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DEPARTMENT OF BIOMEDICAL ENGINEERING

THE EFFECT OF MICROFIBER MORPHOLOHY AND CELL DENSITY ON THE LOCALIZATION OF YAP PROTEIN IN MESENCHYMAL STEM CELLS

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A thesis submitted in partial fulfillment of the requirements for baccalaureate degrees in Biomedical Engineering and Mechanical Engineering with honors in Biomedical Engineering in addition to the requirements for a master's degree in Bioengineering

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ABSTRACT

Mesenchymal stem cells (MSCs), a non-hematopoietic adult derived stem cell found in the bone marrow of mammals, are capable of differentiating into many cell types of the mesenchyme, including osteoblasts, chondrocytes, adipocytes, and myoblasts. The ability to easily harvest and grow these stem cells has caused MSCs to become a large focus of research in regenerative medicine. Past studies have shown that combinations of chemical and mechanical stimulus, including specific growth factors and extracellular fiber morphology, can be used to influence the differentiation of MSCs into specific cell types. However, the underlying signaling pathways controlling how MSCs respond to different extracellular signals are not as well understood. Recently, Yes-associated protein (YAP), a well-known regulator and coactivator in the Hippo Signaling Pathway, has been shown to function as a nuclear transducer of mechanical signals. YAP is now known to transduce many mechanical signals, including ECM rigidity, cell density, and tensional forces, into cell responses.

In this study, the effect of microfiber morphology and cell confluence on the localization and concentration of YAP protein in mouse mesenchymal stem cells (mMSCs) was investigated. mMSCs were grown on both flat PMMA surfaces and PHEMA surfaces coated in PMMA microfibers at different cell densities. The cells were allowed to incubate for 24 hours, then were examined using immunofluorescence and DIC microscopy, as well as Western Blot to quantify YAP expression and localization. In a follow up experiment, the effect of different fiber diameters as well as cell confluency on YAP expression and localization was examined. In addition to immunofluorescence and DIC microscopy, samples were separated into cytoplasmic and nuclear components. Western blotting was then used to quantify changes in nuclear and cytoplasmic YAP expression as a function of fiber diameter. Results suggested that cell-cell contact and cell microenvironment have an effect on mMSC YAP localization. Cell microenvironment was also shown to affect the overall YAP concentration in mMSCs.

TABLE OF CONTENTS

| LIST OF FIGURES | .iv |
|--|-------|
| LIST OF TABLES | . vii |
| ACKNOWLEDGEMENTS | .ix |
| Chapter 1 Literature Review | . 1 |
| A Brief History of Tissue Engineering | .1 |
| Embryonic Stem Cells | .3 |
| Adult Derived Stem Cells | .4 |
| Mesenchymal Stem Cells | .5 |
| Controlling MSC Properties through ECM Physical Mechanisms. | .7 |
| YAP and TAZ | .8 |
| YAP and TAZ as Mechanotransducers | .9 |
| YAP and TAZ Mesenchymal Stem Cell Mechanobiology | .10 |
| Chapter 2 Methods | .12 |
| Procedures | .12 |
| Polymer Selection | .12 |
| Solution Preparation | .14 |
| Surface Creation | .15 |
| Fiber Creation | .17 |
| Cell Thawing | .20 |
| Cell feeding: | .21 |
| Cell Passaging | .21 |
| Cell Seeding: | .23 |
| Sample Lysing: | .24 |
| Cell Staining: | .25 |
| Immunofluorescence Microscopy | .26 |
| Cell Fractionation | .27 |
| Western Blot | .27 |
| Experiment 1: Effect of Cell Microenvironment and Cell Density on YAP Expression | .33 |
| Experimental Setup | .33 |
| Quantification | .35 |
| Statistical Analysis | .35 |
| Experiment 2: Effect of Fiber Morphology and Cell Density on YAP Expression and | |
| Localization | .37 |
| Experimental Setup | .37 |
| Quantification | .38 |
| Statistical Analysis | .39 |
| - | |
| Chapter 3 Results | .42 |

Experiment 1: Effect of Cell Microenvironment and Cell Density on YAP Expression.42

| Eccentricity | 46 |
|---|----|
| YAP | 48 |
| Experiment 2: Effect of Fiber Morphology and Cell Density on YAP Expression and | 1 |
| Localization | 53 |
| Fiber Size | 56 |
| Area58 | |
| Eccentricity | 60 |
| YAP | 63 |
| Chapter 4 Discussion | 70 |
| Experiment 1 | 70 |
| Experiment 2 | 72 |
| Sources of Variance and Error | 74 |
| Improvements and Future Works | 77 |
| Chapter 5 Conclusion | 79 |
| BIBLIOGRAPHY | 80 |

LIST OF FIGURES

Figure 1: This figure details the spin-coater used to prepare polymer surfaces for both

| Figure 2: Electrospinning Setup used to prepare fiber samples. An electric field draws polymer from the syringe tip towards the copper plate, where they are deposited onto the cover slip as microfibers |
|--|
| Figure 3: Illustrations of the surface samples prepared in the experiments. Figure 1(a) illustrates a coverslip evenly coated with PMMA. This creates a flat 2D surface for the cells to adhere to. In contrast, Figure 1(b) depicts a coverslip evenly coated with PHEMA, which is in turn coated with PMMA fibers. The PHEMA coating discourages cell binding to the flat surface, allowing for the isolation and analysis of cell behavior when binding to 3D microfibers. 19 |
| Figure 4: Western blot setup used in all experiments. The proteins are forced through the gel by an electric current, which separates the proteins by size. The Laemmli band can be seen running down the gel. Once the target bands approach the bottom of the gel, the running phase it stopped and the gel is prepared for the transfer of proteins to the membrane28 |
| Figure 5: mMSCs seeded on PMMA fibers imaged for DNA (blue), actin (red) and YAP protein (green). Images in the top row display cells seeded at a high density, while images on the bottom row display cells seeded at a low density. Images in the second and third columns are the YAP and DNA channels, respectively |
| Figure 6: mMSCs seeded on flat PMMA surfaces imaged for DNA (blue), actin (red) and YAP protein (green). Images in the top row display cells seeded at a high density, while images on the bottom row display cells seeded at a low density. Images in the second and third columns are the YAP and DNA channels, respectively |
| Figure 7: Area interval plots displaying the samples means for fiber and flat samples seeded at both high and low cell densities. The bars are one standard error from the mean |
| Figure 8: Eccentricity Interval Plots displaying the samples means for each test group. The bars are one standard error from the mean |
| Figure 9: Outlines of the Nucleus and Cell Membrane overlaid onto the YAP channel of a high confluency fiber sample. The YAP intensity was adjusted in ImageJ to aid in the identification of YAP nucleus localization |
| Figure 10: Fraction of mMSCs with significicant nucleus YAP intensities for each sample group.49 |
| Figure 11: Western Blot stains for YAP and tubulin. Membrane 1 represents cells seeded on fiber samples, while membrane 2 represents cells seeded on flat samples. YAP and tubulin bands can be seen for the high cell density groups for both the fiber and flat samples. YAP and tubulin bands are not visible on the low cell density samples due to insufficient concentrations of the YAP and tubulin proteins |

