ABSTRACT

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Agusta Thuridur Vigfusdottir, M.S., 2007

Directed By: Dr. Adam Hsieh, Assistant Professor
Fischell Department of Bioengineering

Intervertebral disc degeneration has been identified as a main cause for low back pain, a large health problem in the Western world today with 60%-80% of people affected at some point during their lives. Current treatments alleviate the symptoms rather than repair the tissue, but tissue engineering the intervertebral discs to stem degeneration or to repair damaged tissues may be a realistic alternative.

Adult bone marrow derived mesenchymal stem cells are undifferentiated, multipotent cells that have the ability to differentiate into the chondrocyte-like cells found within the nucleus pulposus of the adult intervertebral disc. This study examines one determinant of the cellular mechanotransduction pathway, an important aspect of cell-based therapies. Specifically, we investigate the role of cytoskeletal proteins on resisting mesenchymal stem cell deformation. An improved understanding of the relative contributions of microfilaments and microtubules to cell deformation characteristics will aid in the interpretation of cellular mechanotransduction mechanisms in future studies.

EFFECTS OF CYTOSKELETAL PROTEIN DISRUPTION ON THE DEFORMATION OF MESENCHYMAL STEM CELLS DURING CHONDROGENESIS

by

Agusta Thuridur Vigfusdottir

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Advisory Committee:
Professor Adam Hsieh, Chair
Professor William E. Bentley
Professor Sameer Shah

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Chapter 1: Introduction

Intervertebral disc (IVD) degeneration is one of the main causes of low back pain. Low back pain is one of the most common causes of disability, with 60% to 80% [1] of people affected at some point during their lives. Current treatments are non-operative or surgical. Non-operative only addresses the symptoms but do not prevent degradation and do not restore the tissue. Surgical treatments for IVD degeneration are disc excision or disc immobilization [1][6], procedures that do not repair the tissue. One approach aiming to slow down degeneration or to repair degenerated discs is tissue engineering of the IVDs [1]. Adult bone marrow derived mesenchymal stem cells are undifferentiated, multipotent cells that have the ability to differentiate into a number of cell types, including the chondrocyte-like cells found within the nucleus pulposus of the intervertebral disc [2] .

Cell biomechanics approaches provide tools for the characterization of important cell and tissue behaviors. In particular, understanding of mechanotransduction or the biological response of cells to their biomechanical environment, would foster further understanding of how cellular responses correlate to tissue level characteristics. The study of mechanotransduction has many implications in biology, engineering and medical science, because it can lead to the elucidation of disease etiologies, and the formulation of repair and tissue engineering strategies. The first step to understanding the phenomenon of mechanotransduction involves characterization of the mechanical environment in and directly around single cells [3]. Intervertebral disc cells are a good example where the

determination of the material properties of single cells is crucial to understanding their in vivo biomechanical environment.

Chapter 2: Background

2.1 Intervertebral disc's function and degeneration

The intervertebral discs (IVDs) lie between the vertebral bodies, linking them together. Their primary function is to confer flexibility to the spine under intrinsic loads, due to gravity and muscular forces [4]. The IVD is comprised of a central gelatinous nucleus pulposus (NP) surrounded by a more highly organized ring of predominantly type I collagen fibrils, the annulus fibrosus (AF). Within the NP, rounded chondrocyte-like cells are embedded in a disorganized matrix of a mainly type II collagen and aggrecan [2], supplemented with other collagens and other proteoglycans, including versican [5]. Aggrecan is highly hydrophilic molecule. The function of aggrecan in the NP is such that by imbibing water it generates sufficient swelling pressure to force loaded vertebrae apart, a force resisted by the AF [2]. Spinal stability relies, in part, on the balance of these two opposing forces. The mechanical properties of the IVD are highly dependent on the water content of the disc [4]. The NP, acts as an incompressible pillow of water, absorbs and distributes load. In the normal IVD the NP exerts a hydrostatic pressure against the constraining AF, which allows the disc to maintain flexibility between adjacent vertebrae, while absorbing compressive forces. The NP performs this role because of its hydrophilic gel-like structure [5]. During degenerative disc disease, the NP is characterized by enhanced enzymatic breakdown and decreased production of extracellular matrix components, in particular proteoglycans, leading to reduced swelling pressures in the NP and results in a more fibrillar tissue [6]. Consequently, there is a loss of the water content of the NP, increased instability, and can lead to traumatic damage to the disc and surrounding structures. Even though there have been recent developments in aggressive therapies to treat IVD degeneration, such as physical therapy, nerve root

blocks, disc excision and arthordesis[7], these treatments alleviate the symptoms rather then repair the tissue. Naturally, the ideal solution would be to repair the degenerated tissue using autologous IVD cell implantation. However, high cell numbers are desired [1] in cell therapy and tissue engineering approaches but as with articular cartilage, the IVD is relatively acellular and healthy IVD cells cannot be harvested without compromising other discs, making the source of cells one of the main problems for any tissue repair strategy. One approach that has recently been explored is transplanting mesenchymal stem cells (MSCs) into the intervertebral space of a degenerated disc. Such a cell-based approach is theoretically attractive, as MSCs are an ideal cell source with high proliferative capacity with the ability to follow one of several mesenchymal cell lineages, including chondrogenic differentiation [2]. While studies have already been undertaken to demonstrate feasibility of this technique, there remain unanswered questions as to how MSCs would respond to the harsh mechanical and biological environment to which the intervertebral disc is subjected.

2.1.1. Potential uses of chondrocyte-like cells in disc tissue engineering

The IVD has a limited vascularity and capacity for self-repair. Transplantation of autologous intervertebral disc cells as well as mesenchymal stem cells opens up an alternative therapeutical approach for degenerative intervertebral disc repair.

It has been shown that transplanting autologous nucleus pulposus tissue into denucleated rat discs slowed down the progression of degeneration [5]. Obtaining these cells has practical limitations in the clinical setting because of graft procurement and harvest site morbidity. Harvesting the patient's own cells can damage adjacent discs which is likely

to induce further degeneration. Also, as the IVD is relatively acellular it would require culture expansion which is a slow process for disc cells [2]. MSCs are therefore superior for tissue engineering the IVD because high cell numbers can be produced in a reproducible manner [8].

2.2 Mechanical deformation

The application of compressive load to hydrated soft tissues induces cell deformation, changes in hydrostatic or fluid pressure and deformation of the charged extracellular matrix, with associated localized alterations in osmolality and pH. These initial signals may activate specific intracellular mechanotransduction pathways [23]. Cell deformation is typically associated with changes in cell volume and/or surface area.

The cytoskeletal organization is known to be critical for normal cell metabolism and may therefore be involved in mechanotransduction.

It is now believed that the application of mechanical stimuli to cells in vitro, which emulate the forces applied to the tissue, will lead to the production of functional tissues. Mechanical stresses are an important factor of cellular function in musculoskeletal tissues as they stimulate them to increase the synthesis of ECM components. Similar to observations made in intervertebral discs, in vivo studies have reported that reduced synthesis of proteoglycans results in the degeneration of cartilage and ultimately loss of joint function. This is due to a reduction in loading of the articular cartilage illustrating the need for mechanical forces to modulate the biomechanical and the biochemical properties of the tissue. Many studies have shown that mechanical forces can be used to

stimulate the synthesis of cartilaginous and fibrocartilaginous ECM, and may even enhance the mechanical properties of the developing tissue.

2.3 Mesenchymal stem cells

Adult human stem cells have been isolated from a wide variety of tissues and, in general, their differentiation potential may reflect the local environment. They lack tissue-specific characteristics but under the influence of appropriate signals they can differentiate into specialized cells with a phenotype distinct from that of the precursor.

Mesenchymal stem cells (MSCs) can be isolated from the adult bone marrow and expanded with high efficiency while maintaining their multipotence. MSCs have the capacity to differentiate along osteogenic, chondrogenic or adipogenic lineages [39] under defined culture conditions. Previous studies have shown that MSCs can differentiate into chondrocyte-like cells if given the proper environment. One approach aiming to repair degenerated IVDs is implanting chondrogenic MSCs to the IVD. Because of the limitations of obtaining NP cells, tissue engineering based on them is therefore unlikely to prove successful and instead studies have focused on the use of stem cells for tissue engineering cartilage

2.3.1 Chondrogenic differentiation of MSCs

Chondrogenic differentiation occurs when MSCs are grown under conditions that include a three-dimensional culture format, a serum-free defined nutrient medium and the addition of a member of the TGF-β super family. When these conditions are met the

cells rapidly lose their fibroblastic morphology and begin to initiate expression of a number of cartilage-specific extracellular matrix components [11].

Chondrogenic differentiation of MSCs taken from adult bone marrow results in rapid biosynthesis of glycosaminoglycan and deposition of an integrated extracellular matrix and is accompanied by a dramatic alteration in cell morphology. Three isoforms of TGF-ß have the ability to induce this response, and under the conditions of culture described the initial appearance of mRNA coding for cartilage matrix components becomes evident within 24 hours. Both TGF-ß2 and TGF-ß3 are more effective than TGF-ß1 in promoting chondrogenesis, causing a twofold greater accumulation of glycosaminoglycan and earlier and more extensive deposition of type II collagen [9].

