.120$; $p=0.17$. However, there was a significant effect due to Compression: $F_{(1,10)}=6.059$; $p=0.034$. * indicates $p<0.05$ compared to its own control.

Chapter 5 – Discussion

In recent reports, there has been a call to researchers to attempt to understand the role the MAPK pathways play in the differentiation of stem cells into chondrogenic, adipogenic and osteogenic lineages. This study has shown these cells have the ability to differentiate into chondrocytes and the major MAPK pathway involved in the differentiation of these cells into chondrocytes. Secondly, it has also shown the PDLs behave much like hMSCs under the same conditions of pellet culture with TGF-β3 treatment as well as under the application of dynamic unconfined compression in the absence of exogenous growth factors in a fibrin 3D matrix.

In order to determine if the differentiation of the PDLs into chondrocytes was possible, a pellet culture was maintained for 14 days both with and without the addition of exogenous TGF-β3. This procedure was carried out in the same fashion as many others have done for hMSCs in the past with similar results such as the increase in aggrecan and type II collagen gene expression (24;43;44). Additionally, it has been demonstrated that hMSCs express aggrecan constitutively after 14 days in culture without any stimulation due to cell-cell contact interactions (43;45). The appearance of aggrecan in the unstimulated culture displays a similar response which hMSCs exhibit as well as what we have shown in PDLs. The complete absence of type II collagen gene expression in the unstimulated cultures supports the findings of Lee, et al. which noted expression of type II collagen does not appear until after 5 days of treatment with TGF-β3

and slowly increases until day 14, which was the termination point of their study as well.

In a study performed in our laboratory, rabbit MSCs (rMSCs) were encased in agarose gels and compressed in a custom built bioreactor and resulted in the expression of chondrogenic markers (35). Another study of ours exhibits evidence that the act of compressing the cells in the fibrin gels helped maintain the viability of the cells in the gels. The compression of the cells for 4h per day maintained the viability of the hMSCs in the fibrin gels for up to 72h in culture (26) which is a longer duration than this study. Compressions promoted proliferation of the cells while the number of viable cells in the uncompressed gels decreased by approximately 50%. These previous results support our belief the PDLs in the fibrin gels are viable and proliferating cells. Furthermore, it has also been independently verified that compressions in agarose gels in a similar bioreactor under very similar conditions maintains the viability of terminally differentiated chondrocytes (46), removing the possibility the mechanical force is detrimental to the differentiated PDLs.

The application of a biomimetic force onto stem cells in the effort to differentiate them into chondrocytes is not a novel idea. The application of force to these specific adult stem cells under these conditions is. Many groups, including our own, have successfully shown the presence of chondrogenic markers in hMSCs after the application of this type of compressive force (16-18;21;22;47). The main goal of this study is to elucidate the mechanism of the

interpretation of the mechanical force into a chemical signal in PDLs under dynamic compression.

The MAPKs have been implicated in the differentiation of hMSCs into chondrocytes (18-22). It has previously been mentioned (25) and then later reiterated (22) that the role of MAPKs in the differentiation of stem cells into chondrocytes is very poorly understood. As stated in Chapter 2, most studies involving mechanical stimulation of the cells only examine the effects of exogenous growth factors and/or mechanical force. Additionally, the individual roles of each of the MAPKs are currently under debate. This is largely in part to the many different conditions and cell types which are being utilized.

This brings to light the reasoning behind our methodology. By removing all exogenous growth factors and animal proteins, it is possible to examine the effects of compression alone on these cells and to focus on each of the three major MAPK pathways independently. This design was brought about to some extent by an experiment performed in our laboratory where rMSCs were compressed in the absence of inhibitors (18). The results of these experiments strongly pointed to the involvement of the MAPKs in mechanical differentiation of MSCs, but which one(s) were involved were unclear. It was therefore of interest to investigate their role in the differentiation process. We have shown mechanical stimulation alone increases the activation of the p42/44 MAPK protein (Figure 4.2), and has a very minor effect on p38 MAPK (Figure 4.3) or JNK phosphorylation (Figure 4.4).

