# The Role of IGF-1 and TRPV4 in Regulation of $[Ca^{2+}]_i$ and Actin Organization in ATDC5 Chondrocytes

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Bachelor of Arts in Biological Sciences with Distinction

Spring 2015

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#### ACKNOWLEDGMENTS

I would first like to thank Dr. Randall Duncan for giving me this amazing opportunity to conduct research and to work on my senior thesis. I am grateful for Dr. Duncan's continual support and endless patience over the past year and a half working on this project as well as his life advices. I would also like to thank everybody in Dr. Duncan's lab. I wish to thank Dr. Mary Boggs, Ben Rohe, and Vimal Gangadharan for welcoming me to the lab and helping me along the way with whatever problems I had. Everyone in the lab was close to each other and had great chemistry that it created an amazing environment. I would like to especially thank Lauren Hurd for always being there to help me whenever I was in trouble and for being patient with me as I learned the ways of the lab and cell culture. I know I was not the fastest learner with lab techniques, but hopefully I have not caused too much trouble for her. Besides the lab techniques and biology, I am grateful to Dr. Boggs and Lauren for giving me advices and guidance on the complex journey called life. Therefore, thank you Dr. Duncan and everyone in his lab for being patient, helping, and guiding me along this challenging, but wonderful experience. I will always remember all the great memories I had working in this lab and be grateful for the opportunity. I would also like to thank members of my committee; Dr. Cooper and Dr. van Golen for being patient with me and helping me along this process. Special thanks to Dr. Cooper who was always there with a joke to cheer up the atmosphere in lab and in lab meetings. I am also grateful to all my biology professors I had since freshman year for always being willing to help me whenever I had problems and for teaching all those lectures. I would also like to

thank my friends, especially Joseph, Dyllon, and Henry for their support and helping me de-stress throughout this process. Finally, I would like to thank my family for their support during this process. While they may not have understood what I was doing or why, they were always there willing to do whatever was needed to help me. I am lucky to have such amazing people to help and guide me throughout this process.

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#### ABSTRACT

Cartilage is a thin layer of tissue composed of mainly water, collagen, and proteoglycans that forms a sliding area so that diarthrodial joints can easily function. Chondrocytes are the cells of this tissue that produces and maintains the cartilage of matrix and the phenotype and function of these cells is regulated by the organization of actin cytoskeleton. Two dimensional culture studies have shown that Insulin-like Growth Factor-1 (IGF-1) increased cell stiffness through regulation of actin cytoskeleton, which suppressed the activation of TRPV4. TRPV4, a  $Ca^{2+}$  channel, is an important regulator of intracellular Ca<sup>2+</sup> and is an anabolic factor in chondrocytes for matrix synthesis. I hypothesize that IGF-1 has differential effects on the actin cytoskeletal organization and TRPV4 channel activity in ATDC5 chondrocyte cells in 2D/3D culture environments. ATDC5 chondrocytes were cultured in sodium alginate beads and treated with 4 different treatments: control, IGF-1 treatment, Rac-1 Inhibitor treatment, IGF-1+Rac-1 inhibitor treatment (n=10) and  $[Ca^{2+}]_i$  levels were measured with PTI and FURA-2AM protocol. ATDC5 chondrocytes were also grown in 2D culture environment (collagen II coated coverslips) and in 3D (collagen/agar matrix), treated with 4 different treatments, stained with 488 Alexa Fluor Phalloidin to visualize actin cytoskeleton, and imaged with Zeiss 510 laser scanning microscope. Chondrocytes were then cultured in micromass cultures and treated with the four different treatments to see if there were any changes to levels of proteoglycan synthesis. IGF-1 treated chondrocytes in 3D tended to increase  $[Ca^{2+}]_i$  and exhibited a

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biphasic response, which was different from what 2D studies had predicted. IGF-1 treatment resulted in increased actin fiber formation in both 2D and 3D culture environments, while the effects of Rac-1 inhibition seemed dependent on the dimensions of culture environment. This suggests that IGF-1 increases actin fiber formation through RhoA in 2D culture and through Rac-1 activation in 3D cultures. IGF-1 treated micromass cultures exhibited similar levels of proteoglycan content as control treatments, but Rac-1 inhibition led to lowered proteoglycan content. The effects of IGF-1 are dependent on the dimensions of the culture environment. In 2D the effects of IGF-1 are dependent on Rac-1 activation, but in 3D IGF-1's effects are dependent on RhoA activation.

#### Chapter 1

#### **General Information**

#### **1.1** Introduction to Cartilage and the Importance of Cytoskeletal Architecture

Cartilage is a type of connective tissue that provides a smooth sliding area for proper joint function and is important in the formation, development, and growth of the skeletal system. Cartilage matrix is composed water, collagens, and proteoglycans. The collagen provides the cartilage with tensile strength (force of pulling) and the proteoglycans provides resilience or elasticity. Cartilage itself can be divided into three different types based on its composition and histological appearance. Elastic cartilage consists of elastin fibers (highly elastic), and provides structural support in tissues such as the epiglottis and the outer ear. Fibrocartilage has larger amounts of collagen fibers for increased tensile strength. This type of cartilage is found where bones are connected to tendons and in between the intervertebral discs. Hyaline cartilage, the most common type of cartilage, forms the template for bone formation during endochondral ossification. Hyaline cartilage is also important after the initial formation of bones as it is also found in the epiphyseal growth plates for postnatal longitudinal bone growth (Lin *et al.*, 2006), as well as the gliding surface of diarthrodial or synovial joints.

Chondrocytes are highly specialized cells responsible for making and maintaining the different components of the cartilage matrix. Chondrocytes are formed when mesenchymal stem cells (embryonic cells capable of differentiation) transform

into chondrocytes through a series of differentiation pathways with a number of cytokines and transcription factors (Daniels e Solursh, 1991).

Endochondral ossification, one of the two forms of osteogenesis, refers to the formation of long bones based on a cartilage model. Endochondral ossification consists of hyaline cartilage that is modified to aid in bone formation by osteoblasts. Bones formed through endochondral ossification continue to grow through the epiphyseal growth plates separating the epiphysis and the metaphysis on long bones (Sit e Manser, 2011). Epiphyseal growth plates can be divided into histologically different zones. The resting zone consists of small clusters of chondrocytes. The proliferative zone is where the chondrocytes, arranged in organized columns, are highly metabolically active and undergo rapid mitosis. The zone of maturation or hypertrophy is where chondrocytes grow larger in size, and have ceased proliferation. The zone of provisional calcification is where apoptotic chondrocytes are reabsorbed, and calcification of cartilage occurs. It is also in this zone where blood vessels and osteoblasts enter into the now free spaces. In the ossification zone at the metaphysis, osteoblasts lay down osteoid on the calcified cartilage, which then becomes mineralized. Therefore the proper function of chondrocytes through the maintenance of proper chondrocyte phenotype is essential in the normal development of the skeletal system.