Chondrogenic differentiation is detected by examining the presence of glycosaminoglycan and newly synthesized collagen type II in the extracellular matrix. Articular cartilage is an avascular tissue composed of chondrocytes and ECM, which predominantly consists of collagen type II, aggrecan and hyaluronic acid. In addition to these macromolecules, collagen types VI, IX, XI, decorin, biglycan, COMP and fibromodulin are also present in minor concentrations.

Chondrogenesis is initiated as densely packed precursor cells form appropriate cell-cell contacts. These events are followed by expression of cartilage-specific extracellular matrix molecules [10]. Studies have shown that differentiating MSCs show rapid upregulation of expression of aggrecan, fibromodulin, decorin, and cartilage oligomeric matrix protein (COMP) mRNA and protein. Fibromodulin and decorin messages increased rapidly, whereas aggrecan and COMP show a more gradual increase. Versican, fibromodulin and decorin form the earliest extracellular matrix components during

chondrogenesis with aggrecan and biglycan being incorporated at a later time. The undifferentiated MSC is characterized by the expression of a wide variety of adhesion molecules and growth factor receptors as well as versican, decorin and types I and X collagen mRNA. In early stages of chondrogenic differentiation (the first 6 days) [9], cells express fibromodulin mRNA, representing the first matrix component to be upregulated, followed by the appearance of COMP mRNA. Aggrecan is also expressed in early stage and glycosaminoglycan accumulation initiates. Approximately 7 days after initiation of differentiation, the expression of type II collagen and chondroadherin message is observed and that gradually increases until day 21 [9]. Glycosaminoglycan accumulation occurs during that time period [9]. Collagen type II is one of the major components of the cartilage and plays a key role in maintaining chondrocyte function. In a study where MSCs were cultured in pellets, collagen type II was detected in the central region of the cell pellet after 14-21 days [11]. Aggrecan and Collagen type II were not detected in monolayer or in pellets lacking TGF-ß. This indicates that a three-dimensional culture format is needed and cell-cell and cell-matrix interactions are important factors in chondrogenesis. Pellet culture may promote chondrogenesis because cells assume a compact shape, rather than spreading on a growth surface.

2.4 Pericellular matrix

Cartilage is a connective tissue which is composed of chondrocyte cells. Within the extracellular matrix, chondrocytes are surrounded by a narrow region of tissue termed the pericellular matrix (PCM), which together with the cell has been termed the chondron [12]. The PCM is a narrow region of matrix, immediately surrounding nearly all

chondrocytes in adult cartilage, and is generally characterized as having a higher concentration of proteoglycans and a fine arrangement of collagen fibers as compared to the ECM as well as type VI collagen [37]. Like cartilage tissue, the fibrocartilaginous intervertebral disc contains cells that possess a PCM [38]. The mechanical properties of the pericellular matrix are critical in determining the forces transmitted to cells, both in situ with intact tissues and within cell-agarose constructs. Although the gross mechanical properties of extracellular matrix have been extensively studied [13][14], little is known of the stiffness of the pericellular matrix. How the presence of a limited amount of pericellular matrix might influence cell deformation is not fully understood [15]. In addition, the proteins that make up the PCM directly interact with integrins on the cell surface and, therefore, determine the precise signaling processes involved in mechanotransduction [16]. Thus, the PCM is an important determinant of both cell deformation and signaling mechanisms for cells subjected to mechanical stress.

2.5 Cytoskeleton

The cytoskeleton is of major importance in controlling cell shape and the movement of cells and organelles. The varied functions of the cytoskeleton center on the behavior of three families of protein molecules, which assemble to form three main types of filaments. Each type of filament has distinct mechanical properties and dynamics, but certain fundamental principles are common to them all. The three types of filaments are microfilaments which determine the shape of the cell's surface and are necessary for whole-cell locomotion, microtubules which determine the positions of membrane-enclosed organelles and direct intracellular transport and intermediate filaments which

provide mechanical strength and resistance to shear stress. Chondrocytes cannot move because they are locked within a stiff extracellular matrix (ECM). They can be passively deformed during compressive loading but they do not change their shape except during hypertrophy. The cytoskeleton of chondrocytes is especially important in chondrogenesis and maintaining the chondrogenic phenotype, cell division and intracellular trafficking, and the response of cells to external forces [17][18].

The cytoskeleton is also believed to contribute to the biomechanical properties of the chondrocyte, which also influence the interactions between the cell and its pericellular and extracellular matrices [19].

The strong association of the cell cytoskeleton with the plasma membrane in many cell types suggests that mechanical functions of the membrane may depend on cytoskeleton organization.

Compressive loading results in cell deformation, hydrostatic pressure gradients, fluid flow, and deformation of the charged extracellular matrix with associated changes in osmolarity and pH. These extracellular events may influence cell metabolism through the activation of specific intracellular events including nucleus deformation, modulation of the cytoskeleton [26].

2.5.1 Actin

Actin filaments are two-stranded helical polymers of the protein actin. They appear as flexible structures, with a diameter of 5-9 nm, and they are organized into a variety of linear bundles, two-dimensional networks, and three-dimensional gels. Although actin

filaments are dispersed throughout the cell, they are most highly concentrated in the cortex, just beneath the plasma membrane [18].

2.5.2 Microtubules

Microtubules are long, hollow cylinders made of the proten tubulin. With an outer diameter of 25nm, they are much more rigid than actin filaments. Microtubules are long and straight and typically have one end attached to a single microtubule-organizing center called a centrosome [18].

2.5.3 Disruptive agents

2.5.3.1 Cytochalasin D

Drugs that interfere with actin polymerization are frequently used for probing the role of the actin cytoskeleton in various biological events. Cytochalasin D has been extensively used for assessing the role of the actin cytoskeleton in different biological processes [20]. Cytochalasin D inhibits actin subunit addition to the fast-growing or "barbed" end of actin filaments. This inhibitory action apparently involves binding to high affinity binding sites or F-actin [21].

2.5.3.2 Colchicine

The assembly of microtubules can be altered using the reagent colchicine to inhibit tubulin polymerization. Colchicine binds to the β subunit of the tubulin heterodimer, forming a tubulin-colchicine complex and inhibiting tubulin polymerization into

microtubules. Because of this inhibitory effect, cells cultured in the presence of colchicine are prevented from forming mitotic spindles and entering mitosis [22].

Chapter 3: MSC deformation

3.1 Introduction

The objective of this study was to determine the role of cytoskeletal proteins on resisting mesenchymal stem cell deformation during chondrogenesis.

Lower back pain is one of the largest health problems in the western world today, affecting 60% to 80 percent [1] of people at some point during their lives. Intervertebral disc degeneration has been identified as a main cause. Current treatments are symptomatic but cell-based tissue engineering methods are realistic alternatives for tissue regeneration [2]. The most striking alterations in the properties of the disc with degeneration occur in the nucleus pulposus, with an eightfold increase in shear modulus and substantial loss of swelling pressure [24]. Generating a suitable population of NP cells in order to tissue engineer the IVD has limitations in the clinical setting because of graft procurement and harvest site morbidity [2]. MSCs are therefore a suitable option because of their multipotency with ability to differentiate into number of cell types, including the chondrocyte-like cells found within the NP of the IVD.

3.2 Materials and Methods

3.2.1 Cell culture

Human mesenchymal stem cells (hMSC's) were obtained (Poietics hMSCs, Cambrex Corp, NJ) and cultured in 75 cm^2 cell culture flasks according to manufacturer's suggested protocol using growth media. Growth media consisted of mesenchymal stem cell basal medium (MSCBM) containing 10% mesenchymal cell growth supplement (MCGS), L-glutamine (2%), and penicillin/streptomycin (0.1%) (Cambrex Corp., NJ).

Once cells reached ~75% confluency, they were removed from the flask with 0.25% Trypsin-EDTA solution and suspended in 2% (w/v) alginate solution at a concentration of $1x10^6$ cells/mL. The alginate-cell suspension were introduced dropwise into a 102 µM $CaCl_2$ solution using a 22 gauge needle, and were allowed to cure for 10 minutes. We estimate that there were approximately 5,000 cells per bead. Cells were cultured in alginate beads under two different media conditions. Half of the beads continued their culture in growth media. The other half was cultured in chondrogenic differentiation medium, which consisted of chondrogenic basal medium supplemented with dexamethasone, ascorbate, ITS+supplement, penicillin/streptomycin, sodium pyruvate, proline and L-glutamine (Cambrex Corp., NJ). Transforming growth factor beta 3 (TGF-ß3; Cambrex Corp., NJ) was added to the chondrogenic differentiation medium immediately before use at every media change. Beads were cultured in 12-well plates up to two weeks with 2 timepoints, 1 week and 2 weeks. All media changes were performed three times per week.

3.2.2 Cytoskeletal disruption and specimen preparation

At every timepoint 4 beads from each media condition were treated with either 20µM [25] colchicine or 98.5 µM cytochalasin D for 3 hours in the incubator at 37°C, 5%CO_2 and 20% O_2. Following the treatment the beads were dissolved in 100mM sodium citrate+30mM EDTA containing the same concentration of respective disrupting agent. The solution was centrifuged at 650xg for 6 minutes, afterwhich the supernatant was removed. Once released, cells were stained with cell membrane stain CM DiI (Molecular Probes/Invitrogen, Carlsbad, CA), intracellular stain Cell Tracker Green CMFDA

(Molecular Probes/Invitrogen, Carlsbad, CA). The same concentration of the respective agent was kept throughout the staining process. Stained cells were suspended in a 4% [25] (w/v) agarose-PBS solution containing the appropriate concentrations of the disrupting agent and allowed to polymerize for 10 minutes in a 6mm x 6mm x 10mm mold. For control, 4 beads from each media condition were released from alginate gels, fluorescently stained, and re-embedded in 4% agarose in an identical manner, but without cytoskeletal disrupting agents. For each culture condition experiments were repeated three times.