The lack of a change in the amount of p-JNK protein expression during chondrogenesis of mesenchymal progenitor cells has been previously demonstrated in mice (48) and was later shown in human cells (21). Also, interruption of JNK signaling in genetically engineered mice show defects in the nervous and immune systems (49), strengthening the possibility that the JNK signaling cascade has little or no participation in chondrogenesis.

Although there is an increase in the expression of p-p38 MAPK when phosphorylation of p42/44 MAPK is inhibited, it appears to be more of a stress response from the cell and an attempt to compensate for the lack of p-p42/44 MAPK. A reaction of this type has been reported before in human cells (42), however, this response is irrelevant due to the lack of an increase in the expression of the chondrogenic markers as shown in Figures 4.7 & 4.8. This further enforces the conclusion p-p42/44 MAPK is the major component involved in the differentiation of these cells by mechanical stimulation.

Furthermore, the data collected shows the inhibition of the p38 MAPK and JNK pathways does not change the levels of p-p42/44 MAPK from compressions alone, enforcing the fact the p38 MAPK and JNK cascades are not involved in the differentiation process. In the presence of PD98059, the inhibitor of the activation of p42/44 MAPK, there is no increase in the expression of p-p42/44 MAPK after compression, proving the efficacy of the inhibitor and the concentration used (Figure 4.2). Additionally, the lack of expression of the chondrogenic genetic markers further substantiates this theory.

The chondrogenic markers utilized for this study were chosen for very important reasons. The first is SOX-9 and aggrecan are expressed after 4 hours of compression (26) and a second is TGF-β3 cannot activate aggrecan expression after only 4 hours of exposure. This biochemical process requires multiple days of treatment. Another motive for these choices is SOX-9 and aggrecan are expressed at the same time in chondroblasts and proliferating chondrocytes (25). The steady expression of these chondrogenic markers without inhibitors as related to inhibition of the p38 MAPK and JNK pathways is a fact which supports the theory the p42/44 MAPK pathway is the major pathway involved in the differentiation of the PDLs into chondrocytes.

TGF-β3 is a well known effecter for the chondrogenesis of hMSCs and has been shown to promote the expression of aggrecan and type II collagen in PDLs (Figure 4.1), however there are no changes in the expression of both TGF-β3 message (Figure 4.6) or TGF-β3 protein (data not shown). These data suggest TGF-β3 does not play a part in the chondrogenesis of PDLs due to mechanical stimulation in either an autocrine or paracrine capacity.

The upregulation of the c-fos message is linked to the increase of the p-42/44 MAPK protein expression (50) and was expected as a result of the increased expression of the p-p42/44 MAPK protein levels (Figure 4.2 and 4.5A). Increases in the levels of c-jun mRNA have been attributed to increases in the levels of JNK protein, however since there is no change in the expression of JNK (Figure 4.4) and c-jun is transcribed due to a variety of different cellular stimuli,

this elevation of c-jun message must be due to one of these other stimuli (Figure 4.5B).

In conclusion, the introduction of a new cell type for use in tissue engineering applications is not a simple objective. It is our hope this report will serve as both a beginning to understanding the translation of a mechanical stimulus to a biological response, and as an introduction of new adult stem cells which are not intended to be a replacement for other adult stem cells, but as a reasonable alternative which should be further explored.