| Figure 12: This interval plot shows the YAP intensity of each sample when normalized to its corresponding tubulin intensity. The error bars are one standard error from the mean. The figure illustrates the fiber samples had a Normalized YAP concentration greater than those of the PMMA surface samples |
|--|
| Figure 13: mMSCs seeded on flat samples imaged for DNA (blue), actin filaments (red), and YAP protein (green). The top row is seeded at a high cell density, while the bottom row is seeded at a low cell density |
| Figure 14: mMSCs seeded on large diameter fibers imaged for DNA (blue), actin filaments (red), and YAP protein (green). The top row is seeded at a high cell density, while the bottom row is seeded at a low cell density |
| Figure 15: mMSCs seeded on medium diameter fibers imaged for DNA (blue), actin filaments (red), and YAP protein (green). The top row is seeded at a high cell density, while the bottom row is seeded at a low cell density |
| Figure 16: mMSCs seeded on small diameter fibers imaged for DNA (blue), actin filaments (red), and YAP protein (green). The top row is seeded at a high cell density, while the bottom row is seeded at a low cell density |
| Figure 17: mMSCs seeded on the smallest diameter fibers imaged for DNA (blue), actin filaments (red), and YAP protein (green). The top row is seeded at a high cell density, while the bottom row is seeded at a low cell density |
| Figure 18: Fiber diameter interval plots displaying the sample means for all fiber groups used in this experiment. The error bars are one standard error from the mean |
| Figure 19: Area interval plots displaying the sample means for all surface types seeded at both high and low cell densities. The bars are one standard error from the mean |
| Figure 20: Eccentricity interval plots displaying the samples means for all surface types seeded at both high and low cell densities. The bars are one standard error from the mean |
| Figure 21: Fraction of mMSCs with significant nucleus YAP intensities for each sample group. 64 |
| Figure 22: Membranes stained for YAP and tubulin in experiment 2. Green bands are tubulin, while red bands are YAP. For both membranes, the first 8 lanes are two flat samples, two large fiber samples, two medium fiber samples, and two small fiber samples. The samples alternate between nuclear and cytoplasmic extracts. In membrane 1, the last lane is the smallest fiber nuclear extract, while in membrane 2, the last lane is the smallest fiber cytoplasmic extract |
| Figure 23: This interval plot displays the tubulin intensity of each sample group for experiment 2. The error bars are one standard error from the mean |
| Figure 24: This interval plot displays the YAP intensity normalized to cytoplasmic tubulin intensity of each sample group for experiment 2. The error bars are one standard error from the mean |

Figure 25: This figure depicts 4 cells with differing levels of nuclear YAP localization......75

LIST OF TABLES

| Table 1: Immunofluorescence Microscopy Parameters 26 |
|--|
| Table 2: 10% running gel recipe. All values are in ml |
| Table 3: Stacking gel recipe. All values are in ml. 30 |
| Table 4: PMMA Electrospinning Settings Used to Prepare Fiber Samples in Experiment 133 |
| Table 5: This table details a summary of experiment parameters for experiment 1, including samples groups, independent variables, and number of samples for each group |
| Table 6: Electrospinning Parameters for Large, Medium, Small, and Smallest PMMA fibers for Experiment 2 37 |
| Table 7: This table details a summary of experiment parameters for experiment 2, including samples groups, independent variables, and number of samples for each group |
| Table 8: Summary of mMSC area data obtained from the Cell Profiler analysis |
| Table 9: Tukey Pairwise Comparisons: Response = Area (μm ²), Term = Surface. Means that do not share a letter are significantly different45 |
| Table 10: Tukey Pairwise Comparisons: Response = Area (μm ²), Term = Cell Density. Means that do not share a letter are significantly different |
| Table 11: Summary of eccentricity data from the cell imaging 46 |
| Table 12: Tukey Pairwise Comparisons: Response = Eccentricity, Term = Surface. Means that do not share a letter are significantly different |
| Table 13: Tukey Pairwise Comparisons: Response = Eccentricity, Term = Cell Density. Means that do not share a letter are significantly different |
| Table 14: This table shows the number of cells with significant nuclear localized YAP concentrations in each sample group. 49 |
| Table 15: Summary of the fiber diameter measurements obtained from the measurements taken from each fiber type. 56 |
| Table 16: Tukey Pairwise Comparison: Response =Diameter, Term=Surface. Means that do not share a letter are significantly different |
| Table 17: Summary of mMSC area data obtained from the Cell Profiler analysis for experiment 258 |
| Table 18: Tukey Pairwise Comparisons: Response = Area (μm ²), Term = Surface. Means that do not share a letter are significantly different |
| Table 19: Tukey Pairwise Comparisons: Response = Area (μ m ²), Term = Cell Density. Means that do not share a letter are significantly different 60 |

| Table 20: Summary of mMSC eccentricity data obtained from the Cell Profiler analysi experiment 2 | s for 60 |
|---|----------------|
| Table 21: Tukey Pairwise Comparisons: Response = Eccentricity, Term = Surface. Me not share a letter are significantly different | ans that do |
| Table 22: Tukey Pairwise Comparisons: Response = Eccentricity, Term = Cell Density that do not share a letter are significantly different | v. Means 62 |
| Table 23: This table shows the number of cells with significant nuclear localized YAP concentrations in each sample group for experiment 2 | 63 |
| Table 24: Pairwise Comparisons: Response = Normalized Cytoplasmic YAP Intensity, Surface. Means that do not share a letter are significantly different | Term = 69 |

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Chapter 1

Literature Review

Tissue engineering is a field defined by the utilization of living cells, biocompatible materials, as well as both biochemical and physical factors to create tissue like structures. The primary goal of tissue engineering is to either repair an injury in the body or to replace the function of a failing organ. Tissue engineering differs from other medical therapies in the use living cells, either alone or in combination with nonliving materials, as opposed to utilizing nonliving materials alone. In addition to implanting constructs directly into tissue, tissue engineering also has applications in extracorporeal life support systems, such as bio-artificial livers and kidneys [1]. Mesenchymal Stem cells are a popular subject of research due to their ability to differentiate into many tissues of the mesenchyme and their accessible presence in adult mammals, including mice and humans. YAP has shown some ability to transduce signals from the cell environment to the cell nucleus, where it affects the cell's phenotypic expression. This phenomenon is heavily investigated in this paper.