The chondrocyte phenotype and function can be regulated by many factors including the organization of the actin cytoskeleton. Actin, one of the three types of cytoskeleton filaments, determines the shape of cell surfaces and is important for whole cell locomotion. Actin filaments are usually found near the plasma membrane of cells, where filaments are important to the plasma membrane's strength and

stability. During chondrocyte differentiation there are noticeable changes in the cells' morphology that results from actin rearrangement. Brown and Benya identified that it was the microfilaments that acted as the principal affected cytoskeletal element and showed that modification rather than disruption of the actin cytoskeleton was sufficient for re-expression of the chondrocyte phenotype (Brown e Benya, 1988). Another study found that flattened chondrocytes formed elongated actin stress fibers, which occurs in dedifferentiation, led to decreased synthesis of type II collagen and aggrecan expression (Blain, 2008). Others have also shown that addition of cytochalasin D, a drug that cuts filamentous actin into shorter chain, stimulated the rounding of de-differentiated chondrocytes which returned to normal expression of chondrogenic markers such as expression of type II collagen and aggrecan (Woods, Wang e Beier, 2007; Blain, 2008). There is also evidence that actin may have an influence in epiphyseal growth plate function. It was found that overexpression of adservin, an actin binding protein, led to rearrangements of the actin cytoskeleton, changes in cell shape, increased cell volume, and up regulation of collagen type X all suggestive of chondrocyte differentiation (Nurminsky et al., 2007). Therefore these studies point to actin playing an important role in maintaining the chondrocyte phenotype.

The 3-dimensional properties of the growth plates and the body are important for the differentiation of mesnchymal stem cells into chondrocytes. A number of studies have shown that there are differences between 2-D (monolayer) and 3-D (alginate beads/hydrogels) cultures that led to differences in chondrocyte differentiation and expression of chondrogenic markers. In one of these studies, Gründer found that seeding cells in alginate beads supported chondrogenesis, maintained the

chondrogenic phenotype of cells, and that chondrocytes in the alginate beads synthesized cartilage matrix components similar to those in native tissue (Gründer *et al.*, 2004). Chondrocytes seeded in monolayers (2D) had decreased collagen type II and aggrecan expression, and an increase in collagen type I expression (Gründer *et al.*, 2004; Hwang *et al.*, 2005; Albrecht *et al.*, 2008; Caron *et al.*, 2012). By putting chondrocytes into alginate beads, a number of studies have found that the 3D culture environment was more conducive to maintaining the chondrocyte phenotype (Albrecht *et al.*, 2008; Caron *et al.*, 2012). In contrast, culturing chondrocytes in 2D caused a loss of the chondrocyte phenotype (de-differentiation) with the loss of collagen II and aggrecan expression. While it is shown that maintaining proper cytoskeletal architecture is essential for chondrogenesis, new molecular players in the regulation of the signaling mechanisms are continually being elucidated.

#### 1.2 Effects of IGF-1 and Rac-1 on Actin Cytoskeleton

One of the differentiation factors that affect the development of cartilage and the skeletal system is growth hormone (GH) secreted by somatotropes, specialized cells in the anterior pituitary. It is now known that growth hormone may not directly interact with tissues in the body, but rather uses intermediate substances to cause many of the physiological responses. One of the most important of these intermediate substances is called Somatomedin C, or Insulin-like Growth Factor-1 (IGF-1). It has been found that IGF-1 increases the chondrogenic potential of bone marrow derived mesenchymal stem cells by stimulating proliferation, regulating apoptosis, and inducing expression of chondrogenic markers (Longobardi *et al.*, 2006). Studies suggest that IGF-1 regulates chondrogenesis in mesenchymal cells, cartilage, bone development, and maintenance of cartilage homeostasis through activation of PI3K, subsequent activation of PKC $\alpha$  and p38 kinase, and inhibition of ERK1/2 (Oh e Chun, 2003). The activation of PI3K, PKC $\alpha$ , and p38 kinase are all important signaling events for IGF-1 induced chondrogenesis, but the exact mechanism of how these events interacted in IGF-1 induced chondrogenesis is unknown. It has also been reported that IGF-1 induced chondrogenesis led to chondrocytes that were able to express key chondrogenic markers like collagen type II similar to primary chondrocytes (Longobardi *et al.*, 2006). Therefore IGF-1 is also an important anabolic factor in chondrocytes as it stimulates increased production of cartilage matrix components like collagen and proteoglycans.

IGF-1 also has the ability to regulate the actin cytoskeleton in chondrocytes. Data from our lab has shown that treatment of chondrogenic cells with IGF-1 can alter the actin stress fiber formation and increase the stiffness of the cell (Gardinier, et al., in preparation). Figure 1 shows that a time course of IGF-1 treatment for 3 hours caused an in increase in actin stress fiber formation (Gardinier, et al., in preparation). This is important because chondrocytes are constantly experiencing a changing extracellular osmotic environment during normal development and joint loading. Several in vitro studies have shown that the formation and cytoskeletal architecture and the chondrocyte phenotype are interdependent (Kerr *et al.*, 2008). Therefore it is important for chondrocytes to adapt to changes in the environment through cytoskeletal rearrangements in order for them to function properly.



Figure 1: Time Course of IGF-1 treatment over 3 hours showing an increase in actin stress fiber formation over a period of three hours. Figure from (Gardinier, et al., in preparation).

The IGF-1 signaling pathway is one of the signaling pathways that are involved in the cytoskeletal rearrangement response in chondrocytes. A part of the IGF-1 signaling pathway involves Rho GTPases, a group of GTP-binding proteins that function as signaling molecules. The Rho-GTPases have been documented in activating pathways and other molecules required for the assembly, disassembly, and organization of actin filaments (Ridley, 1995; Albertinazzi, Cattelino e de Curtis, 1999; Sit e Manser, 2011). Rho-GTPases act as switches, cycling through active (GTP-bound) and inactive (GDP-bound) configurations, which then can bind to a large number of different downstream signaling pathways causing different responses such as assembly or disassembly of actin fibers. The Rho-GTPase family of proteins are composed of three types of molecules that causes different events in the cell. RhoA controls stress fiber formation, Rac-1 induces membrane ruffling and lamelipodia, and cdc42 stimulates filopodia formation(Ridley, 1995). In chondrocytes, the activities of Rac-1 and Cdc42 activities are important for chondrocyte hypertrophy, as both are required for expression of collagen type X, a hypertrophic marker (Wang e Beier, 2005). Activation of the Rho-GTPases are believed to occur one at a time as activation of one suppresses the activity of another Rho-GTPase. For example, activation of RhoA suppresses the activity of Rac-1 and vice versa (Gardinier, et al., in preparation).