3.2.3 Cell deformation, imaging, and measurement

Gels were made one at a time and placed in a custom-designed chamber (Fig 3.1 and 3.2) that was mounted on top of an Olympus IX81-based Nipkow disk confocal microscope (Olympus America, Inc., Center Valley, PA) (see Fig. 3.3) along with PBS to keep the gel hydrated. An identical initial low tare strain was applied to every gel to keep the gel in place. This strain was considered negligible in comparison with the magnitude of the applied load. The cells in the agarose gel were viewed perpendicular to the plane of loading (see Fig. 3.1). Widefield differential interference contrast (DIC) and fluorescent confocal image stacks under both 100x and 400x magnification were taken. The images consisted of a series of 25-40 serial confocal sections at intervals of 0.5µm. All gels were then compressed 0.6mm (10%) by manual turning of the plunger and the cells manually tracked. The same sets of images were captured, followed by 1.2mm (20%) compression, cell tracking, and image capture. Samples were tested at room temperature.

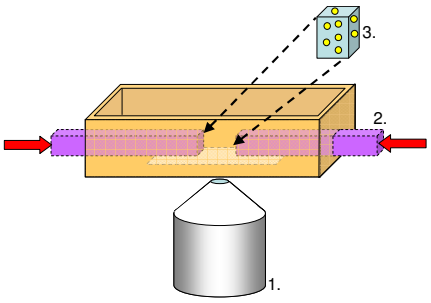

Fig.3.1 Schematic diagram of testing chamber. 1.Microscope objective 2. Loading plunger 3.Cell-agarose construct

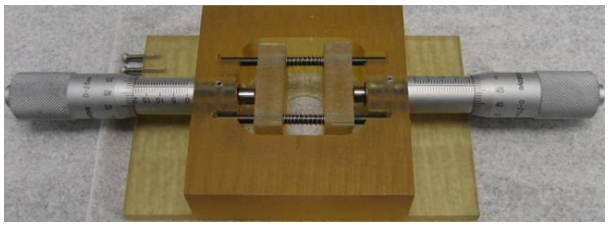

Fig.3.2 Actual picture of chamber

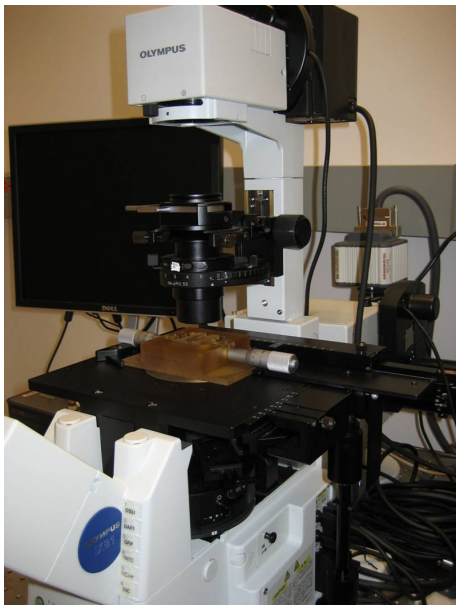

Fig.3.3 Testing chamber mounted on top of a confocal microscope

The cell diameters parallel and perpendicular to load directions were measured by using ImageJ [27]. To define the cell boundary the confocal images were changed from RGB color image to 8-bit image and subjected to computer thresholding. The diameters

parallel (X) and perpendicular (Y) to direction of compression were measured (see Fig. 3.4). Cell diameter strains, ε_X and ε_Y, were calculated using the following equations.

$$\varepsilon_X = \frac{X_S - X_u}{X_u} \times 100\% \quad \text{(Equation 3.1)}$$

$$\varepsilon_Y = \frac{Y_S - Y_u}{Y_u} \times 100\% \quad \text{(Equation 3.2)}$$

Cell diameter ratio = $\frac{X}{Y}$ was used to describe the overall shape or roundness of the cell with 1.0 (100%) being a perfect circle.

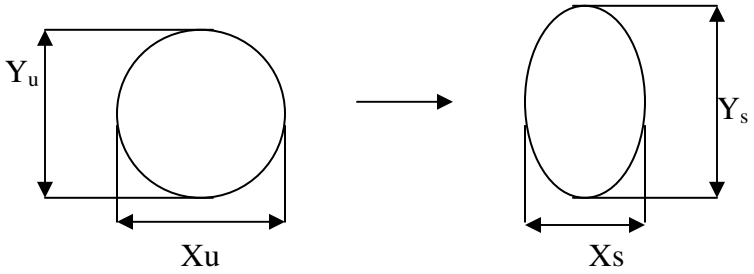

Fig.3.4 A schematic of how cell diameters are measured. Xu and Yu measure unstrained diamters. Xs and Ys measure strained diameters.

3.2.4 Cytoskeletal visualization

The structure of microfilaments and microtubules were visualized to assess the effects of cytochalasin D and colchicine treatments, respectively, compared to control. One bead from each treatment was retrieved at each timepoint and fixed with one overnight incubation of 4% paraformaldehyde solution. The bead was then subjected to a second overnight incubation in a 30% sucrose solution, and embedded in OCT. Sections 16µm thick were cut using an HM550 series cryostat (Richard Allen Scientific, Kalamazoo, MI) and placed onto slides coated with aminoalkylsilane.

To visualize microtubules, sections were incubated for 5 minutes with 0.1% Triton X-100, washed with PBS and incubated for 30 minutes with primary antibody against

tubulin-ß (Lab Vision, Fremont, CA). Following a PBS wash, sections were first incubated for 30 minutes with biotinylated IgG secondary antibody (Vector Laboratories, Burlingame, CA), washed again, and incubated for another 30 minutes with Texas Red conjugated streptavidin (Vector Laboratories, Burlingame, CA). Washed slides were mounted with Prolong Gold antifade reagent with DAPI (Molecular Probes/Invitrogen, Carlsbad, CA).

To visualize actin, sections were incubated with 0.1% Triton X-100 for 5 minutes, incubated with 1% Bovine serum albumin for 20 minutes and incubated with Alexa Fluor 555 phalloidin for 20 minutes. The sections were washed twice with PBS and mounted with Prolong Gold antifade reagent with DAPI (Molecular Probes/Invitrogen, Carlsbad, CA).

All incubations were performed at room temperature, and sections were immediately visualized using confocal microscopy (Olympus America, Inc., Center Valley, PA) under 600x magnification.

3.3 Results

3.3.1 Cell morphology

By looking at the cell diameter ratio of X (parallel to axis of compression) to Y (perpendicular to axis of compression), X/Y, it was apparent that after deformation the cells went from having spherical to ellipsoidal morphology (see Fig. 3.5 and Fig.3.6). A value of 100% for the diameter ratio indicates a perfectly round cell. We assumed that deformations in the Z-direction was equal to those in the Y-direction.

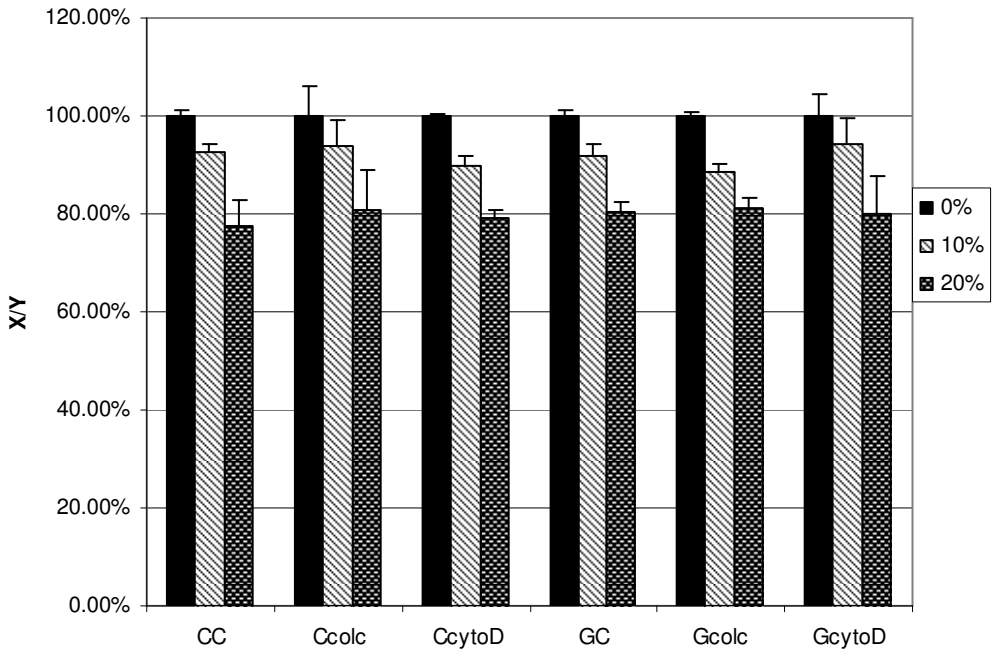

Fig.3.5 Diameter ratio X/Y for all culture conditions after 1 week in culture. Each value represents the mean and standard error for n=3. The graph shows normalized ratio.