A Brief History of Tissue Engineering

First developed and produced in the late 1970s and early 1980s, skin grafting techniques were some of the first tissue-based therapies. This period is believed by many to be the start of modern tissue engineering [1]. One of the first successful artificial skin products was Epicel, developed by Howard Green at Harvard Medical School. Epicel is used to treat burn victims that do not possess enough viable skin to be treated with traditional autografting techniques. Keratinocytes, isolated from a biopsy from the patient, are grown in vitro by co-culturing with a feeder layer of mouse mesenchymal cells [2]. This allows the patient cells isolated from the biopsy to grow several thousand-fold in weeks. Sheets of the grown keratinocytes are placed over the burn sites, facilitating tissue regrowth. Because Epicel is manufactured with and contains residual amounts of murine cells, the FDA considers it a xenogeneic product, the first of its kind in the field [1].

In the 1990s, artificial skin products like Epicel, as well as some cartilage products, were successfully commercialized. The success of these early tissue engineering applications generated huge enthusiasm in the field, encouraging research into treatments and therapies for many other body tissues. Unfortunately, the results were less fruitful than earlier skin and cartilage-based research. Skin and cartilage do not require extensive vascularization. As a result, simply combining the cells and matrix, then implanting them was successful for these therapies. In contrast, most other body tissue, such as neural and bone tissue, require significant vascularization in order to survive in vivo. As a result, the research failed to obtain the same level of success displayed in skin and collagen applications.

In the early 2000s, the tissue engineering research boom subsided, resulting in a decline in the industry. However, this decline was offset by growth in the field of regenerative medicine, a specific branch of tissue engineering that utilizes stem cells instead of already differentiated cells as a source. In particular, two types of stem cells were investigated. Embryonic stem cells (ESCs), isolated from the inner cell mass of a developing blastocyst, are pluripotent, possessing the potential to produce every type of cell type and tissue in the body [3]. In contrast, mesenchymal stem cells (MSCs), a type of adult derived stem cell, are isolated from the bone marrow. They are multipotent, having the potential to different cell types has caused them to become the focus of many researchers in the field of regenerative medicine. However, many of the ethical limitations of embryonic stem cells have caused MSCs to become the primary focus of therapy targeted research in recent years.

Embryonic Stem Cells

Scientists were aware of the existence of embryonic stem cells since the mid-20th century. Murine (mouse) embryonic stem cells were first isolated in 1981 from the inner cell mass of a developing mouse blastocyst, then successfully grown in vitro [5]. Embryonic stem cells have since been shown to differentiate into all cell lineages. Additionally, though the presence of undifferentiated embryonic stem cells is short lived in the embryo, they have been shown to propagate indefinitely in an undifferentiated state when they are grown in the presence of leukemia inhibitory factor in vitro [6]. The isolation of mouse ESCs cultivated a new path for tissue engineering. If embryonic stem cells could be derived for humans, their multilineage differentiation capacity could be used for cell-based therapies where the desired cell type could be produced from the source ESCs in lab. Human stem cells were derived in 1998, a milestone that brought the idea of using regenerative medicine to treat human disease closer to reality [7].

However, the use of human embryonic stem cells in research and treatment has sparked significant debate regarding its morality. For research, human ESCs are typically derived from the 'spare' embryos left over after in vitro fertilization. These embryos, created with the informed consent of the parents for the purpose of reproduction, are then used in research [8]. The controversy stems from the destruction of the embryos during research. Many groups, both religious and otherwise, believe that human life begins at conception, making the destruction of an embryo analogous to killing a human being. Many arguments have been made as to whether the embryos should have the same rights as fully developed humans. This has complicated the support for commercializing embryonic stem cell therapies. Alternative methods of obtaining human embryonic stem cells, including somatic nuclear transfer (cloning), bypasses the need to destroy a 'naturally' created embryo. This method was first successfully completed in sheep, where the nucleus of a somatic cell was transferred into an enucleated oocyte, leading to the normal development of an embryo in a significant proportion of the cases [9]. This practice has since been completed on several other mammals, including mice, goats, and cows [10-12]. Using cloned

embryo as a source of ESCs has the potential to expedite the process of regenerative medicine, as it would circumvent the problems associated with cell rejection that typically plagues tissue engineering. However, the creation of cloned human embryos specifically for use in therapies and research is even more ethically loaded than using spare embryos from in vitro fertilization procedures. This is because some of the cloned embryos created in animal trials developed into live young when they were implanted into host mothers [10-13]. The possibility of generating live human clones is a global ethical controversy sufficient to get the application of somatic nuclear transfer using human cells banned in most western countries [14].

The use of embryonic stem cells in regenerative medicine is wrought with controversy. Utilizing embryonic stem cells obtained from in vitro fertilized fetuses introduces the risk of rejection of the foreign cells by the host, while obtaining patient specific embryonic stem cells via cloning has been deemed too ethically controversial to pursue. Fortunately, there are other types of stem cells within the body. Mesenchymal stem cells, a type of adult derived stem cell found in the bone marrow, are multipotent, meaning they are able to differentiate into a number of different cell types. Due to their ability to be harvested directly from patients, combined with their multipotent differentiation potential, many labs have turned to adult derived stem cells as the future of regenerative medicine.

Adult Derived Stem Cells

Adult stem cells can be broadly defined as undifferentiated cells found among the differentiated cells in tissues and organs [15]. Like embryonic stem cells, they can renew themselves indefinitely, albeit with some phenotypic drift overtime. Additionally, adult derived stem cells are multipotent, meaning they can differentiate to yield some or all of the major cell types in the tissue or organ in which they are found [15]. All adult stem cells are diploid cells of the body. As a result, they are often referred to as somatic stem cells. Their role is thought to be to maintain and repair the tissues in which they are found.