#### **1.3 TRPV4, a non-selective cation Channel**

Chondrocytes and the cartilage matrix are important for proper development of the skeletal system and for proper joint function and calcium ( $Ca^{2+}$ ) is an essential component to chondrocyte function. It has been shown that one crucial regulator of intracellular calcium [ $Ca^{2+}$ ]<sub>i</sub>, is a member of the Transient Receptor Potential (TRP)

superfamily of ion channels, TRPV4 (transient receptor potential vanilloid 4 channel). TRPV4 is a  $Ca^{+2}$  and  $Mg^{+2}$  permeable non-selective cation channel that is involved in many different cellular functions. The TRPV4 channels are in charge of regulating calcium signaling in cells through the influx of calcium, and controlling calcium release (Gardinier, et al. in preparation). The channel is involved in many cellular processes such as mechanosensation, osmosensation, and thermosensation, and is widely expressed throughout the body (Leddy et al., 2014). TRPV4 channels can be activated a number of different ways. The TRPV4 channel can be activated by moderate heat (24-27°C), but the exact mechanism of heat activation is not understood (Nilius et al., 2004; Everaerts, Nilius e Owsianik, 2010). The channel can also be activated in a cell's response to hypotonic swelling (HTS) as mechanical stress is detected by phospholipase A<sub>2</sub> which leads to the formation of epoxyeicoastrienoic acids (EETs) that can activate the channel (Watanabe *et al.*, 2003). The non-selective cation channel TRPV4 plays an important role in regulation of volume of chondrocytes, which is important for chondrocyte hypertrophy for endochondral ossification (Wang e Beier, 2005).

Some studies have also shown that TRPV4 interacts with the cytoskeleton since many functions involving TRPV4 require cytoskeletal involvement, but the exact mechanisms are not well understood. It has been shown that the structure of TRPV4 has domains on its carboxy and amino-terminal ends where cytoskeletal elements like actin and microtubule can bind (Albertinazzi, Cattelino e de Curtis, 1999; Umlauf *et al.*, 2010; Leddy *et al.*, 2014). One study has reported that IGF-1 increases cell stiffness in ATDC5 cells through the regulation of the actin cytoskeleton, which suppresses the activation of the TRPV4 channel during

mechanical stimulation(Gardinier, et al., in preparation), which can be seen in Figure 2. This suggested that IGF-1 has the ability to control the activity of TRPV4 channels through regulation through increasing polymerization of the actin cytoskeleton. The increase in the actin cytoskeleton polymerization then exerts a large influence on how sensitive cells are to mechanical stimuli (Gardinier, et al., in preparation).



Figure 2: IGF-1 treatment suppressed the Ca<sup>2+</sup> response following HTS. Treatment with cytochalasin D, which disrupts the F-actin, rescues the Ca<sup>2+</sup> response through decrease in actin cytoskeleton. Figure from (Gardinier, et al., in preparation).

#### **1.4** Purpose of Study

This study looked at the effects of IGF-1 on the actin organization and TRPVchannel activity in ATDC5 cells in 2D and 3D culture environment. This study included experiments in 3D culture environments because 2D culture environments may not faithfully capture the physiological behavior of cells in vivo. (Baker e Chen, 2012) My hypothesis is that IGF-1 has differential effects on the actin cytoskeletal organization and TRPV4 channel activity in ATDC5 chondrocytes in 2D/3D culture environments. To test this hypothesis, I proposed two aims. The first aim is to look at the effects of IGF-1 treatment and Rac-1 inhibitor treatment on the intracellular concentration of  $[Ca^{+2}]_i$  and TRPV4 activity after hypertonic swelling. The second aim was to determine the effect of IGF-1 treatments on actin stress fiber formation, the cellular morphology, and chondrocyte phenotype in 2D and 3D environments. Completion of these aims will contribute knowledge to the differences in chondrocyte function in different dimensions, as well as knowledge to interactions between IGF-1, RhoGTPases, TRPV4, and actin cytoskeleton.

#### Chapter 2

#### **Materials and Methods**

#### 2.1 Cell Culture of ATDC5

ATDC5 chondrocytes were cultured in T-75 corning flasks with Dulbecco's Modified Eagle Medium (Corning Cellgro, Manassas, VA) with the addition of 10% Fetal Bovine Serum (Gemini BioProducts, Sacramento, CA) and 1% pen-step (Thermo Scientific, Logan, Utah). Chondrocytes were grown to 70% confluence in  $37^{\circ}$ C in 95% air/CO<sub>2</sub> for at least 48 hours before use for experiments.

#### 2.2 Treatments:

The control treatment used in all the experimental methods except for micromass cultures was made of DMEM (10%FBS+1%PS) and then incubated for 3 hours at 37°C in 5% CO<sub>2</sub>. The IGF-1 treatment used was made up of 300ng/mL IGF-1(Sigma Life Sciences, St. Louis, MO) added to DMEM (0.2%FBS+10%PS) and incubated for 3 hours. The Rac-1 inhibitor treatment consisted of 100uM/mL Rac-1 Inhibitor (EMD Millipore, Billerica, MA) added to DMEM(0.2%FBS+10%PS) and incubated for 3 hours. The combined IGF-1 and Rac-1 Inhibitor treatment was made of Rac-1 Inhibitor(100uM/mL) combined with IGF-1(300ng/mL) and added to DMEM(w/o phenol red+0.2% FBS) and then incubated for 3 hours.

The control treatment used to treat micromass cultures was DMEM (10% FBS) +1% ITS liquid media supplement (Sigma Life Sciences). The IGF-1 treatment was 300ng/mL IGF-1+DMEM (0.2% FBS ) + 1% ITS. Rac-1 inhibitor treatment consisted of 100uM/mL Rac-1 Inhibitor+ DMEM (0.2%FBS) + 1% ITS. Rac-1 inhibitor and IGF-1 treatment was made of Rac-1 Inhibitor(100uM/mL)+IGF-1(300ng/mL)+ DMEM(0.2%FBS) + 1% ITS.

#### 2.3 Sodium Alginate Bead Culture

ATDC5 cells were cultured and incubated for at least 48 hours before being placed into sodium alginate bead cultures. A 1.2% solution of sodium alginate was prepared by first adding 0.906g of NaCl (Fisher Scientific, Fairviews, NJ) into a flask with 100mL dH<sub>2</sub>O, then placed on stir plat and warmed for at least five minutes. Then 1.2g of Alginic Acid Sodium Salt (Sigma Life Science) was added to the flask, and the mixing process continued for 3-4 hours. Once dissolved, the sodium alginate solution is filtered through 0.45µm filters into sterile containers, and stored at 2-8°C. When the ATDC5 cells reach a confluence of 80%, the cells were counted so that there would be 400,000 cells/mL in each sample. After the spinning process, the cell pellet was brought up with the 1.2% sodium alginate solution. The ATDC5/sodium alginate solution was then added drop wise fashion (needle and syringe) into 15mL conical test tubes with 8 mL of 102 mM CaCl<sub>2</sub>·2H<sub>2</sub>O (Arcros Organics, New Jersey). For the best bead results, the ATDC5/sodium alginate solution were added drop wise with the needle just above the 102 mM CaCl<sub>2</sub>·2H<sub>2</sub>O solution. After every 10th to 15th drop, the conical tube was gently shaken to make sure that the beads did adhere. The ATDC5 culture beads were allowed to rest for about 3 minutes before being washed with 155mM NaCl. After the 155mM NaCl wash, 8mL DMEM with the addition of 10% FBS and 1% PS without phenol red was added, and the bead cultures were placed in an incubator at 37°C in 5% CO<sub>2</sub> for three days before treatments were administered.