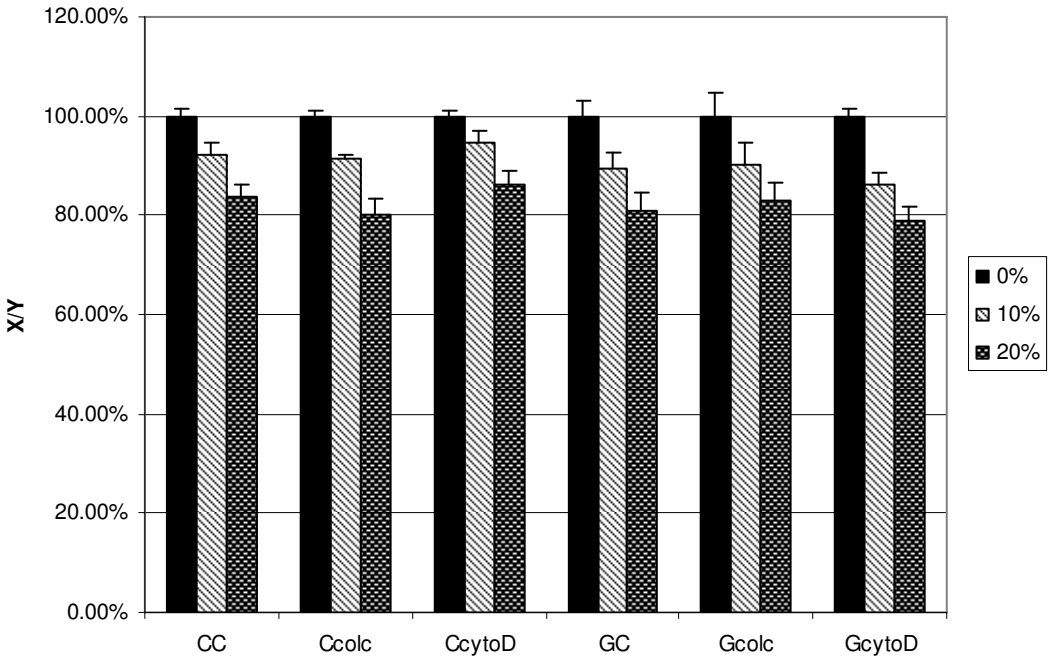

Fig. 3.6 Diameter ratio X/Y for all culture conditions after 2 weeks in culture. Each value represents the mean and standard error for n=3. The graph shows normalized ratio.

3.3.2 Cell deformation

Cells were imaged at 0%, 10% and 20% strain (see Fig.3.7). 20% compression resulted in cell deformation from a spherical to an ellipsoid morphology. The cell deformation was characterized by reduction in X diameter and corresponding elongation in Y diameter of the cell (see Fig.3.7 (g)).

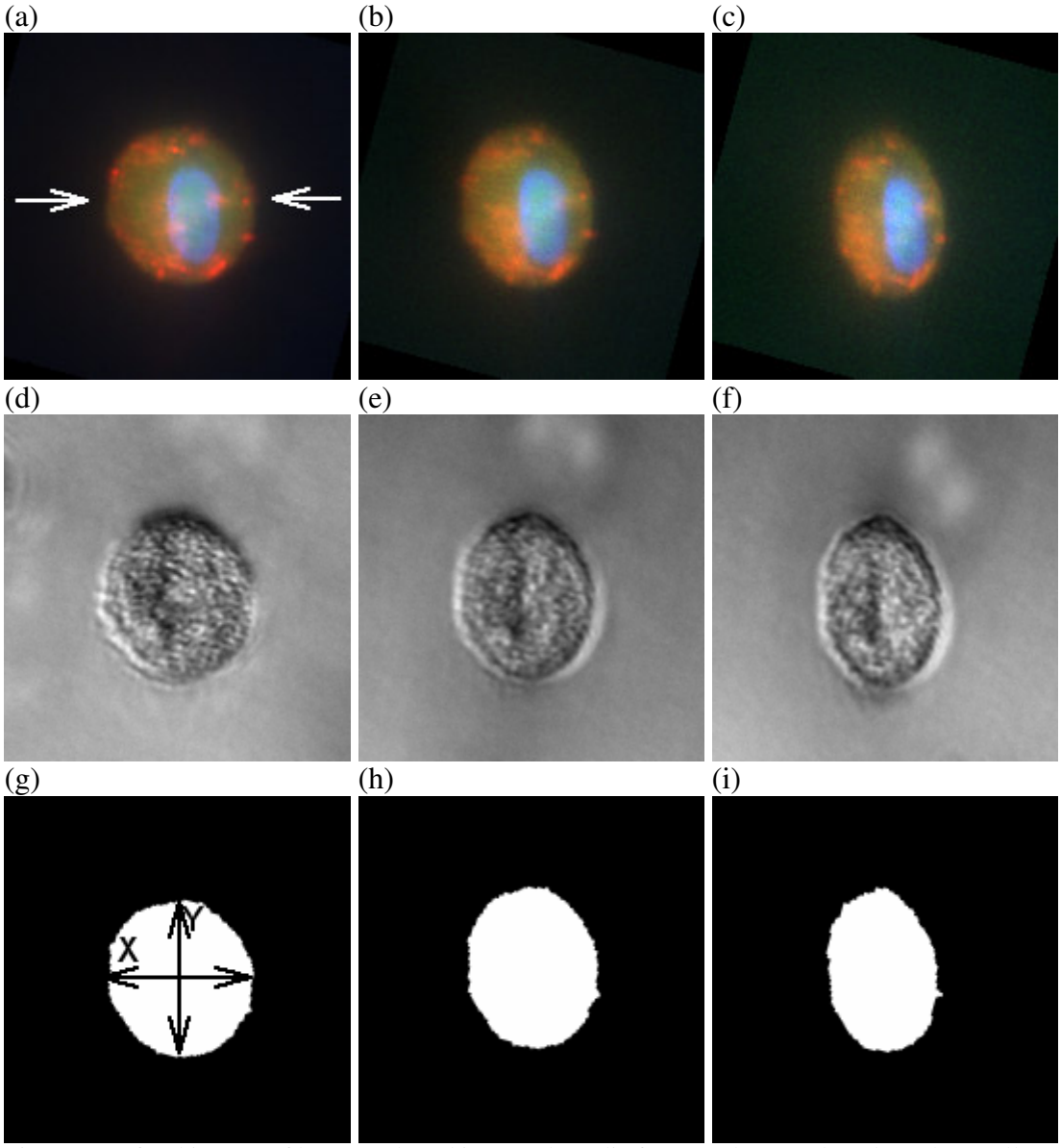

Fig. 3.7 Confocal images of hMSC cultured in growth media for 1 week. (a), (d) and (g) show cell at 0% strain, (b),(e) and (h) at 10% strain and (c),(f) and (i) at 20% strain. (a)-(c):Fluorescent image of cells; (d)-(f); DIC images; (g)-(i): Intensity threshold has been applied to each image to clarify the cell boundary to allow measurement of cell diameters X and Y (g). (a) shows direction of compression.

3.3.2.1 Deformation of Control cells

Deformation of all control cells (i.e. cells with intact cytoskeleton cultured in chondrogenic media (CC) and growth media (GC) at 1 and 2 weeks, and time zero control cells) were compared. At 10% strain there is no obvious difference between

samples. Samples with 20% strain applied exhibit differences between weeks.(Fig 3.8 and 3.9) The most obvious difference at 20% strain is between deformations of time zero control cells and deformations of cells at other timepoints. Results are shown in Table 3.1. Figs.3.8 and 3.9 reveal a slight difference between timepoints at week 1 and week 2 for both CC and GC. Importantly, at 20% strain time zero control cells exhibited decreases in X diameter (20.0 ± 2.5 %) that approached the magnitude of applied strain, suggesting that the decreases in cell deformation in other conditions are representative of cellular changes during

culture.

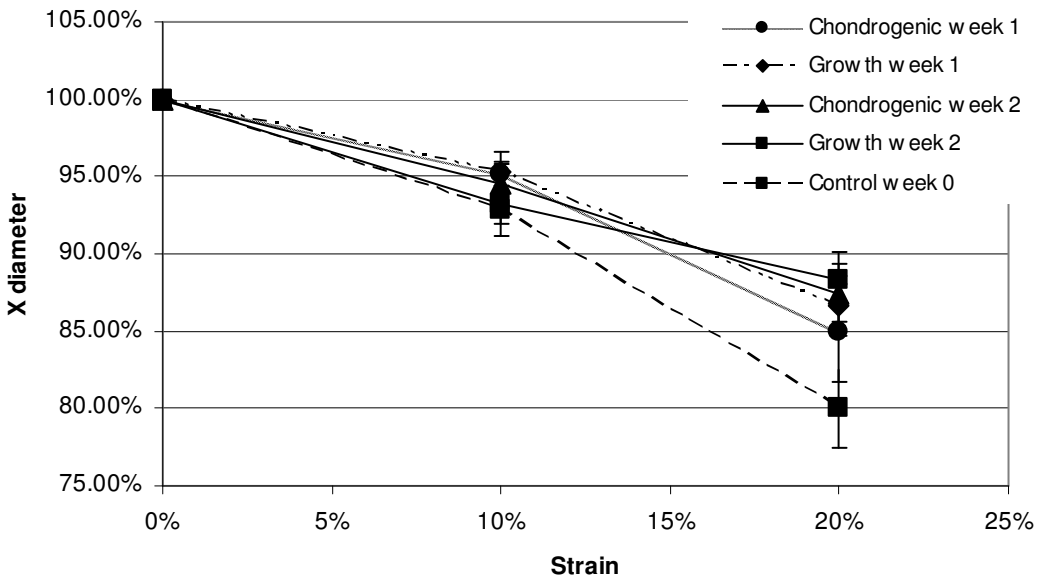

Fig. 3.8 Reduction in X diameter for control cells at week 0,1 and 2. Results are shown as mean ± S.E.M. for n=6