Furthermore, their ability to differentiate into multiple tissue types has led researchers and clinicians to ponder whether adult stem cells can be used in autologous regenerative medicine and organ repair.

Adult derived stem cells were first discovered in the bone of mice in the late mid 1970s [16]. Since then, adult stem cells have also been discovered in organs and tissues throughout the body, including the brain, bone marrow, peripheral blood, skeletal muscle, teeth, heart, and liver. A number of stem cell types can be found in the bone marrow. Hematopoietic stem cells give rise to all of the red blood cells, white blood cells and platelets in the body. In contrast, mesenchymal stem cells, a nonhematopoietic stem cell, can differentiate into many of the cell types of the mesenchyme, including bone, cartilage, and fat cells [17]. This study focuses primarily on mesenchymal stem cell.

Mesenchymal Stem Cells

The theory that nonhematopoietic stem cells exist in the bone marrow was first proposed in the late 1800s by Conheim, whose work suggested that bone marrow may be the source of the fibroblasts that deposit collagen fibers during wound repair [17]. Starting with the work of Friedenstein in the mid-1970s, these cells were shown to differentiate into other mesenchymal cells. Using mouse bone marrow as a source, the samples were placed in culture dishes, and the hematopoietic cells were removed. The remaining cells were heterogeneous. However, the most tightly adherent cells were spindle shaped and remained inactive for 2 to 4 days. After which, they began to divide rapidly. It was also observed that these spindle shaped cells could differentiate into colonies of bone or collagen cells [16]. Follow up experiments from Friedenstein and other researchers concluded that these cells were multipotent and could differentiate into osteoblasts, chondrocytes, adipocytes, and myoblasts [18, 19]. The term mesenchymal stem cell (MSC) was adopted for these cells, due to their ability to differentiate into many of the different cells of the mesenchyme. Human mesenchymal stem cells (hMSCs) were later isolated from bone marrow in 1999, opening up the potential uses of MSCs in regenerative medicine [20, 21].

Of particular interest in our studies are the utilization of human mesenchymal stem cells in the generation of osteogenic tissue. Many techniques can be employed to stimulate hMSCs to differentiate into osteoblasts, as well as other tissue types in vitro. A number of growth factors have been shown to affect the activity of MSCs in vitro. However, it should be noted that the effects of specific growth factors can vary between species tested (mouse, rat, human, etc.). In order to encourage differentiation of MSCs into osteoblasts in vitro, the cells are traditionally incubated as a monolayer of confluent cells with ascorbic acid, Beta-glycerophosphate and dexamethasone for approximately two to three weeks. It has been observed that the cells form aggregates and increase their expression of alkaline phosphate [19]. In addition, calcium accumulation can also be observed with time, another indicator osteoblast formation. Similarly, to promote adipogenic differentiation, MSCs are cultured with the dexamethasone, insulin, isobutyl methyl xanthine, and indomethacin [19]. Using other combinations of chemicals, MSCs have been shown to differentiate into chondrocytes, tenocytes, skeletal myocytes, neurons, and even endothelial cells. The ability to chemically guide the differentiation pathways of MSCs has further increased their relevance in the growing field of regenerative medicine.

However, the use of chemicals to control differentiation presents a number of obstacles when applied to an in vivo environment. Many of the chemicals used to direct the differentiation of MSCs in vitro are not conducive to the environments in vivo. In addition, it is unlikely that these chemicals provide the same signals MSCs receive naturally in vivo that promote osteogenesis [19]. Alternative methods need to be pursued in order to create a method of stimulating desired cell phenotypic expression in a living extracellular environment. As a result, scientists began to explore the effect of the extracellular environment of MSCs on their differentiation and phenotypic expression.

Controlling MSC Properties through ECM Physical Mechanisms.

Understanding the response of human mesenchymal stem cells to the shape and orientation of their environment would be a huge benefit to the development of artificial extracellular environments, which would in turn have many applications in the field of regenerative medicine. Patient derived hMSCs could be seeded onto specific extracellular matrix scaffolds. The geometry of the scaffold would facilitate a specific differentiation path from the hMSCs. Implanting the scaffold along with hMSCs at the site of injury would encourage the growth of the desired type of tissue.

As stated above, chemicals can be used to stimulate MSCs to differentiate into osteoblasts, chondrocytes, tenocytes, skeletal myocytes, neurons, and endothelial cells. However, many of the chemicals used to stimulate MSC phenotypic expression and differentiation are not conducive to an in vivo environment. Fortunately, studies have shown that by altering the shape of an artificial extracellular environment, MSC responses can be elicited that are similar to those achieved by chemical stimulation [22]. The ability of cells to respond to the shape of their environment has been termed contact guidance. Responses in MSCs include migration, adhesion, cytoskeletal organization and gene regulation [22]. Currently, the focus of many researchers is to understand why specific cues from the extracellular environment stimulates specific cell responses.

It has been shown that the extracellular environment presents a number of physical mechanisms which serve to regulate the cells in terms of their shape, plasticity, as well as phenotypic and genotypic expression. Such physical mechanisms include cell-cell interactions, as well as extracellular matrix (ECM) geometry at both the micrometer and nanometer scale, ECM elasticity, and mechanical signals transmitted from the ECM to the cells [23]. However, the exact mechanism responsible for the mechanotransduction of the cell microenvironment into specific MSC responses remains a mystery. Currently, the MSC can be viewed as a "black box". An input is applied to the cell in the form of a physical mechanism from the environment. The cell then transduces this physical signal into an output in the form of a cell response. However, the exact mechanisms and signals that occur inside the cell, from the interaction of surface proteins to the output, are not well understood. An improved understanding of the mechanisms involved in the interactions of MSCs with their extracellular environment will serve to increase the precision and accuracy of scientists in dictating the desired phenotypic expression of mesenchymal stem cells. Recently, YAP and TAZ, initially identified as signaling proteins of the Hippo Signaling pathway, have been identified as nuclear transducers that play a significant role in linking mechanical forces to cell fate [24].