# 2.4 Measurement of intracellular calcium concentrations in ATDC5 alginate bead cultures

ATDC5 alginate bead cultures were analyzed for intracellular calcium concentrations with the use of the PTI Photomultiplier Detection System 810 Fluorescence Masters System (Photon Technology International, Edison, NJ) and associated FelixGx (Photon Technology International) software. Cuvettes were first filled with 1.5 mL of 3% agar (Becton, Dickinson, and Company, Sparks, MD) and allowed to solidify for 45 minutes. At the end of the treatment period, the treatment solutions were removed and ATDC5 alginate beads were washed two times with Hanks' Balanced Salt Solution (HBSS) with calcium and magnesium (Corning Cellgro, Manassas, VA). Then 1mL of ATDC5 alginate bead samples were added to the cuvettes with 1.5mL of solidified 3% agar. Cuvettes were then placed into the PTI machine and a FURA-2 AM protocol was ran using the FelixGx program. Measurements were collected for 300 seconds, then paused so that 1mL of a 50% hypotonic swelling solution (HTS) was added, and measurements were collected for another 300 seconds. Data provided by the FelixGx program was then exported into Microsoft Excel for analysis.

#### 2.5 Proteoglycan Production in a 3D environment (Micromass procedure)

ATDC5 cells were grown to a confluence of 70%, then centrifuged and counted so that there would be 500,000 cells/  $\mu$ L. Cells were then reconstituted with DMEM + 10% FBS + 1% PS + 1% ITS liquid media supplement (Sigma Life Sciences). The amount of media used to reconstitute the cells depended on how many samples there were; ideally there would be 10  $\mu$ L of media per sample. Cells were then plated into 24 well plates and incubated at 37°C in 95/5% air/CO<sub>2</sub> for 1 to 2 hours for the micromass cultures to settle down. Following the incubation period, different

treatments were added to the samples in the wells. The samples were then placed back in the incubator and treatment solutions were changed every other day. After four days, the treatments were removed, and samples were carefully washed with DBPS. Samples were then fixed with a solution of 10% formalin (Sigma Life Sciences) and .5% cetylpyrridiniumc chloride (Sigma Life Sciences) for 20 minutes at room temperature. After 20 minutes, samples were then rinsed with 3% glacial acidic acid, and then stained for proteogylcans with 0.5% Alcian Blue (Lifeline Technologies Frederick, MD) in 3% glacial acetic acid overnight. Alcian blue stain was removed the following day, and samples were rinsed two times with acetic acid (pH 2.5). Samples were then allowed to air dry at room temperature.

#### 2.6 Actin cytoskeletal changes in ATDC5 cells in 2D:

Once ATDC5 cells reached approximately 70%, they were trypsinized, centrifuged, and counted so that 15,000 cells would be plated onto 35mm coverslips coated with collagen type II (Sigma Life Sciences). Cells were then incubated for 3 days before treatments were applied. After treatments were applied, samples were then placed into an incubator at 37°C for the duration of the treatment period. After this, the treatments were removed, washed with DPBS (Corning Cellgro), then fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), and permeablized with 0.1% triton X-100 (Fisher Scientifics, Fairviews, NJ) for 20 minutes at 4°C. After 20 minutes, samples were washed with DPBS and blocked over night with 3% Bovine Serum Albumin (Sigma Life Sciences). Samples were then stained with 488 Alexa Fluor Phalloidin (to visualize the actin cytoskeleton), covered with tin foil, and placed in a shaker at 35°C for 4 hours. Following the staining process, the phalloidin stain was removed, and DPBS was used to wash the cells. Afterwards, the coverslips were carefully mounted onto microscope slides with 15uL of the nuclear stain DAPI Prolong Gold (Life Technologies, Eugene, OR), and allowed to cure for three days before imaging.

#### 2.7 Actin cytoskeletal changes in ATDC5 cells in 3D

Once ATDC5 cells reached 70% confluence, cells were centrifuged and the supernatant was removed. Cells were then reconstituted in a 2.5mg/mL collagen type II solution composed of collagen II (Sigma Life Sciences) and DPBS (Corning Cellgro). The cell/collagen mixture was then diluted by 50% with a solution of 1% agar and DPBS for a final concentration of 250,000 cells/ µL. The cell/collagen-agar mixture was then quickly plated into 8 well Nunc<sup>TM</sup> chambers and incubated for at least 48 hours with DMEM (w/o phenol red+10% FBS) in 37°C and 95/5% air/CO2. After at least 48 hours, the appropriate treatments (stated at end of methods section) were applied to the cells. Following treatment period of 3 hours, the cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) and 0.1% triton X-100 (Fisher Scientifics) for 1 hour at 4°C and then washed with DPBS. Cells in the agar/collagen II matrix were then stained with 488 Alexa Fluor Phallodin to visualize the actin cytoskeleton for 4 hours covered with tin foil and at room temperature (25°C). After staining and DPBS wash, 350uL of DPBS was added to each well, which was then wrapped with Para-film, plastic wrap, and tin foil and placed at 4°C fridge prior to imaging.

#### 2.8 Confocal Microscopy

After cells have been stained with 488 phalloidin and DPBS added; 2D/3D immunofluorescence images were obtained using Zeiss LSM 510 Multiphoton

confocal microscope at 40x. Images were then analyzed with Zen Software (Zeiss, Thornwood, NY) and ImageJ.

#### 2.9 Statistical Analysis

Intracellular calcium concentration measured in the alginate beads were pooled together and averaged according to treatment groups. One-way analysis of variance (ANOVA) with Tukey-Kramer posthoc tests were used to determine the statistical significance of differences between the treatment groups. Significance was defined by a p-value <0.05.

#### Chapter 3

#### Results

# 3.1 IGF-1 and Rac-1 Inhibition on [Ca<sup>2+</sup>]<sub>i</sub> in ATDC5 cells grown on sodium alginate beads

To determine the effects of IGF-1 and Rac-1 inhibition on the  $[Ca^{2+}]_i$  response in ATDC5 cells grown on sodium alginate beads, I first used the PTI imaging system. The PTI system detected two different wavelengths of emitted light, 340nm and 380nm, which were then converted to a ratiometric unit (340nm/380nm) called intensity for this study. The intracellular  $[Ca^{+2}]_i$  in the ATDC5 sodium alginate bead samples can be seen in Figure 3. Throughout the whole imaging time period (10 minutes), control samples maintained a baseline intracellular  $[Ca^{2+}]_i$  of approximately 0.7 with a slight rise following addition of 1mL of hypotonic swelling. Addition of IGF-1 to chondrocytes found the baseline intracellular  $[Ca^{2+}]_i$  intensity was approximately 0.6. However, following the addition of 1mL of hypotonic swelling solution (HTS), there was a slight increase in the measured intracellular  $[Ca^{+2}]_{i}$ , followed by a return to baseline levels, before exhibiting a secondary increase in intracellular  $[Ca^{2+}]_i$ . Following this secondary increase, intracellular  $[Ca^{2+}]_i$  decreased to levels similar to those seen with the control treatment. Chondrocytes treated with Rac-1 inhibitor maintained a baseline intracellular  $[Ca^{2+}]_i$  around 0.5 intensity. Addition of HTS resulted in an increase in intracellular  $[Ca^{2+}]_i$  to around 0.55 intensity, which was maintained throughout the experiment. Chondrocytes treated with IGF-1 and Rac-1 inhibition exhibited a baseline intracellular [Ca<sup>2+</sup>]<sub>i</sub> slightly higher