Future experimentation will be required in order to fully understand the mechanisms which are occurring in PDLs during dynamic unconfined compression under serum free conditions. For instance, it is known the addition of TGF-β3 to these cells will cause them to differentiate along a chondrogenic lineage. However, it is not known if TGF-β3 plays a role in the differentiation of the cells during mechanical stimulation. One hypothesis is the cells may release TGF-β3 on their own early on in the compression session and returns to basal levels after 4h. To test this hypothesis, the addition of a competitive inhibitor or small interfering RNA (siRNA) to TGF-β3 or its receptors would be the best choice to observe this interaction. Along this same line, inhibition of other members of the TGF-β family would assist in the complete understanding of the mechanism involved in the differentiation process. This is necessary due to the increases in the early response genes, c-jun and c-fos, which form the AP-1 early response transcription factor necessary to activate transcription of other TGF-β family genes (51). Other experiments which would be helpful in understanding

the biochemical reactions occurring during compression would be to perform a time course analysis of the genes and proteins selected in this study after 15, 30, 60, and 120 min of compression. Finally, performing compressions in the presence of pairs of the inhibitors to the phosphorylation of the MAPKs and then all three together would certainly clarify which of the MAPK pathways is dominant in the chondrogenesis of PDLs under dynamic compression.

References

1. Athanasiou, K. A., Shah, A. R., Hernandez, R. J., and LeBaron, R. G. (2001) *Clin. Sports Med.* **20,** 223-247

2. Buckwalter, J. A. (2002) *Clin. Orthop. Relat Res.* 21-37

3. Dale Daniel M, Pedowitz Robert A, O'Connor John J, and Akeson Wayne H (2003) *Daniel's knee injuries : ligament and cartilage structure, function, injury, and repair*, Lippincott Williams & Wilkins, Philadelphia

4. Pearle, A. D., Warren, R. F., and Rodeo, S. A. (2005) *Clin. Sports Med.* **24,** 1-12

5. Ulrich-Vinther, M., Maloney, M. D., Schwarz, E. M., Rosier, R., and O'Keefe, R. J. (2003) *J. Am. Acad. Orthop. Surg.* **11,** 421-430

6. Hunziker, E. B. (2002) *Osteoarthritis. Cartilage.* **10,** 432-463

7. O'Connor, W. J., Botti, T., Khan, S. N., and Lane, J. M. (2000) *Orthop. Clin. North Am.* **31,** 399-410

8. Benya, P. D. and Shaffer, J. D. (1982) *Cell* **30,** 215-224

9. Saadeh, P. B., Brent, B., Mehrara, B. J., Steinbrech, D. S., Ting, V., Gittes, G. K., and Longaker, M. T. (1999) *Ann. Plast. Surg.* **42,** 509-513

10. Shapiro, F., Koide, S., and Glimcher, M. J. (1993) *J. Bone Joint Surg. Am.* **75,** 532-553

11. Cheung, H. S., Cottrell, W. H., Stephenson, K., and Nimni, M. E. (1978) *J. Bone Joint Surg. Am.* **60,** 1076-1081

12. Johnson, L. L. (1986) *Arthroscopy* **2,** 54-69

13. Fritz, J. R., Pelaez, D., and Cheung, H. S. (2009) *Current Rheumatology Reviews* **5,** 8-14

14. Ward, D. F., Jr., Salasznyk, R. M., Klees, R. F., Backiel, J., Agius, P., Bennett, K., Boskey, A., and Plopper, G. E. (2007) *Stem Cells Dev.* **16,** 467-480

15. Bahuleyan, B., Cheung, H. S., and Huang, C. Y. (2009) *Current Rheumatology Reviews* **5,** 34-39

16. Finger, A. R., Sargent, C. Y., Dulaney, K. O., Bernacki, S. H., and Loboa, E. G. (2007) *Tissue Eng* **13,** 1151-1158

17. Campbell, J. J., Lee, D. A., and Bader, D. L. (2006) *Biorheology* **43,** 455-470

18. Huang, C. Y., Reuben, P. M., and Cheung, H. S. (2005) *Stem Cells* **23,** 1113-1121

19. McMahon, L. A., Prendergast, P. J., and Campbell, V. A. (2008) *Biochem. Biophys. Res. Commun.* **368,** 990-995

20. Motomura, H., Niimi, H., Sugimori, K., Ohtsuka, T., Kimura, T., and Kitajima, I. (2007) *Biochem. Biophys. Res. Commun.* **357,** 997-1003