YAP and TAZ

Yes-associated protein 1, also known as YAP1 or YAP, is a transcriptional regulator protein responsible for activating the transcription genes that result in increased cell proliferation, as well as suppressing apoptotic genes. YAP and TAZ are well known to be key downstream effectors of the Hippo Signaling Pathway, a kinase cascade activated by cell-cell contact that slows cellular proliferation and facilitates apoptosis, resulting in the controlled size of organs. Furthermore, the cascade ends up inhibiting YAP and TAZ downstream via phosphorylation, resulting in a negative feedback loop that regulates YAP and TAZ expression [25]. In some mutations affecting the Hippo signaling pathway, YAP and TAZ can become upregulated, leading to the overgrowth of tissue. Overexpression of YAP has been identified in a number of human cancers, which are marked by uncontrolled cell proliferation and lack of apoptosis [26]. High YAP and TAZ concentrations have also been shown to result in higher proliferation rates in cells, including MSCs. [25] However, a number of recent studies and reports have shown that a significant proportion of YAP and TAZ regulation is due to mechanotransduction [24].

YAP and TAZ as Mechanotransducers

Many recent studies have demonstrated that YAP and TAZ also function as nuclear transducers of mechanical information. The activation of YAP and TAZ is described by the localization of the proteins in the nucleus, where they function as transcriptional co-regulators that utilize TEAD factors as DNA-binding platforms. This results in increased transcription of specific genes [24]. In contrast, YAP/TAZ are inactivated when they are in the cytoplasm, where they are the target of proteasomal degradation [25]. YAP and TAZ have been shown to transduce many mechanical signals into cell responses, including cell-cell contact, ECM rigidity and topology, cell and tissue geometries, tensional forces, as well as macroscopic forces, such as blood flow and muscle contractions. In cells experiencing low mechanical signaling, such as rounded cells attached to a soft ECM, YAP/TAZ are primarily localized in the cytoplasm. In contrast, in cells cultured on a stiff ECM, YAP/TAZ are primarily localized in the nucleus. Furthermore, cells that cover a larger adhesive area have more YAP localized in the nucleus then cells covering a small adhesive area. Additionally, decreased cell-cell interactions, stiff 3D matrices, and cellular stretching have all been shown to increase the localization of YAP protein in the nucleus [24]. All of these external mechanical forces are detected at the cell surface, primarily via cell adhesion molecules such as integrins and cadherins. In addition to external signals mentioned above, YAP and TAZ have also been implicated as transducers of intracellular mechanical signals, as actin contractility and increased actin cytoskeleton tension results in increased YAP/TAZ activation [24].

9

YAP and TAZ Mesenchymal Stem Cell Mechanobiology

In adults, cells with high nuclear YAP concentrations are typically found in regions with high concentrations of either somatic stem cells or progenitor cells. This suggests that YAP/TAZ signaling is elevated in stem cells. Furthermore, the proteins appear to be dispensable for the functioning of normal tissue homeostasis, as levels of mechanical stimulation of normal epithelial tissue are below the threshold necessary to facilitate YAP/TAZ responses. However, in mesenchymal stem cells, YAP and TAZ are believed to function as mechanotransducers that guide MSC differentiation [24].

It is well accepted that differentiation of MSCs into various cell types can be dictated by mechanical inputs from the cell's microenvironment [27]. This can be demonstrated by observing differentiation patterns when MSCs are allowed to stretch and spread out, which result in osteoblast formation, or by encouraging round cell formation by confinement, which results in adipocyte formation [24]. The contractility of the actomyosin skeleton has been shown to play a key role in the determination of mechanically driven cell fate, as inhibiting actomyosin contractile units blocks osteogenesis in favor of adipogenesis. As stated above, the elasticity of the underlying substrate was also shown to play a role in guiding differentiation. Until recently however, the nuclear effectors guiding MSC mechanosensitivity have remained a mystery. In regard to this, recent studies have shown that YAP and TAZ depletion significantly impairs MSC osteogenic differentiation, even when they are seeded on a stiff substrate, which typically favors osteogenic differentiation. Additionally, overexpression of YAP and TAZ have been shown to encourage osteogenic differentiation of MSCs plated on soft substrates, which typically encourage adipogenic differentiation [28]. These findings suggest that YAP and TAZ are a key part of the pathway required for mechanically induced osteogenic and adipogenic differentiation. That said, further research and investigation is required to fully elucidate the role and function of these two effector proteins in the mechanotransduction of MSCs.

In addition to mechanical signals from their corresponding ECM or substrate, MSC fate is also mechanically regulated via contact with other cells. This has been demonstrated by the engagement of cadherin 2, a cell adhesion molecule involved in cell-cell contact, which results in a reduced contractile state of the cell. This state is in turn paired with reduced YAP and TAZ activation in MSCs [29]. With few exception, MSCs engage in both cell-cell and cell-ECM contact throughout their development and life. It would be interesting to see which signal type, ECM or cell-cell, tends to dominate the mechanotransduction pathways of MSCs in the presence of both.

The overall goal of the research presented in this thesis was to investigate the localization of YAP protein in response to both changes in three-dimensional cellular environments as well as cell confluency. Utilizing a combination of electrospinning and spin coating techniques, PMMA microfiber and surface environments were seeded with mesenchymal stem cells. Samples were seeded at both high and low cell densities to observe how the combination of cell-cell and cell-ECM signals effect YAP localization and expression. Employing analysis techniques including immunofluorescence microscopy, cell fractionation, and western blotting, the effect of fiber presence, fiber size, fiber orientation, and fiber density, as well as cell confluency, on YAP concentration and localization in mesenchymal stem cells was investigated. Using the results of these experiments, we aimed to draw conclusions regarding the role of YAP within the mechanotransduction pathways of MSCs, as well as suggested possible methods of influencing YAP localization to guide MSC fate.

Chapter 2

Methods

This thesis consists of two similar but distinct experiments. The first experiment focuses on examining the effects of mMSC cell-cell contact and cell microenvironment structure on YAP expression. mMSCs were seeded on both PMMA fiber and flat samples at both high and low cell densities. Immunostaining and western blotting techniques were used to characterize and quantify data. The second experiment examines the effects of varying fiber morphology and cell densities on YAP expression. Cells were seeded on PMMA fibers of different diameters and consistent fiber densities. Samples from each group were analyzed through immunofluorescence microscopy, while the remaining samples were lysed and separated into cytoplasmic and nuclear extracts. The extracts were then analyzed via western blotting to determine how cytoplasmic and nuclear YAP concentrations varied with fiber diameter and cell confluence.

Procedures

This section describes in detail the material selection processes and procedures used in both experiments. For more information on specific experiments, please refer to their corresponding subsection in the Methods section below.