than the Rac-1 inhibitor treatment. Chondrocytes subjected to hypotonic swelling following treatment with IGF-1 and Rac-1 inhibitor exhibited an increase in intracellular  $[Ca^{2+}]_i$  followed by a decrease in intracellular  $[Ca^{2+}]_i$  that reached levels similar to those with Rac-1 inhibitor treatment alone after 6 minutes. The means +SE of the baseline and  $\Delta$ HTS (changes after hypotonic swelling)  $[Ca^{2+}]_i$  of the 4 different treatments are presented in Figure 4. Comparisons among the different treatment groups in  $\Delta$ HTS revealed no significant differences There was a significant difference in the average baseline measurements between control treatment and Rac-1 inhibitor treatment (Tukey–Kramer method, P>0.05). Other pairs of comparisons in average baseline measurements were not significant. Differences in average  $\Delta$ HTS measurements were also not significant.



Figure 3: Intracellular Calcium [Ca<sup>2+</sup>]<sub>i</sub> levels of ATDC5 cells seeded in 3D sodium alginate bead culture following staining with Fura-2AM. The different groups all were exposed to their respective treatments for 3 hours and incubated before proceeding with staining. ATDC5 alginate bead samples were hypnotically swelled at 300s.



![](_page_30_Figure_1.jpeg)

# **3.2 IGF-1 and Rac-1 inhibitor effects on Actin stress fiber formation in 2D culture environment**

To determine the effects of IGF-1 and Rac-1 inhibitor on actin stress fiber formation in 2D, ATDC5 chondrocytes were treated, stained, and then imaged with Zeiss LSM 510 Multiphoton confocal microscope at 40x. Figure 5 shows the immunofluorescence images of ATDC5 chondrocytes plated on 2D culture environment after being stained with Alexa Fluor Phalloidin. These images show the organization of the actin stress fibers stained in ATDC5 cells on collagen II coated coverslips. The bright green striations seen in the ATDC5 cells are organized actin stress fibers that formed following three hour control treatment (Fig 5A). Compared to the control treatment, 3 hour IGF-1 treatment resulted in an increase in actin stress fiber formation (Fig 5B). ATDC5 chondrocytes treated with Rac-1 inhibitor showed in less organized actin fiber formation, but did not appear to be different from the stress fiber formations seen in other treatments (Fig 5C). IGF-1 and Rac-1 inhibitor combined treatment resulted in the appearance of organized actin stress fiber formation as seen in both the IGF-1 and control treatments (Fig 5D).

![](_page_32_Picture_0.jpeg)

(A) Control Treatment

![](_page_32_Picture_2.jpeg)

(B) IGF-1 Treatment

![](_page_32_Picture_4.jpeg)

(C) Rac-1 Inhibitor Treatment

![](_page_32_Picture_6.jpeg)

(D) Rac-1 Inhibitor + IGF-1 Treatment

Figure 5: Immunofluorescence images of ATDC5 Chondrocyte Cells plated on 2D Culture Environment A. Organization of actin fibers (green) in ATDC5 cells following control treatment. B. Organization of actin stress fibers following IGF-1 treatment. C. The organization of actin stress fibers following Rac-1 Inhibitor treatment. D. The organization of the actin stress fibers within ATDC5 cells following Rac-1 Inhibitor + IGF-1 treatments combined.

# **3.3 IGF-1 and Rac-1 inhibitor effects on Actin stress fiber formation in 3D culture environment**

To determine the effects of IGF-1 and Rac-1 inhibitor on actin stress fiber formation in 3D, ATDC5 chondrocytes were cultured in 3D, stained, and imaged with Zeiss LSM 510 Multiphoton confocal microscope. Figure 6 shows the immunofluorescence images of ATDC5 cells seeded in the 3D collagen II/agar culture environment after being stained for actin stress fibers with phallodin. Figure 6A shows the organization of actin fibers of ATDC5 cells in a 3D culture environment after control treatment. It is important to note that compared to the 2D images, stress fibers are not formed and instead show that cells in 3D culture environment exhibit staining for cortical actin around the periphery of the cells. Figure 6B shows the cortical actin organization of ATDC5 cells after three hour IGF-1 treatment. Figure 6C shows the cortical actin organization of ATDC5 cells after three hour Rac-1 inhibitor treatment, and Figure 4D shows the organization of cortical actin following three hour of combined IGF-1 and Rac-1 inhibitor treatment. Inhibition of Rac-1 in 3D culture appeared to have less organized cortical actin as compared to the control and IGF-1 treatments, which is different from its effects seen in 2D culture environments. The three hour combined IGF-1 and Rac-1 inhibitor treatment resulted in the interesting appearance of punctuated staining as seen in the Fig 6D.

![](_page_34_Picture_0.jpeg)

(A) Control Treatment

![](_page_34_Picture_2.jpeg)

(C) Rac-1 Inhibitor Treatment

![](_page_34_Picture_4.jpeg)

(B) IGF-1 Treatment

![](_page_34_Picture_6.jpeg)

(D) Rac-1 Inhibitor + IGF-1Treatment

Figure 6 :Immunofluoresence images of ATDC5 Cells seeded in 3D Agar/Collagen II Culture Environment.( A )Organization of cortical actin in ATDC5 cells following control treatment. Note the cortical actin arrangement. (B.) The cortical actin following the 3 hour IGF-1 treatment (C.)Cortical actin organization following 3 hour treatment of Rac-1 Inhibitor. (D.) Cortical actin organization in ATDC5 cells following the combined treatment of Rac-1 and IGF-1.