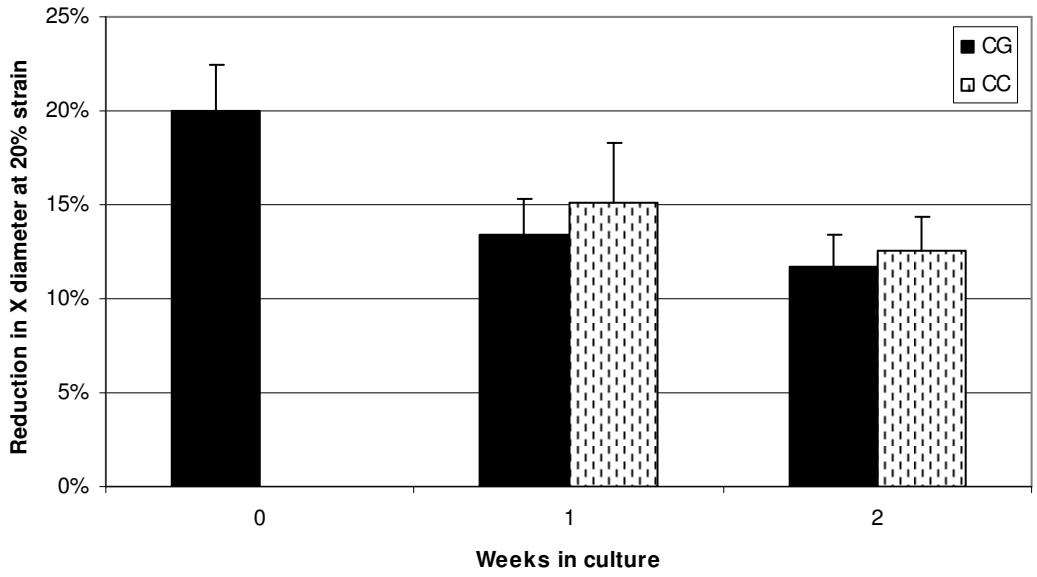

Fig. 3.9 Reduction in X diameter of control cells at timepoint 0, 1 week and 2 weeks. The graph shows both culture conditions at week 1 and 2. Results are shown as mean ± S.E.M. for n=6

Growth media				
Days in culture	10% strain		20% strain	
	ε_x	ε_y	ε_x	ε_y
0*	-7.12±1.7%	1.4±0.2%	-19.99±2.5 %	3.8±1.3%
7	-4.70±1.25%	4.09±0.9 %	-13.40±1.95 %	7.47±1.0 %
14	-6.80±1.3 %	4.19±1.2 %	-11.70±1.8 %	8.97±1.4 %

Chondrogenic media				
Days in culture	10% strain		20% strain	
	ε_x	ε_y	ε_x	ε_y
0*	-7.12±1.7%	1.4±0.2%	-19.99±2.5 %	3.8±1.3%
7	-4.80±0.7 %	2.73±1.2 %	-15.10±3.2 %	11.24±4.2 %
14	-5.50±1.5 %	2.27±0.4 %	-12.50±1.8 %	4.80±0.9 %

Table 3.1 Changes in X- and Y- diameters for all control cells Results are shown as mean ± S.E.M. for n=6
*Control cells at timepoint 0 were not cultured in alginate beads with neither growth nor chondrogenic media.

Under both growth and chondrogenic culture conditions, cells were treated with either 20µM colchicine or 98.5 µM cytochalasin D for 3 hours before they were embedded into

4% (w/v) agarose gel and deformed under 10% and 20% strain. Colchicine and cytochalasin D disrupts microtubules and actin filaments, respectively.

3.3.2.2 Effects of colchicine on deformation

In all uncompressed constructs, treatment with colchicine did not result in detectable changes in cell height, width, volume or shape.

After one week of culture in chondrogenic media, reduction in X diameter was very similar between untreated control cells and cells treated with colchicine at both 10% and at 20% strain (see Table 3.2 and Fig. 3.14). After two weeks in chondrogenic culture colchicine treated cells deformed more at both applied strains (see Table 3.2 and Fig. 3.15) when compared to control. Colchicine treated cells were the only cells that did not deform less at two weeks but change in X diameter was similar between weeks. Same results are shown for the reduction in X/Y diameter ratio (Fig. 3.10 and Fig. 3.11). These findings indicate that microtubules may have a strong role in the inhibition of deformation for cells during chondrogenesis. The lack of effect on cells after 1 week may reflect the evolving interactions between the PCM and cell cytoskeleton.

When cultured in growth media there was no significant difference in change in cell diameters between colchicine treated and untreated cells. This may suggest that cells cultured in growth media lack the PCM-cytoskeleton synergism similar to one week in chondrogenic media.

Chondrogenic media	10% strain		20% strain	
Days in culture	Control	Colchicine	Control	Colchicine
	ε_x	ε_x	ε_x	ε_x
7	4.84±0.72%	4.25±0.96%	15.08±3.2%	15.37±2.47%
14	5.53±1.46%	7.21±0.19%	12.53±1.82%	15.88±2.55%

Table 3.2 Cell strain in X direction of cells cultured in chondrogenic media. Cells are either treated with 20 µM colchicine or untreated. Values are mean from n=6 for control and n=3 for colchicine treated.

Growth media	10% strain		20% strain	
Days in culture	Control	Colchicine	Control	Colchicine
	ε_x	ε_x	ε_x	ε_x
7	4.68±1.25%	6.26±0.80%	13.39±1.95%	12.06±0.54%
14	6.79±1.23%	7.22±0.76%	11.67±1.75%	12.89±1.60%

Table 3.4 Cell strain in X direction of cells cultured in growth media. Cells are either treated with 20 µM colchicine or untreated. Values are mean from n=6 for control and n=3 for colchicine treated

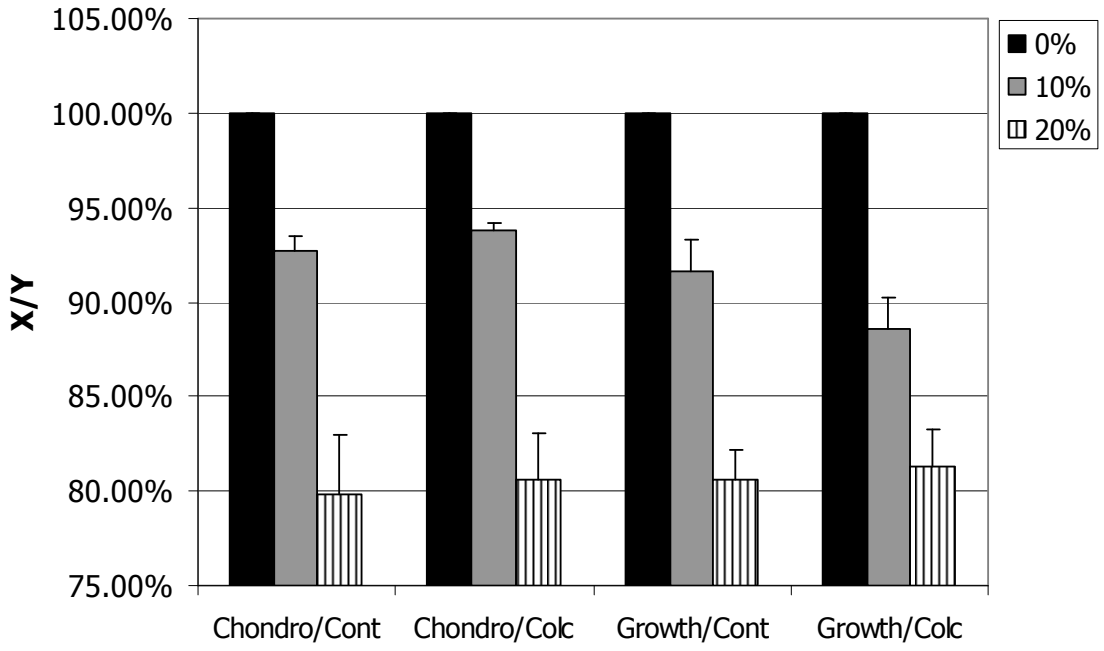

Fig.3.10 Reduction in X/Y diameter ratio. Colchicine (Colc) treated cells compared to control (Cont) cells after 1 week in culture.