Polymer Selection

In both experiments, Poly-methyl methacrylate (PMMA), also known as acrylic or "Plexiglass", was used to create the extracellular environments on which the mMSCs were seeded. Though PMMA is

formed by polymerizing methyl methacrylate monomers, which are known to be both an irritant and a possible carcinogen, the PMMA polymer is extremely biocompatible. The polymer is resistant to degradation via chemical interactions, body temperatures, and cellular actions of human tissue [30]. Its biocompatibility was initially discovered by English ophthalmologist Sir Harold Ridley, who observed that the plastic caused little to no signs of irritation or rejection in the eyes of WWII RAF pilots, who had been riddled with the polymer from the windows of their submarine Spitfire aircraft [31]. Today, the polymer is commonly used as bone cement in orthopedic surgery and is used to manufacture both contact and intraocular lenses [30]. Additionally, using processes such as spin-coating and electrospinning, PMMA can be used to create both 2D surfaces and 3D microfiber structures on coverslips. Due to its biocompatibility and ability to be manipulated into desired 2 dimensional and 3 dimensional geometries, PMMA was chosen as the material used to synthesize both the 2D surface and 3D fibers in the presented experiments.

In addition to PMMA, Polyhydroxyethylmethacrylate (PHEMA), a material often used to make soft contact lenses, was also used in this experiment. Mesenchymal stem cells display a high level of attachment and adherence when seeded on glass. Consequently, if PMMA fibers were introduced directly to glass coverslips surfaces, then seeded with MSCs, the cells would adhere to both the glass and PMMA fibers. This would be detrimental to the experiments, as the glass adherence would directly impede the ability to analyze the effect of the PMMA fibers on MSC morphology and YAP localization. However, PHEMA does not normally support the attachment of mammalian cells [32]. As a result, the polymer was used to create a 2D coating on the glass coverslips before the fibers were introduced to the sample. The PHEMA coating prevents MSCs from adhering to the glass coverslip, allowing the effects of the PMMA fibers on the cells to be directly observed with significantly less interference.

Solution Preparation

In order to utilize PMMA to create surfaces and fibers through spin-coating and electrospinning, respectively, the polymer must be dissolved in a solvent. PMMA consists of polar ester groups coupled with a nonpolar hydrocarbon backbone. This molecular structure makes PMMA hydrophobic, making it hard to dissolve in water, a polar solvent. However, the polymer can be dissolved in organic solvents such as nitromethane. As a result, nitromethane was used as the solvent to create the PMMA solutions for these experiments. Similarly, the PHEMA polymer must also be dissolved in solvent to be utilized in surface creation through spin-coating. In both experiments, PHEMA was dissolved in a solution of 95 percent ethanol and 5 percent water.

Different concentrations of solutions were required throughout the experiments. 4-weight percent PHEMA solution was prepared for creating 2D PHEMA surfaces. Similarly, 10-weight percent PMMA solution was prepared for creating the 2D PMMA surface slides. A number of different PMMA concentration solutions were utilized in electrospinning PMMA fibers, including 15%, 17.5%, 20%, and 22.5% solutions. Solutions were created be measuring an amount of polymer, then dividing the measured amount by the desired concentration to obtain the amount of solvent needed to create the desired solution concentration. A sample calculation for determining the required volume of nitromethane needed to create a 20% PMMA solution is provided below.

$$\frac{1.1076 \text{ g PMMA}}{\left(\frac{0.2 \text{ g PMMA}}{1 \text{ ml Nitromethane}}\right)} = 5.538 \text{ mL Nitromethane}$$

The polymer was then transferred to a labelled glass vile. Using a micropipette, the calculated volume of solvent was added to the vile and was vortexed for 1 minute to facilitate the dissolving of the polymer. The solution was then heated for at least 12 hours to further encourage the dissolving of the polymer into the solution. The solution was then allowed to sit for at least two days before use. The solution was checked to assure the polymer was fully dissolved before it was used in other processes.

Surface Creation

To start sample preparation, 2D polymer coatings were introduced onto glass coverslips. The 2D surfaces were created using a spin-coater. Spin coating is a process used to deposit uniform thin films of material onto flat substrates. The spin-coating setup used to prepare samples for the experiments are depicted in figure 1 below.



(a)

(b)

Figure 1: This figure details the spin-coater used to prepare polymer surfaces for both experiments. The compressed air drives a vacuum, which fixes the cover slip to the spinning apparatus. Polymer solution is deposited on the coverslip and is spread across the glass by centrifugal forces as the spinning apparatus rotates.

To begin, glass coverslip slides were sprayed with ethyl alcohol to remove any dust residue, then were wiped with a Kim-wipe and allowed to dry. A lab marker was used to write sample identifying information on the back of the coverslip. Writing on the back side assured the label was not removed when the polymer solution was added to the slide.

Next, the PHEMA and PMMA spin coated slides were prepared. 4% PHEMA solution and 10% PMMA solution were used for the creation of the PHEMA and PMMA spin coated slides, respectively. The slide was placed ink side down on the spin coater and affixed to the spinning apparatus using a

vacuum. Using a micropipette, 120 microliters of the PMMA or PHEMA solution was deposited onto the center of the coverslip. Using the tip of the micropipette, the solution was evenly spread around on the coverslip until it was evenly coated with the solution. Next, the spin-coater lid was shut and the sample was spun at 3500 rpm for 15 seconds. During this time, the centrifugal force pushed the fluid off the edges of the coverslip until the sample was fully dry, leaving a polymer coating on the glass cover slip. Upon completion of the cycle, the vacuum was turned off and the sample was inspected to assure an even polymer coating was obtained. The PMMA coated slides were used as experiment controls, while the PHEMA coated slides were used as a surface on which to seed PMMA microfibers.

After the polymer surfaces were prepared, electrospinning was used to seed the PHEMA surfaces with microfibers. Fibers were seeded on the PHEMA coated cover slips to prevent the cells from adhering to the glass in addition to the PMMA fibers. Electrospinning is a fiber production method that utilizes an electric force to draw charged threads of polymer solution into fibers ranging from hundreds of nanometers to micrometers in diameter [33]. The electrospinning setup used to prepare fiber samples for both experiments is depicted in figure 2 below.



Figure 2: Electrospinning Setup used to prepare fiber samples. An electric field draws polymer from the syringe tip towards the copper plate, where they are deposited onto the cover slip as microfibers.