# 3.4 IGF-1 and Rac-1 Inhibitor effect on proteoglycan production in ATDC5 cells

To determine the effects of IGF-1 and Rac-1 inhibitor on proteoglycan synthesis, chondrocytes were cultured micromasses. Images of micromass cultures were taken and then converted to 8-bit grayscale with the use of ImageJ before analyzing pixel intensity to quantify the data. Figure 5 shows the resulting grayscale images of the micromass cultures. Figure 5A shows ATDC5 cells that were treated with the control treatment. The ATDC5 cells are located around the darker shaded areas, which represented the presence of proteoglycans as stained by Alcian Blue. The intensity of the shades of gray represented the amount of proteoglycans made by the cells. ATDC5 cells treated with the control treatment had an average pixel intensity of 105,757 pixels (Fig7). ATDC5 cells that were treated with the IGF-1 treatment also resulted in darker shades of gray (Fig 7B). IGF-1 treated ATDC5 chondrocytes had a lower average pixel intensity of approximately 93,629 pixels (Fig 8). With Rac-1 inhibitor treated chondrocytes it was observed that there were lower levels of proteoglycan as compared to the control and IGF-1 treatments based on the lighter intensity of the staining (Fig7C). Chondrocytes treated with Rac-1 inhibitor exhibited a lower average pixel intensity of about 90,599 pixels (Fig 8). Chondrocytes treated with IGF-1 and Rac-1 inhibitor exhibited a darker shade of gray as compared to Rac-1 inhibitor by itself, but lighter than either control or IGF-1 treatments (Fig 7D). IGF-1 and Rac-1 inhibitor treatment resulted in an average pixel intensity of 103,829 pixels (Fig 8). Differences between in the average pixel density between the treatment groups were not significant (P>.05).

![](_page_36_Picture_0.jpeg)

(A) Control Treatment

![](_page_36_Picture_2.jpeg)

(C) Rac-1 Inhibitor Treatment

![](_page_36_Picture_4.jpeg)

(D) IGF-1 + Rac-1 Inhibitor Treatment

Figure 7: ATDC5 Micromass cultures following treatment and Alcian Blue Staining following conversion to 8-bit grayscale image (A) Proteoglycan (gray) levels in ATDC5 micromass culture with control treatment. (B)
Proteoglycan content (blue) in ATDC5 micromass culture with IGF-1 treatment. (C) Proteoglycan (blue) levels in ATDC5 micromass culture with Rac-1 inhibitor treatment. (D) Proteoglycan content (blue) in ATDC5 micromass culture with IGF-1 plus Rac-1 inhibitor together.

![](_page_37_Figure_0.jpeg)

Figure 8: Average pixel intensity measure from ATDC5 chondrocytes among different treatments groups. Images of micromasses were obtained and then converted to 8-bit grayscale images and average pixel intensity was measured through ImageJ. Errors bars represent standard error.

#### **Chapter 4**

#### Discussion

The first aim of the study was to look at the effects of IGF-1 and Rac-1 inhibitor treatments on the intracellular concentration of  $[Ca^{2+}]_i$  and TRPV4 activity following hypotonic swelling. Based on the graph shown in Figure 1, it was observed that the different treatment groups all had differential effects on the intracellular  $[Ca^{2+}]_{i}$ . The differences seen between IGF-1 and Rac-1 inhibitor suggested that both had different effects on the TRPV-4 channel. Based on 2D studies of chondrocyte response (Gardinier, et al. in preparation) that showed IGF-1 lowered the Ca<sup>+2</sup> response through increased cell stiffness and actin cytoskeleton organization following HTS. I predicted measurements of intracellular  $[Ca^{2+}]_i$  from ATDC5 cells on sodium alginate beads should have lowered  $[Ca^{2+}]_i$  changes following HTS. However, this was not the case as ATDC5 chondrocytes treated with IGF-1 had baseline intensity measurements that was below the control, but exhibited an increase in  $[Ca^{2+}]$ following HTS. There then was a drop in intensity to levels similar to the baseline, followed by a secondary increase in  $[Ca^{2+}]_i$  near the end of the measurement period (Fig 3). Rac-1 as a member of RhoGTPase, has been linked to the formation of meshwork of actin fibers at the cell periphery below the cell plasma membrane. Therefore Rac-1 inhibition should have resulted in lowered baseline and  $\Delta HTS$  [Ca<sup>2+</sup>] levels because inhibition of Rac-1 would have prevented TRPV4 from being inhibited by actin fibers. Rac-1 inhibitor treated samples maintained an average baseline measurement below that of control and IGF- 1treatments before an increase in  $[Ca^{2+}]$ following HTS (Fig 3). The difference between what was expected to happen and

what actually happened with Rac-1 inhibition treatment may be attributed to the Rac-1 inhibitor used. There are alternative splice variants of Rac-1 such as Rac-1 B (Fiegen *et al.*, 2004). The Rac-1 inhibitor used in the experiment did not specify which variant of Rac-1 it inhibited, which could have impacted the Rac-1 inhibition results seen. The higher levels of intensity and the secondary increase in  $[Ca^{2+}]_i$  seen with IGF-1 treatment was not expected (Fig 3). It is also worth noting that the results in Figure 1 were obtained in room temperature (around 23°C) and TRPV4 channels are activated by moderate heat around 24-27°C (Watanabe *et al.*, 2003; Nilius *et al.*, 2004). Therefore the temperature of the room at which the experiment was conducted may have played a role in influencing the results. However, the results still showed that there are differences in chondrocyte  $[Ca^{2+}]_i$  response to HTS possibly due to the dimensions of the culture environment. The data from the first aim suggests that the effect of IGF-1 could be mediated by the dimensions of the culture environment.

The second aim of the study was to determine the effect of IGF-1 and Rac-1 inhibitor treatments on the formation of actin stress fibers and the cellular morphology in 2D and 3D culture environments. Here treatment of ATDC-5 chondrocyte treated with Rac-1 inhibitor blocks the effects of IGF-1 on the organization of actin in cells in 3D culture environment, but not in a 2D culture environment. The images from Figures 5 and 6 shows that there are differences to how these treatments impacted the organization of the actin cytoskeleton depending on the dimensions of the culture environment. From both figures showed that treatment with IGF-1 resulted in an increase in actin stress fibers in the 2D culture environment (Fig 5B), and an increase in cortical actin in the 3D culture environment (Fig 6B). This increase formation of actin stress fibers supports our lab's and other studies, that suggested that that IGF-1

can alter he formation of actin fibers to increase the stiffness of the cell (Taya *et al.*, 2001). The switch from stress fibers seen in the 2D culture environment to cortical actin in 3D culture environment has more to do with the dimensions of the culture environment chondrocytes were cultured in. These data suggest that the effects of IGF-1 are not limited by the dimensions of the culture environment because in both 2D and 3D environments, because it increased the formation of actin stress fibers and cortical actin respectively.

The opposite occurred with Rac-1 inhibitor treatment, which should have disrupted the formation of actin fibers. The Rac-1 inhibitor treatment had different effects on the ATDC5 cells depending on the dimensions of the physical environment. It was observed that Rac-1 inhibitor treatment did not alter the formation of cortical actin in ATDC5 cells grown in 3D culture environment, but there was a noticeable decrease in the organization of the cortical actin as compared to either control or IGF-1 treatments (Fig 6). However, in the 2D culture environment Rac-1 inhibitor treatment blocked actin stress fiber formation as seen with either the control treated or IGF-1 treated cells (Fig 5C). This difference between the 2D and 3D culture could mean that Rac-1 does play a role in formation of actin fibers, but only in 3D environment since treatment with Rac-1 inhibitor should have disrupted the organization of the cortical actin. The dimensional differences between IGF-1 and Rac-1 inhibitor suggested that IGF-1 may increase actin fiber formation through RhoA activation and not Rac-1 activation in 2D, and Rac-1 activation in 3D. RhoA is another member of the Rho GTPases family of proteins that, just like Rac-1, can bind to a number of different effector molecules such as some actin binding proteins. RhoA, B, and C, once activated can activate a downstream kinase target protein called

ROCK (Rho-associated protein kinase), which has been shown to phosphorylate a number of actin regulator proteins (Woods, Wang e Beier, 2005; Spiering e Hodgson, 2011). Studies have indicated that IGF-1 could bind with Rho GEFs (Guanine exchange factors) forming a IGF-1/Rho GEF complex that could activate the Rho-kinase signaling pathway leading to increase formation of actin stress fibers (Taya *et al.*, 2001). This might explain the results seen with the IGF-1 and Rac-1 inhibitor combined treatment.