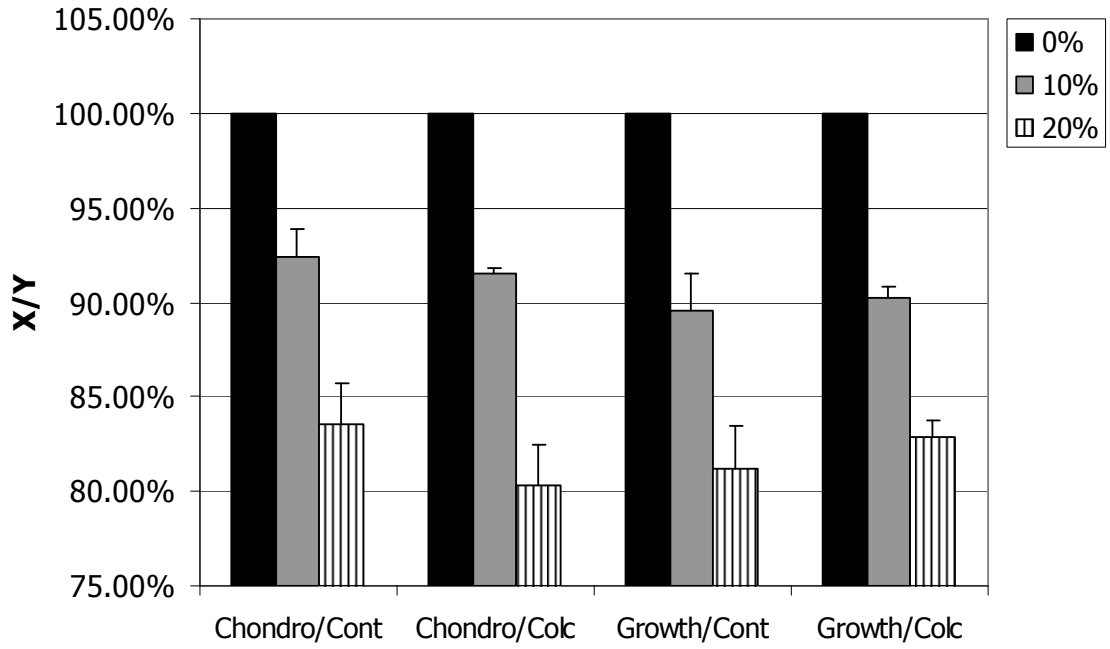

Fig.3.11 Reduction in X/Y diameter ratio. Colchicine (Colc) treated cells compared to control (Cont) cells after 2 weeks in culture.

3.3.2.3 Effects of cytochalasin D on deformation

In all uncompressed constructs, treatment with cytochalasin D did not result in detectable changes in cell height, width, volume or shape.

After one week of culture, cytochalasin D has some effect on deformation of cells cultured in chondrogenic media (see Fig. 3.14 and Table 3.3) for both 10% and 20% strain. After two weeks of culture cytochalasin D was not found to have any effect when compared to control samples (see Fig 3.15 and Table 3.3). Cytochalasin D treated cells deformed less at week two than at week one, but deformation of treated cells was unchanged compared to the control group.

Cytochalasin D was not found to have significant effect on the cells cultured in growth media and deformed similarly compared to control at 10% strain after one week in culture. At 20% strain cytochalasin D treated cells deformed more (15.76%) than untreated cells (13.39%). Cytochalasin D was found to have some effect at two weeks compared to control both at 10% and at 20% strain (see Table 3.5 and Fig. 3.15). Control cells deformed less at two weeks than at one week but reduction in cell diameter for cytochalasin D treated cells was similar between weeks. Same results are shown for the reduction in X/Y diameter ratio (Fig. 3.12 and Fig. 3.13).

Overall, when the cells have not differentiated fully or are cultured in growth media cytochalasin D appears to have a more pronounced effect on cell deformation. On the other hand when cells are further along in chondrogenesis microfilaments do not seem to have any effect. This discrepancy may be explained by the degree of PCM elaboration.

Chondrogenic media	10% strain		20% strain	
Days in culture	Control	CytoD	Control	CytoD
	ε_x	ε_x	ε_x	ε_x
7	4.84±0.72%	8.42±1.91%	15.08±3.2%	16.58±1.81%
14	5.53±1.46%	5.92±2.02%	12.53±1.82%	12.89±2.00%

Table 3.3 Cell strain in X and Y direction of cells cultured in chondrogenic media. Cells are either treated with 98.5 µM cytochalasin D or untreated. Values are mean from n=6 for control and n=3 for cytochalasin D treated

Growth media	10% strain		20% strain	
Days in culture	Control	CytoD	Control	CytoD
	ε_x	ε_x	ε_x	ε_x
7	4.68±1.25%	3.28±0.82%	13.39±1.95%	15.76±6.76%
14	6.79±1.23%	9.44±1.15%	11.67±1.75%	16.10±1.34%

Table 3.5 Cell strain in X and Y direction of cells cultured in chondrogenic media. Cells are either treated with 98.5 µM cytochalasin D or untreated. Values are mean from n=6 for control and n=3 for cytochalasin D treated

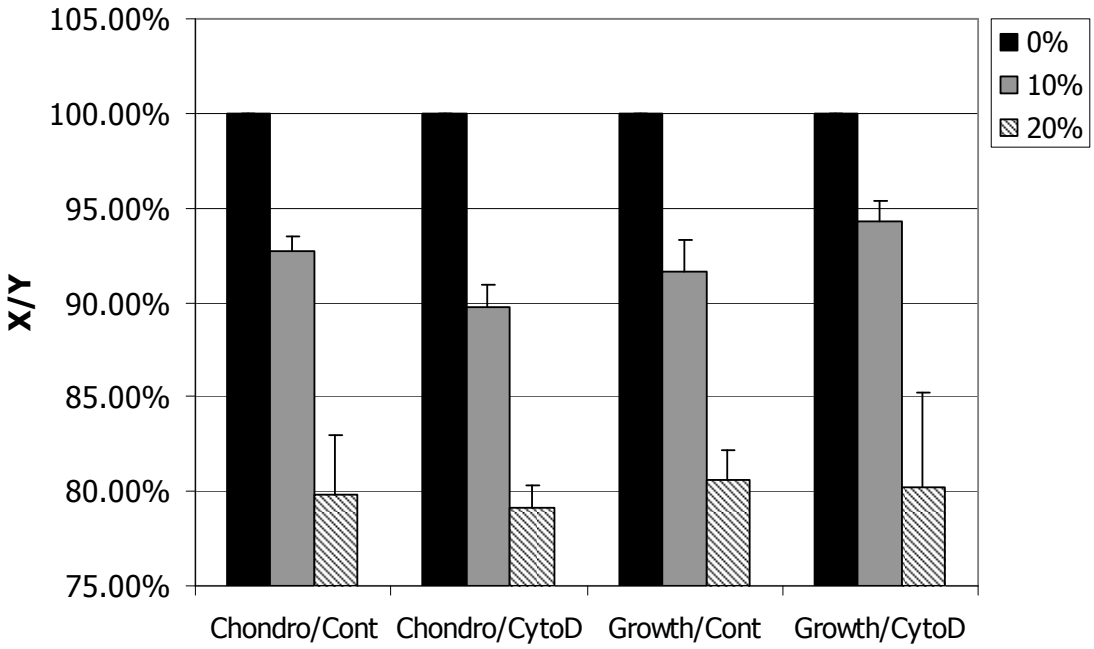

Fig.3.12 Reduction in X/Y diameter ratio. Cytochalasin D (CytoD) treated cells compared to control (Cont) cells after 1 week in culture.

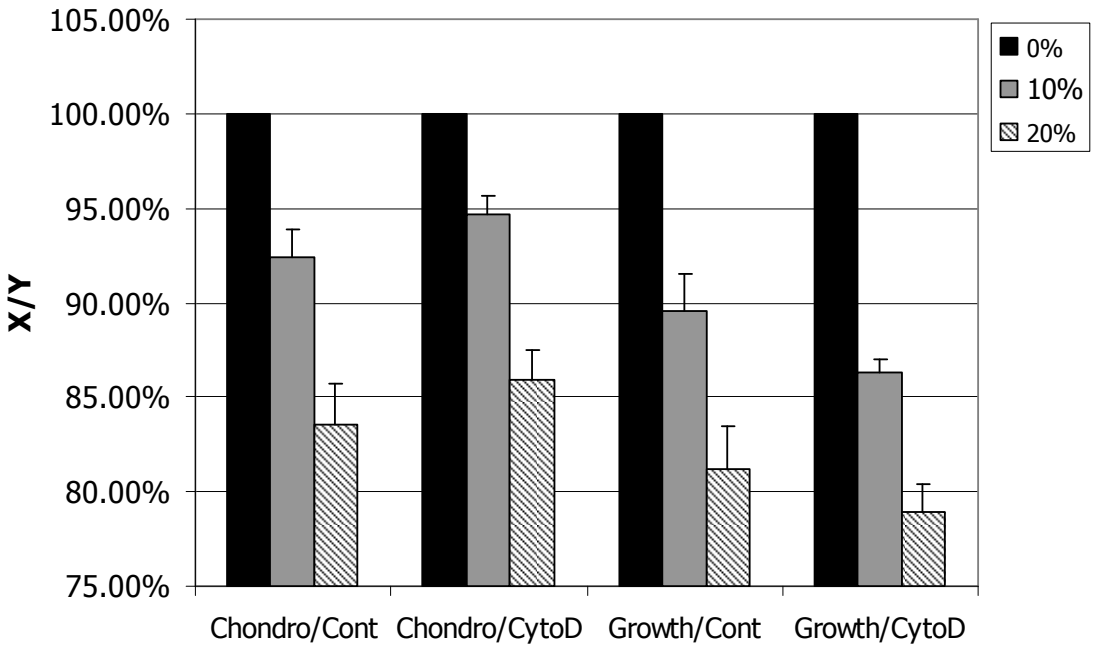

Fig.3.13 Reduction in X/Y diameter ratio. Cytochalasin D (CytoD) treated cells compared to control (Cont) cells after2 weeks in culture.