21. Tuli, R., Tuli, S., Nandi, S., Huang, X., Manner, P. A., Hozack, W. J., Danielson, K. G., Hall, D. J., and Tuan, R. S. (2003) *J. Biol. Chem.* **278,** 41227-41236

22. Bobick, B. E. and Kulyk, W. M. (2008) *Birth Defects Res. C. Embryo. Today* **84,** 131-154

23. Yanagisawa, M., Suzuki, N., Mitsui, N., Koyama, Y., Otsuka, K., and Shimizu, N. (2007) *Life Sci.* **81,** 405-412

24. Lee, J. W., Kim, Y. H., Kim, S. H., Han, S. H., and Hahn, S. B. (2004) *Yonsei Med. J.* **45 Suppl,** 41-47

25. Stanton, L. A., Underhill, T. M., and Beier, F. (2003) *Dev. Biol.* **263,** 165-175

26. Pelaez, D., Huang, C. Y., and Cheung, H. S. (2009) *Stem Cells Dev.* **18,** 93-102

27. Gay, I. C., Chen, S., and MacDougall, M. (2007) *Orthod. Craniofac. Res.* **10,** 149-160

28. Koyama, N., Okubo, Y., Nakao, K., and Bessho, K. (2009) *J. Oral Maxillofac. Surg.* **67,** 501-506

29. Ng, L. J., Wheatley, S., Muscat, G. E., Conway-Campbell, J., Bowles, J., Wright, E., Bell, D. M., Tam, P. P., Cheah, K. S., and Koopman, P. (1997) *Dev. Biol.* **183,** 108-121

30. Bi, W., Deng, J. M., Zhang, Z., Behringer, R. R., and de, C. B. (1999) *Nat. Genet.* **22,** 85-89

31. Murakami, S., Kan, M., McKeehan, W. L., and de, C. B. (2000) *Proc. Natl. Acad. Sci. U. S. A* **97,** 1113-1118

32. Lafyatis, R., Lechleider, R., Kim, S. J., Jakowlew, S., Roberts, A. B., and Sporn, M. B. (1990) *J. Biol. Chem.* **265,** 19128-19136

33. Hu, P. P., Harvat, B. L., Hook, S. S., Shen, X., Wang, X. F., and Means, A. R. (1999) *Mol. Endocrinol.* **13,** 2039-2048

34. Liu, G., Ding, W., Neiman, J., and Mulder, K. M. (2006) *J. Biol. Chem.* **281,** 29479-29490

35. Huang, C. Y., Hagar, K. L., Frost, L. E., Sun, Y., and Cheung, H. S. (2004) *Stem Cells* **22,** 313-323

36. Singhatanadgit, W., Donos, N., and Olsen, I. (2009) *Tissue Eng Part A*

37. Wei, X., Wu, L. P., Ling, J. Q., and Liu, L. (2008) *Zhonghua Kou Qiang. Yi. Xue. Za Zhi.* **43,** 495-499

38. Di, N. M., Carlo-Stella, C., Magni, M., Milanesi, M., Longoni, P. D., Matteucci, P., Grisanti, S., and Gianni, A. M. (2002) *Blood* **99,** 3838-3843

39. Le, B. K., Tammik, L., Sundberg, B., Haynesworth, S. E., and Ringden, O. (2003) *Scand. J. Immunol.* **57,** 11-20

40. Le, B. K., Tammik, C., Rosendahl, K., Zetterberg, E., and Ringden, O. (2003) *Exp. Hematol.* **31,** 890-896

41. Wada, N., Menicanin, D., Shi, S., Bartold, P. M., and Gronthos, S. (2009) *J. Cell Physiol* **219,** 667-676

42. Byun, H. J., Hong, I. K., Kim, E., Jin, Y. J., Jeoung, D. I., Hahn, J. H., Kim, Y. M., Park, S. H., and Lee, H. (2006) *J. Biol. Chem.* **281,** 34833-34847