Since both IGF-1 and Rac-1 inhibitor had different effects on the organization of the actin cytoskeleton, I looked at the physiological effects of these treatments. Culturing ATDC5 cells in micromass cultures and then staining with Alcian Blue, allowed for visualization of the levels of proteoglycan synthesized by the ATDC5 cells. The micromass culture was used as a 3D culture environment for the ATDC5 cells, especially the cells located near the center of the culture. The ATDC 5 cells near the center of the culture would have been in contact with other cells all around them, similar to the conditions in real life. Figure 7 showed how IGF-1 and Rac-1 inhibitor treatments affected the levels of proteoglycans. IGF-1 treated chondrocytes exhibited the levels of proteoglycan production that were similar to that seen with the control treatment (Fig 7B), but had lowered average pixel intensity (Fig 8). Many previous studies have shown that IGF-1 increases the synthesis of extracellular matrix proteins like collagen and proteoglycan (Starkman et al., 2005; Longobardi et al., 2006; Zhang et al., 2009). The exact mechanism behind this interaction between IGF-1 and proteoglycan synthesis is not well understood, but it has been suggested that IGF-1 may stimulate the PI3K signaling pathway in chondrocytes to increase proteoglycan synthesis (Starkman et al., 2005). Figure 3C showed that with ATDC5 micromass

culture treated with just Rac-1 inhibitor showed lowered levels of proteoglycan synthesis compared to both the control and IGF-1 treatments. Rac-1 inhibitor treated cells also exhibited lowered average pixel intensity than the other treatments (Fig 8). The organization of the actin cytoskeleton is important for expressing and maintaining chondrocyte phenotypes like production of ECM matrix materials (Haudenschild et al., 2009). One study found that flattened chondrocytes formed elongated actin stress fibers, which occurs in dedifferentiation, led to decreased synthesis of type II collagen and aggrecan expression (Blain, 2008). Since the actin cytoskeleton is also a common downstream target for many signaling pathways (Novakofski, Boehm e Fortier, 2009), changes to the actin cytoskeleton may eventually have impacts on changes to a cell's phenotype. This could have been the reason why Rac-1 inhibitor treated micromass cultures expressed suppressed proteogylcan synthesis even though inhibition of Rac-1 did not measurably alter the actin cytoskeletal organization. It was an interesting observation that the combined IGF-1 and Rac-1 inhibitor treatment showed higher level of proteoglycan synthesis (Fig 7D) and higher average pixel intensity (Fig 7) compared to those treated with just Rac-1 inhibitor, but not as high as the micromass cultures of IGF-1 or control treatments. This suggested that IGF-1 may have countered the effects of Rac-1 inhibitor on organization of the cortical actin and ultimately the synthesis of proteoglycan by the ATDC5 cells in the micromass cultures.

#### Chapter 4

#### Conclusion

My original was that IGF-1 has differential effects on the actin cytoskeletal organization and TRPV4 channel activity in ATDC5 chondrocyte cells in 2D/3D culture environments. Overall I found that IGF-1 does have differential effects on the actin cytoskeleton and TRPV4 activity depending on the dimensions of the environment. The first aim was to determine the effects of IGF-1 treatment on intracellular [Ca<sup>2+</sup>]<sub>i</sub> and TRPV4 activity following hypotonic swelling. I found that IGF-1 treatment tended to increase  $[Ca^{2+}]_i$  and resulted in a biphasic response despite my prediction that IGF-1 should have lowered  $[Ca^{2+}]_i$  based on 2D studies. Rac-1 inhibition resulted in lowered baseline and  $\Delta$ HTS measurements, when I predicted that it would have resulted in increased  $[Ca^{2+}]_{i}$  based on 2D studies. These data suggested that the effect of IGF-1 could be dependent on the dimensions of the culture environment. The second aim was to determine the effects of IGF-1 on actin stress fiber formation, cellular morphology, and chondrocyte phenotype. I found that IGF-1 treatment resulted in an increase of actin stress fibers and cortical actin in 2D and 3D respectively. The effects of Rac-1 inhibition seemed to be dependent on the dimensions of the culture environment. These data suggest that IGF-1 increased actin fiber formation through RhoA activation in 2D and Rac-1 activation in 3D environments. I also found that IGF-1 treated micromass cultures exhibited similar levels of proteoglycan synthesis to control treatments, supporting previous studies that have shown IGF-1 was an anabolic factor for chondrocytes. Micromasses treated with

Rac-1 inhibitor exhibited suppressed proteoglycan synthesis. Together, this information will contribute to our understanding of how chondrocytes function within a 3D culture environment, and IGF-1's differential effects. Future work should focus on identifying the punctuated staining seen IGF-1 + Rac-1 inhibitor treatment in 3D environment, as well as how temperature differences may affect changes to the actin cytoskeleton in 2D/3D culture environments. Work should also be done to more closely examine the baseline level activities seen in Figure 1.

#### References

Albertinazzi, C.; Cattelino, A.; de Curtis, I. Rac GTPases localize at sites of actin reorganization during dynamic remodeling of the cytoskeleton of normal embryonic fibroblasts. **J Cell Sci**, v. 112 ( Pt 21), p. 3821-31, Nov 1999. ISSN 0021-9533. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/10523517</u> >.

Albrecht, C. et al. Impact of 3-D culture on the expression of differentiation markers and hormone receptors in growth place chondrocytes as compared to articular chondrocytes. **International Journal of Molecular Medicine,** v. 23, p. 347-355, 2008.

Baker, B. M.; Chen, C. S. Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues. **J Cell Sci**, v. 125, n. Pt 13, p. 3015-24, Jul 2012. ISSN 1477-9137. Disponível em: < <a href="http://www.ncbi.nlm.nih.gov/pubmed/22797912">http://www.ncbi.nlm.nih.gov/pubmed/22797912</a> >.

Blain, E. J. Involvement of the cytoskeletal elements in articular cartilage homeostatis and pathology. **International Journal of Experimental Pathology**, v. 90, p. 1-5, 2008.

Brown, P. D.; Benya, P. D. Alterations in chondrocyte cytoskeletal architecture during phenotypic modulation by retinoic acid and dihydrocytochalasin B-induced reexpression. **J Cell Biol**, v. 106, n. 1, p. 171-9, Jan 1988. ISSN 0021-9525. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/3276712</u> >.