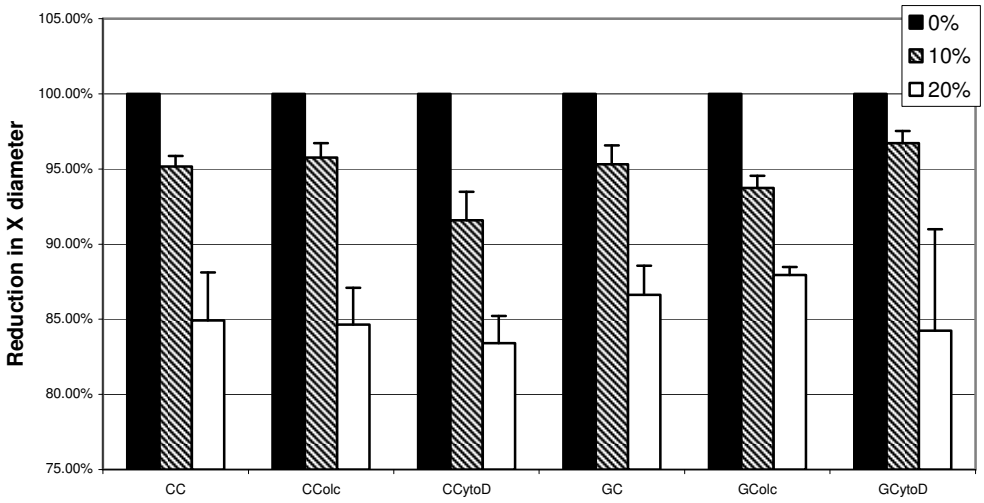

Fig. 3.14 Reduction in X diameter for cells deformed after 1 week in culture. Results are shown as mean ± S.E.M. n=6 for control cells and n=3 for treated cells

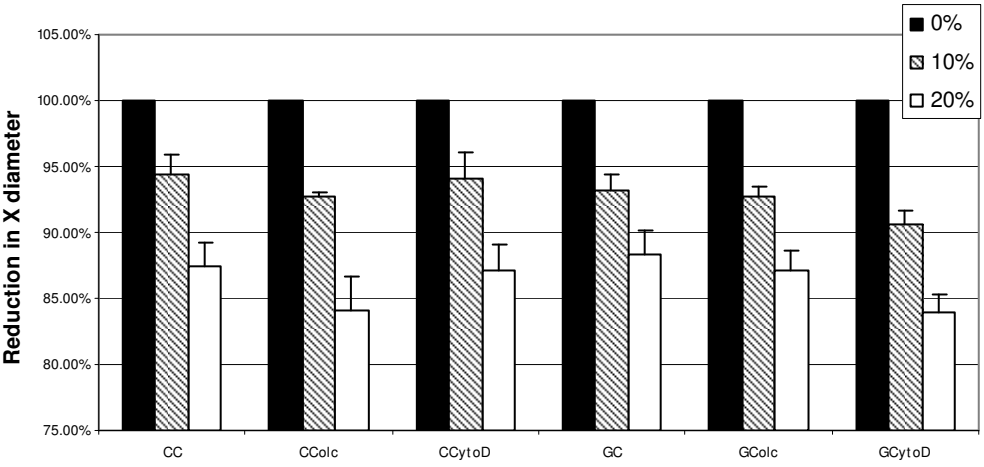

Fig. 3.15 Reduction in X diameter for cells deformed after 2 weeks in culture. Results are shown as mean ± S.E.M. n=6 for control cells and n=3 for treated cells

3.3.2 Visualization of cytoskeletal proteins

To confirm whether the disrupting agents produced results as initially proposed, the cells were examined by fluorescence microscopy with antibodies directed against respective

cytoskeletal proteins. Fluorescent immunolabeling showed significant effects of the cytoskeletal disrupting agents on distribution of microfilaments and microtubules.

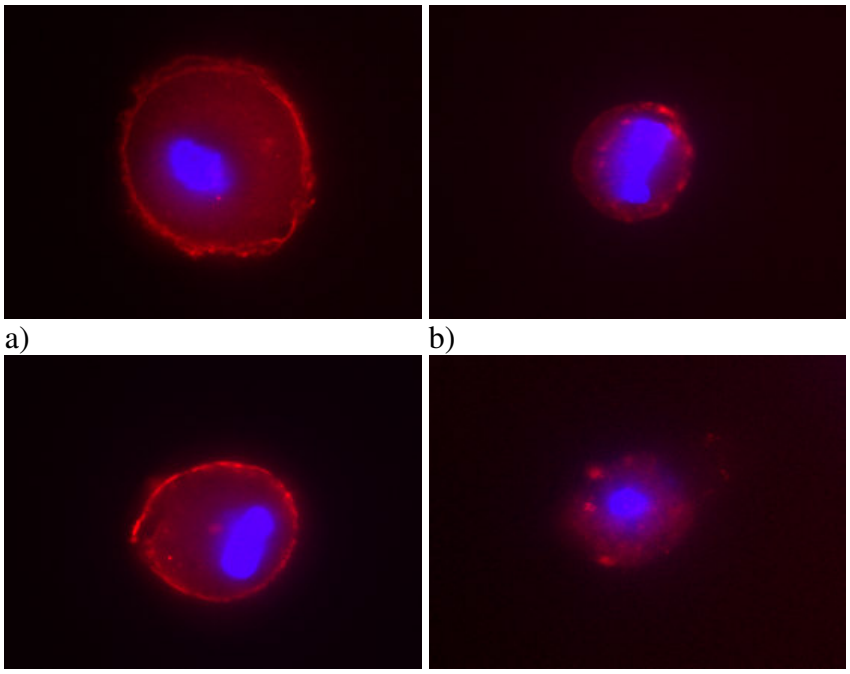

Fig.3.16 Effects of 98.5 µM cytochalasin D on microfilament structure on hMSCs cultured for 1 week in growth media (a-b) and chondrogenic differentiation medium (c-d). (a) control cell cultured in growth medium; (b) cell cultured in growth medium treated with 98.5 µM cytochalasin D; (c) control cell cultured in chondrogenic differentiation medium; (d) cell cultured in chondrogenic differentiation medium treated with 98.5 µM cytochalasin D

3.3.2.1 Microfilament structure

Staining with phalloidin control cells revealed a bright solid ring of F-actin around the periphery of the cells (Fig.3.16 a,c). When treated with 98.5 µM cytochalasin D the staining was discontinous (punctuate) and the cortical localization of the microfilaments was no longer apparent (Fig.3.16 b,d).

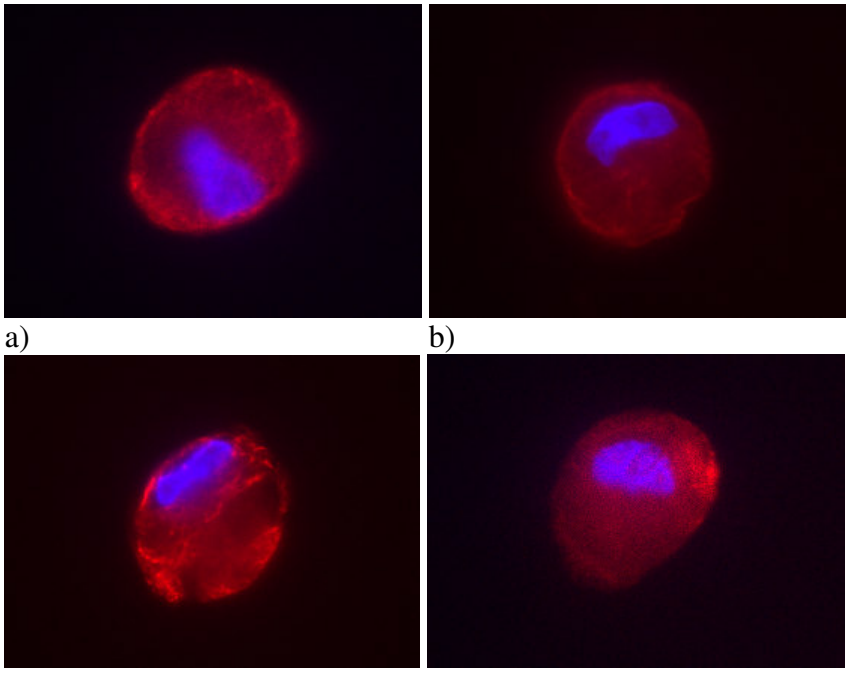

Fig. 3.17. Effects of 20 µM colchicine on hMSCs microtubule structure cultured for one week in growth medium (a-b) and in chondrogenic differentiation medium (c-d). (a) Control cell cultured in growth medium; (b) cell cultured in growth medium treated with 20 µM colchicine; (c) control cell cultured in chondrogenic differentiation medium; (d) cell cultured in chondrogenic differentiation medium treated with 20 µM colchicine

3.3.2.2 Microtubule structure

When stained with tubulin-ß antibody the control cells showed that tubulin was completely distributed throughout the cytosol with clear filamentous structure (Fig 3.17.a,c). When treated with 20 µM colchicine the network structure was disrupted and tubulin was distributed throughout the cell (Fig 3.17 b,d).