43. Mwale, F., Stachura, D., Roughley, P., and Antoniou, J. (2006) *J. Orthop. Res.* **24,** 1791-1798

44. Schmitt, B., Ringe, J., Haupl, T., Notter, M., Manz, R., Burmester, G. R., Sittinger, M., and Kaps, C. (2003) *Differentiation* **71,** 567-577

45. Nishioka, K., Dennis, J. E., Gao, J., Goldberg, V. M., and Caplan, A. I. (2005) *J. Cell Physiol* **203,** 6-14

46. Kelly, T. A., Fisher, M. B., Oswald, E. S., Tai, T., Mauck, R. L., Ateshian, G. A., and Hung, C. T. (2008) *Ann. Biomed. Eng* **36,** 769-779

47. Park, S. H., Park, S. R., Chung, S. I., Pai, K. S., and Min, B. H. (2005) *Artif. Organs* **29,** 838-845

48. Nakamura, K., Shirai, T., Morishita, S., Uchida, S., Saeki-Miura, K., and Makishima, F. (1999) *Exp. Cell Res.* **250,** 351-363

49. Pearson, G., Robinson, F., Beers, G. T., Xu, B. E., Karandikar, M., Berman, K., and Cobb, M. H. (2001) *Endocr. Rev.* **22,** 153-183

50. Nair, D., Misra, R. P., Sallis, J. D., and Cheung, H. S. (1997) *J. Biol. Chem.* **272,** 18920-18925

51. Turner, R. and Tjian, R. (1989) *Science* **243,** 1689-1694

Appendix I – For the tissue engineering impaired

First things first – there is one major principle in cell biology. DNA → mRNA →
proteins. In other words library → messenger → actions. Proteins perform 95% of
all biological activity.

Dynamic unconfined compression – a real fancy way of saying that we allow the
gels with the cells in them to change shape freely when we push down on them
once every second for 4 hours.

DMEM and Media for the cells – When we grow the cells, they are on one of the
flat sides of a plastic bottle. The media contains all the stuff that cells need to live
including proteins and electrolytes. When we change the media, it's like we just
acted as the liver and kidneys for the cells. The T-75 is just a short way of saying
a flat bottle that has 75cm^2 of area to grow cells on.

Fibrin gels – it's just a man made blood clot. All we did was buy the two
components and mix them together with the cells in them. This mimics the 3D
environment of your body.

MAPKs – Mitogen-Activated Protein Kinases. This is a group of three proteins
that take stuff that happens outside the cell and transmits the information into the
cell and into the nucleus of the cell. The three of them are activated by different
things. Information goes into the nucleus to make new proteins to help the cell
adapt. Sometimes these adaptations cause the cell to change into another cell
type, especially when working with stem cells. The MAPKs are like I-95, the
Turnpike and I-75 in Florida. They are all in the same state, they all run in the

same direction and they do not overlap, and they start and end in different places, but they have a little bit of influence on each other.

Phosphorylated-p42/44 – this is the MAPK that I show is responsible for changing the stem cells into cartilage. When it is phosphorylated, it's active. Until then, it's just something else in the soup floating around. This is not unusual, proteins are made inactive a lot because it takes much longer to make a new protein then to just change the shape of one that is already there very slightly.

Growth factors – very powerful proteins which can cause big changes in the cell. When we say exogenous it means it's not from the same species and at a level that is above the natural one.

SOX-9 and aggrecan – these are genes that are turned on when we compress the cells. SOX-9 is required for the change into cartilage and aggrecan shows that the cells really are cartilage. This is how it works in most all projects of this type, genes tell us what type of cells they are or if the cells are reacting to what we are doing to them.

"An alleged scientific discovery has no merit unless it can be explained to a barmaid" – Ernest Rutherford