The electrospinning apparatus consists of a pumping mechanism, polymer solution filled syringe, voltage source, copper plate, and two electrode leads. One of the electrode leads it attached to the metal syringe tip, while the other lead is attached to the copper plate. The pump extrudes the polymer solution out of the syringe at a slow rate. This produces drops at the tip of the syringe. When a sufficiently high voltage is applied across the electrodes, the solution droplet becomes charged. The resulting electrostatic repulsion counteracts the surface tension of the droplet, causing it to stretch. Once the surface tension is

exceeded, a stream of liquid is shot from the syringe through the air. If the molecular cohesion of the solution is high enough, the stream does not break up. and a charged liquid stream is formed between the syringe and the receiving electrode [33]. As the fluid jet travels through the air, the current flow changes from ohmic to convective as the charge migrates to the surface of the stream. At this point, electrostatic repulsion forces between small bends in the fiber stream causes it to bend further as it dries and flies towards the collection plate. This results in the formation of fibers of uniform nanoscale diameters [33].

Throughout the experiments, a number of PMMA solutions of different concentrations were used to create fibers. Fiber diameter tends to increase with the concentration of polymer solution used [33]. Other parameters that were varied to change fiber diameter include syringe tip size and voltage. In all experiments, flow rate, as well as the distance from the syringe tip to the conducting plate, were kept constant.

To begin, a syringe with a metal tip was filled with approximately three milliliters of the selected PMMA solution. The syringe was then inverted and pressed to remove any gas trapped in the syringe with the solution. The syringe was then loaded into the electrospinning apparatus. Once loaded, the pumping mechanism was pressed tight against the back of the syringe. The distance from the syringe tip to the copper plate was then adjusted to 12.5cm. The pump was set to pump solution through the syringe tip at a rate of 0.80 milliliters per hour. The syringe tip was wiped with an acetone dampened Kim-wipe to assure the tip was not clogged. Next, the plastic casing enclosing the electrospinner was closed, the voltage was engaged and the electrospinner was run for 30 seconds. This allowed us to see where the fibers were landing on the copper plate to assist in determining coverslip placement. Once the run was complete, the voltage was turned off and the electrospinner was depolarized by touching one end of a metal wire to the syringe tip and the other end to the copper plate. Care was taken to assure the voltage was turned off before attempting to depolarize the apparatus. If the fibers were hitting the plate either too high or low, the height of the apparatus was adjusted and the calibration process was repeated. Upon

completion of the calibration process, the copper plate was wiped with acetone to remove any PMMA fibers. Using lab tape, a PHEMA coated coverslip was affixed to the copper plate at the location where the fibers were landing. Care was taken to make sure the coverslip was lying as flat on the plate as possible. The syringe tip was then wiped with an acetone Kim-wipe to remove any clogs and excess PMMA dripping from the syringe tip. To further prevent clogs, The PMMA solution was kept flowing out of the syringe tip throughout the setup and transition processes. Next, the desired voltage was set and the electrospinner was run for the desired amount of time. Upon completion, the voltage was turned off and the electrospinner was depolarized. The coverslip was carefully removed and inspected under a microscope to assure a relatively even coating of PMMA fibers of the desired density was obtained. The copper plate was then wiped with acetone to clear any PMMA fibers. Another PHEMA coverslip was affixed to the plate, and the process was repeated until the desired number of fiber coated coverslips were obtained.

Electrospinning was completed using a variety of different PMMA solutions, voltages, run times, and syringe tip sizes. Please refer to the specific experiment in the Methods section below for details on the exact electrospinning parameters used in that experiment. Illustrations of the fiber and flat surface groups used in all experiments are detailed in figure 3 below.



Figure 3: Illustrations of the surface samples prepared in the experiments. Figure 1(a) illustrates a coverslip evenly coated with PMMA. This creates a flat 2D surface for the cells to adhere to. In contrast, Figure 1(b) depicts a

coverslip evenly coated with PHEMA, which is in turn coated with PMMA fibers. The PHEMA coating discourages cell binding to the flat surface, allowing for the isolation and analysis of cell behavior when binding to 3D microfibers.

Cell Thawing

To start, cells were obtained from a cryogenically frozen pellet. It is important to check to assure that the cells are of a low passage number before using. At passage numbers around six and above, mesenchymal stem cells begin to express a fibroblast like phenotype and lose their differentiation potential [34]. As a result, cell populations with low passage numbers should be used. Alpha MEM (aMEM) medium supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (P/S) was warmed to body temperature in an incubator. The Biological Safety Cabinet (BSC) was then wiped down with 70% ethanol to prevent sample contamination. Next, the frozen MSC pellet was removed from the liquid nitrogen tank. The cell line and passage number of the cells were recorded. The cells were thawed by partially immersing the cell tube in a warm water bath and gently moving the cell tube around until the cells were thawed. Care was taken to assure the water never went above the seal on the cell tube. Upon thawing, the cell tube and media were removed from the incubator, wiped with alcohol, and then transferred to the BSC. Equal amounts of the thawed cells and growth media were added to a centrifuge tube. The centrifuge tube was inverted several times to mix, then was centrifuged at 500 rpm (47g) for 5 minutes. Upon completion, the centrifuge tube was wiped and placed back in the BSC. The growth media was vacuumed from the tube, leaving the cell pellet at the bottom of the tube. 4 mL of growth media was added to the tube and pipetted up and down to assure adequate mixing of the growth media and cells. The cell solution was then added to the desired number of petri dishes. The remaining amount of growth media required for the dish size used was then added to each dish. The petri dishes were then covered and gently tilted back and forth to assure the bottom surface of the dishes were uniformly covered with solution. The cell dishes were then removed from the BSC, wiped down with alcohol to prevent contamination, then placed in the cell incubator. The MSC cultures were incubated at

36 to 37 degrees Celsius to facilitate optimal growth. This temperature was chosen to reflect the nominal temperature of the human body.