3.4 Discussion

The main aim of this study was to determine the role of cytoskeletal proteins on resisting mesenchymal stem cell deformation. An improved understanding of the relative contributions of microfilaments and microtubules to cell deformation characteristics will

aid in the interpretation of cellular mechanotransduction mechanisms in future studies. In particular, current results will help distinguish the synergistic processes of cytoskeletal dynamics, pericellular matrix formation, and chondrogenic differentiation in mesenchymal stem cells for potential intervertebral disc tissue engineering applications. The first aim of the study was to determine how best to disrupt cytoskeletal proteins. The methods used are well established [25] and it is clear from the results that they were effective. Incubating cells with respective disrupting agents for three hours was decided upon after several pilot experiments with different incubation times (results not shown) and by visualizing cytoskeletal protein structure after each incubation with epi-fluorescence microscopy. In our previous trials (results not shown), we incubated cells with respective disrupting agents only during the treatment, and did not use disruptive agents throughout the process of staining and deforming of the cells. In this present study the respective disrupting agent were used throughout the process to inhibit repolymerization of the proteins [25]. Treatments with colchicine and cytochalasin D did not result in detectable changes in cell volume or shape. This is consistent with other studies [35].

The cell deformation index is a widely accepted parameter for quantifying the deformation of isolated cells in compressed agarose constructs [29][30][33][36]. Studies have shown that isolated chondrocytes have been found to adopt a spherical morphology (X/Y=1) when seeded in agarose but to deform to an oblate ellipsoid morphology during compression [30]. In this present study, cells were cultured in a three dimensional alginate so that they would adopt a spherical morphology, which is important for chondrogenesis. Cells were cultured in these beads during the differentiation process and

retrieved from alginate for cytoskeletal disruptive treatment and/or staining. The cells were then reembedded in agarose after (i) either colchicine or cytochalasin D treatment or (ii) no treatment which served as a negative control. We expected cells to maintain their spherical morphology in both cases. Fig. 3.4 and Fig. 3.5 show how the morphology changes from being almost spherical to more elliptical for all culture conditions and at both timepoints (1 and 2 weeks).

When looking at control cells – cells that retained an intact cytoskeleton – it is clear that with increasing time in culture, there was a reduction in the level of cell deformation during agarose compression (see Fig. 3.8 and Fig.3.9).

A separate study performed in our lab showed significant (nearly thousand-fold) increase of the Sox-9 gene expression in hMSCs between 1 and 2 weeks in chondrogenic media cultures and a decrease of type VI collagen expression between 1 and 2 weeks for growth media cultures (results not shown). These experiments were performed in alginate, using identical culture procedures as in this present study. Sox-9 is a transcription factor that is closely linked to chondrogenesis. Other studies show that the concentration of cartilage link protein (CRTL1) is dependent on Sox-9 [28] which suggests that the extracellular matrix (pericellular matrix) is growing with time in chondrogenic culture. A previous study shows that glycosaminoglycan content of chondrocytes also increases with time in culture [29]. These findings together with present results suggest that the pericellular matrix should have a greater influence on cell deformation during the second week compared with the first week in chondrogenic culture. And this is also consistent with suggestions that the pericellular matrix provides a protective role for the cells during loading through an adaptive water loss from pericellular matrix proteoglycans.

The similarity between the values of control cells deformed at time zero, X strain and applied gross strain suggests that the compressive moduli of the cells at timepoint 0 is less than or equal to the equilibrium modulus of 4% agarose.

Cells cultured in chondrogenic media deform less at 20% strain at week two than at week one. This is true for all conditions except for cells treated with colchicine. Colchicine treated cells cultured in chondrogenic media deformed more compared to control cells at week two, indicating that microtubules may be important for resisting cell deformation in this scenario. Colchicine treated cells deform very similarly between weeks. Cells treated with cytochalasin D deform less at week two, but there is no significant difference between treated and untreated cells. We have already suggested that the amount of extracellular matrix increases with culture time, these findings could therefore indicate that the pericellular matrix has a stronger role than the microfilaments in resisting deformation.

As mentioned above, type VI collagen expression decreased with culture duration for cells in growth media.

Microtubules seem to have effect on cell deformation when cells have a more elaborated PCM and have undergone more extensive chondrogenesis. When cells are cultured in growth media or are in the first days of chondrogenesis when matrix has not started to accumulate, microtubules do not have any effect.

By disrupting the microfilaments with cytochalasin D, cells deformed more compared to control when less matrix surrounded the cells. With growing matrices, microfilaments did not seem to have much effect, the pericellular matrix seems to be resisting

deformation regardless of microfilaments. Further studies focused on deforming cells with limited amount of or no pericellular matrix should be performed to confirm these results.

3.4.1 Limitations and future directions

In order to carry out the present study, many preliminary steps were needed. Based on these ancillary studies and their respective findings, we believe a strong basis for future studies has been formed.

A specially designed chamber was used to deform the agarose constructs. While suitable for the present series of studies, the chamber is not fully designed and needs further improvements. To mimic the environment within the body it is essential that the testing environment is held constant at 37°C. This could be achieved by either placing the testing chamber into an environmental chamber attached to the microscope which serves like an incubator at desired temperature and CO_2 level (Warner Instruments) or by inserting heat exchanging tubes through the polysulfone near the culture well at temperatures necessary to warm the PBS/media in which the agarose specimen is submerged. If choosing the latter method the chamber also needs to be closed to maintain a sterile environment. That could be achieved by placing a glass top on top of the chamber. In the present study, compression is performed by manually turning one micrometer to displace the loading platen, while the other one is fixed. Studies [30][31] that compress cell-agarose constructs have used a stepping linear actuator which controls the plungers and the construct is compressed at a certain strain rate (certain step size), thereby achieving more uniform compression and steady compression rates. Other studies [30] have also used

two moving plungers instead of one fixed and one moving. By doing so, the displacement of the cells at the center of the agarose construct is minimized so that it is easier to track individual cells in specimens subjected to compression. The one advantage that manual compression of agarose construct offers is greater ease of monitoring cells throughout the compression. Any changes in position in the z-plane can be more readily identified and corrected.

In this study, we used a 4% (w/v) agarose gel in which to embed hMSCs. If the gel is stiffer than the cells, the gel can have too much impact on the cell deformation and the cell could deform more than it would otherwise. To determine the cell stiffness there are direct mechanical perturbation techniques such as micropipette aspiration, atomic force microscopy or cell poking. Nevertheless, these techniques only provide localized properties, which may be primarily influenced by structures closely associated with the cell membrane such as the cortical actin cytoskeleton, rather than gross mechanical properties [31]. A number of studies involving chondrocyte deformation within compressed agarose constructs have used 3% [29][30][32] or 2% [33][34] agarose. Further studies that examine the evolving stiffness changes in cells and how to balance the agarose stiffness with cell stiffness will be performed to obtain a more appropriate agarose stiffness that may allow us to identify cytoskeletal contributions to cell deformation in a more exact manner.

The process from retrieving the cells from alginate to preparing the agarose construct is very time consuming. To limit the number of agarose constructs it would be ideal to track and image more than one cell in each specimen. It is necessary to have the right cell density in the construct to be able to do so. If there are too many cells it can be hard

to distinguish one cell from another, resulting in too much background fluorescence. Too few cells would result in having to move the construct on top of the microscope to track down another cell, which could result in losing the prior one. Ideally there should be just the right density of cells, so little would be needed to do to monitor a few in the same specimen, which would involve keeping the construct in place and mostly just refocus to find other cells. Cell density used in the present study was 150,000 cells/ml with 5000 cells/alginate bead. 4 beads were used for each cell-agarose construct which gives us 20,000 cells in each sample. To be able to monitor more than one cell in each specimen the density needs to be at least double or triple than used in this study. Also, an option worth considering is to compress each sample only once, use one sample for 10% compression and another one for 20% compression. That could give us the option of monitoring more cells at once and minimizing the risk of losing them by sequential compression of the same gel.

To further observe the role of the pericellular matrix in cell deformation, a method for digesting the PCM away needs to be established. Deformation of PCM digested MSCs should be compared to results from this present study. Cells with limited or without PCM and disrupted cytoskeletal proteins should be deformed. A histological study of the pericellular matrix in hMSCs cultured with same method as here, and of hMSCs that have gone through overnight collagenase digestion is currently being pursued in the lab. Another study that observes genes for type VI collagen at different timepoints during chondrogenesis of hMSCs is also being performed. These complimentary studies will

help elucidate the competing and synergistic factors that contribute to mechanotransduction in hMSCs.

3.5 Conclusion

In conclusion, this study examined the effects that microtubules and microfilaments have on cell deformation, both for cells undergoing chondrogenesis and for cells cultured in growth media. The results were consistent with what others have observed in chondrocytes. For all controls, cells changed from being spherical to ellipsoidal with agarose gel compression. Microfilament and microtubule disruption had inverse relationships on cell deformation, that was dependent on culture condition and duration. For 2 weeks of chondrogenic culture, microfilaments didn't have any effect on cell deformation, while microtubules had a strong role in inhibiting cell deformation. Conversely, microfilaments, but not microtubules, were important for cells under other durations and conditions. This discrepancy may be explained by the degree of PCM elaboration, and the likely evolving interactions between the pericellular matrix and cell cytoskeleton during chondrogenesis.

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