Caron, M. M. et al. Redifferentiation of dedifferentiated human articular chondrocytes: comparison of 2D and 3D cultures. **Osteoarthritis Cartilage,** v. 20, n. 10, p. 1170-8, Oct 2012. ISSN 1522-9653. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/22796508</u> >.

Daniels, K.; Solursh, M. Modulation of chondrogenesis by the cytoskeleton and extracellular matrix. **J Cell Sci**, v. 100 ( Pt 2), p. 249-54, Oct 1991. ISSN 0021-9533. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/1757484</u> >.

Everaerts, W.; Nilius, B.; Owsianik, G. The vanilloid transient receptor potential channel TRPV4: From structure to disease. **Progress in Biophysics and Molecular Biology**, v. 103, n. 1, p. 2-17, 2010.

Fiegen, D. et al. Alternative splicing of Rac1 generates Rac1b, a self-activating GTPase. **J Biol Chem,** v. 279, n. 6, p. 4743-9, Feb 2004. ISSN 0021-9258. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/14625275</u> >.

Gründer, T. et al. Bone morphogenetic protein (BMP)-2 enhances the expression of type II collagen and aggrecan in chondrocytes embedded in alginate beads. **Osteoarthritis Cartilage,** v. 12, n. 7, p. 559-67, Jul 2004. ISSN 1063-4584. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/15219571</u> >.

Haudenschild, D. R. et al. Characterization of the chondrocyte actin cytoskeleton in living three-dimensional culture: response to anabolic and catabolic stimuli. **Mol Cell Biomech,** v. 6, n. 3, p. 135-44, Sep 2009. ISSN 1556-5297. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/19670824</u> >.

Hwang, S. N. et al. Effect of Three-Dimensional Culture and Growth Factors on the Chondrogenic Differentiation of Murine Embryonic Stem Cells. **Stem Cells Express**, v. 24, p. 284-291, 2005.

Kerr, B. A. et al. Small GTPase protein Rac-1 is activated with maturation and regulates cell morphology and function in chondrocytes. **Exp Cell Res,** v. 314, n. 6, p. 1301-12, Apr 2008. ISSN 0014-4827. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/18261726</u> >.

Leddy, A. H. et al. Unraveling the mechanism by which TRPV4 mutations cause skeletal dysplasias. **Rare Diseases**, v. 2, n. 1, 2014.

Lin, Z. et al. The chondrocyte: biology and clinical application. **Tissue Eng,** v. 12, n. 7, p. 1971-84, Jul 2006. ISSN 1076-3279. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/16889526</u> >.

Longobardi, L. et al. Effect of IGF-I in the chondrogenesis of bone marrow mesenchymal stem cells in the presence or absence of TGF-beta signaling. **J Bone Miner Res,** v. 21, n. 4, p. 626-36, Apr 2006. ISSN 0884-0431. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/16598383</u> >.

Nilius, B. et al. TRPV4 calcium entry channel: a paradigm for gating diversity. **Am J Physiol Cell Physiol,** v. 286, n. 2, p. C195-205, Feb 2004. ISSN 0363-6143. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/14707014</u> >.

Novakofski, K.; Boehm, A.; Fortier, L. The small GTPase Rho mediates articular chondrocyte phenotype and morphology in response to interleukin-1alpha and insulinlike growth factor-I. **J Orthop Res**, v. 27, n. 1, p. 58-64, Jan 2009. ISSN 1554-527X. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/18634065</u> >. Nurminsky, D. et al. Regulation of chondrocyte differentiation by actin-severing protein adseverin. **Dev Biol,** v. 302, n. 2, p. 427-37, Feb 2007. ISSN 0012-1606. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/17097081</u> >.

Oh, C.-D.; Chun, J.-S. Signaling Mechanisms Leading to the Regulation of Differentiation and Apoptosis of Articular Chondrocytes by Insulin-like Growth Factor-1. **The Journal of Biological Chemistry**, v. 278, n. 38, p. 36563-36571, 2003.

Ridley, A. J. Rho-related proteins: actin cytoskeleton and cell cycle. **Curr Opin Genet Dev,** v. 5, n. 1, p. 24-30, Feb 1995. ISSN 0959-437X. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/7749321</u> >.

Sit, S. T.; Manser, E. Rho GTPases and their role in organizing the actin cytoskeleton. **J Cell Sci**, v. 124, n. Pt 5, p. 679-83, Mar 2011. ISSN 1477-9137. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/21321325</u> >.

Spiering, D.; Hodgson, L. Dynamics of the Rho-family small GTPases in actin regulation and motility. **Cell Adh Migr,** v. 5, n. 2, p. 170-80, 2011 Mar-Apr 2011. ISSN 1933-6926. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/21178402</u> >.

Starkman, B. G. et al. IGF-I stimulation of proteoglycan synthesis by chondrocytes requires activation of the PI 3-kinase pathway but not ERK MAPK. **Biochem J,** v. 389, n. Pt 3, p. 723-9, Aug 2005. ISSN 1470-8728. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/15801908</u> >.

Taya, S. et al. Direct interaction of insulin-like growth factor-1 receptor with leukemia-associated RhoGEF. **The Journal of Cell Biology,** v. 155, n. 5, p. 809-819, 2001.

Umlauf, D. et al. Cartilage biology, pathology, and repair. **Cell Mol Life Sci,** v. 67, n. 24, p. 4197-211, Dec 2010. ISSN 1420-9071. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/20734104</u> >.

Wang, G.; Beier, F. Rac1/Cdc42 and RhoA GTPases antagonistically regulate chondrocyte proliferation, hypertrophy, and apoptosis. **J Bone Miner Res,** v. 20, n. 6, p. 1022-31, Jun 2005. ISSN 0884-0431. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/15883643</u> >.

Watanabe, H. et al. Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. **Nature,** v. 424, n. 6947, p. 434-8, Jul 2003. ISSN 1476-4687. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/12879072</u> >.

Woods, A.; Wang, G.; Beier, F. RhoA/ROCK signaling regulates Sox9 expression and actin organization during chondrogenesis. **J Biol Chem,** v. 280, n. 12, p. 11626-34, Mar 2005. ISSN 0021-9258. Disponível em: < <a href="http://www.ncbi.nlm.nih.gov/pubmed/15665004">http://www.ncbi.nlm.nih.gov/pubmed/15665004</a> >.

Woods, A.; Wang, G.; Beier, F. Regulation of chondrocyte differentiation by the actin cytoskeleton and adhesive interactions. **J Cell Physiol**, v. 213, n. 1, p. 1-8, Oct 2007. ISSN 0021-9541. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/17492773</u> >.

Zhang, M. et al. IGF-1 regulation of type II collagen and MMP-13 expression in rat endplate chondrocytes via distinct signaling pathways. **Osteoarthritis Cartilage,** v. 17, n. 1, p. 100-6, Jan 2009. ISSN 1522-9653. Disponível em: < <u>http://www.ncbi.nlm.nih.gov/pubmed/18595745</u> >.