Cell feeding:

Replacing the cell growth media frequently assured the cells do not deplete the nutrients required for their growth. To maintain a consistent exponential growth profile, the growth medium was replaced every two to three days. αMEM growth medium supplemented with 10% FBS and 1% P/S was used for all MSCs in the experiments. The medium was warmed in the incubator for approximately 8 to 10 minutes. Concurrently, the MSCs were observed using a microscope to determine cell confluence and to check for abnormalities, such as large numbers of dead cells. If the confluence of the dishes were 80 percent or above, the cells were passaged (see next section). Next, the BSC was wiped down with alcohol to reduce the risk of sample contamination. The cell dishes and growth media were also wiped down and placed inside the BSC. The old growth media was aspirated from the dishes, and the required amount of new media was then pipetted into the petri dishes. The dishes were then covered, gently rocked to assure uniform media coverage, then were removed from the BSC and wiped with alcohol. The cell plates were observed under the microscope, then were placed back in the incubator.

Cell Passaging

Once the original culture reached a confluency of 80 percent, the cell population was passaged into additional dishes. Passaging provides the cells with more room to grow so they don't experience the effects of contact inhibition that occur when the cell dishes become over confluent.

To begin, trypsin and α MEM growth media were placed in the warm water bath. Under the BSC, the old growth media was aspirated from the cell culture dishes. The culture was then washed with 5 mL

of PBS. The PBS was aspirated, then 5 mL of prewarmed trypsin was slowly added to the culture. Trypsin is produced naturally in the salivary glands and pancreas of the human gastrointestinal tract. Trypsin is a protease, an enzyme that breaks down proteins. In cell-culture, trypsin breaks down the cell adhesion molecules responsible for the cell adherence to the plate and other cells. After adding the trypsin, the culture was gently rocked to assure uniform coverage of the cell monolayer. The culture was then placed in the incubator. After four minutes, the culture was examined under a microscope to assure that at least 90 percent of the cells were detached from the surface. The bottom of the cell culture was gently tapped to further encourage detachment. If 90 percent of the cells were not detached, the culture was placed back in the incubator and inspected at 1-minute intervals. Upon reaching appropriate levels of detachment, growth media equal to the amount of trypsin used was added to the dish to neutralize the effects of the trypsin. The cell solution was mixed via pipetting, then was transferred into a 50 mL centrifuge tube. Using a centrifuge, the solution spun at 47g for 5 minutes. The cell pellet was isolated from the solution, then re-suspended in 10 milliliters of media and gently mixed. 10 microliters of the new solution was pipetted into a hemocytometer, and the number of cells in each of the 4 x 4 section grids of the hemocytometer were counted. The average cell count for all the grids was then calculated. Each 4 x 4 grid holds 0.0001 mL of solution. As a result, the cell concentration in the original solution was calculated using the following equation:

$$Cell Concentration = \frac{\frac{\# of Cells}{4 x 4 section}}{\frac{0.0001 mL}{4 x 4 section}}$$

Using the calculated cell concentration, the total number of cells in the solution was calculated:

$$Total Cells in Solution = \frac{Cells}{ml} * ml of solution$$

After determining the cell concentration and total number of cells in the solution, the cell solution was gently pipetted to assure adequate mixing. A quantity of cell solution dependent on the desired new

cell concentration was then pipetted to the new petri dishes. Fresh growth media was added to the petri dishes until the total amount of solution equaled the required amount of media for the particular dish size. The dishes were then covered and gently rocked to encourage a relatively even distribution of cells on the dish. The dishes were inspected under the microscope then were placed in the cell incubator to facilitate cell growth.

Cell Seeding:

Next, the polymer coated cover slips were prepared for cell seeding. The selected coverslips were placed polymer side up into the wells of 6-well plate dishes, 1 coverslip to a well. Each dish was labelled with the desired cell density of the sample and the type of polymer surface (flat surface, fibers, fiber size). The well plate dishes were then placed under the ultraviolet light in the BSC for 15 minutes to minimize biological contamination of the samples. After the samples were sterilized, the well plates were covered and placed in a secure location for later use.

Next, the number of cells required for the experiment was determined. For samples sharing the same target cell density, the total number of cells required can be determine used the following equation:

Required Cells = # of wells
$$*\frac{9.5 \text{ cm}^2}{\text{well}} * \frac{\text{desired cell #}}{\text{cm}^2}$$

This equation was solved for both high and low-density sample groups. The resulting cell numbers were added together to obtain the total number of required cells for the experiment.

Next, the cells were passaged as detailed in the cell passaging section above. Cells were counted using a hemocytometer to assure there were enough cells to complete the experiment. Once the cell concentration and cell number were obtained, the amount of cell solution required to obtain the desired cell concentration for each sample well was calculated using the following equation.

$$Required Solution Volume = \frac{desired cells}{cm^2} * \frac{9.5 cm^2}{well} * \frac{1}{\frac{\# of cells}{mL of solution}}$$

The required cell solution volume was then added to each well. α MEM growth media was then added to the wells until the total solution volume in each well was 2 milliliters. Using a metal syringe tip, the edges of each coverslip were gently pushed down in the solution to assure the coverslips were fully immersed in the solution. The well plates were covered and gently rocked to encourage an even distribution of cells. The plates were then taken out of the BSC, inspected under the microscope, then placed in the incubator for 24 hours.

Sample Lysing:

In order to complete a number of additional post-processing procedures on the samples, such as cell fractionation and western blot, some of the samples were lysed. Depending on whether the samples were going to be analyzed by western blot immediately or separated into components using cell fractionation before western blotting, a strong lysis buffer or weak lysis buffer was used. A strong lysis buffer ruptures both the cytoplasmic and nuclear membrane, making it undesirable for use on samples that will be fractionized. In contrast, a weak lysis buffer ruptures only the cytoplasmic membrane, allowing for easier separation of cytoplasmic and nuclear components.

After a 24-hour incubation period, the media was aspirated from the wells of the samples selected for lysing. The samples were washed once with PBS. The PBS was aspirated out, then fresh PBS was added to the samples. Next, the samples were carefully removed from the wells and transferred to a new pre-labelled 6 well plate dish. Next, 200 μ L of the chosen lysis buffer was gently added directly on top of the coverslip. The plates were covered and taped to the lids, then stored in the -80 °C freezer for later use.

Cell Staining:

After the 24-hour incubation period, the media was aspirated from the wells of the coverslips selected for immunostaining. The samples were washed with 1mL of PBS. The PBS was aspirated, then the samples were fixed with a fixation buffer (FB) for 15 minutes. The fixation buffer was aspirated, then the samples were washed with 1 mL of PBS 3 times for 5 minutes per wash. After PBS aspiration, 1 mL of Permeabilization Buffer (PB) was added to each sample and incubated for 45 minutes. The purpose of the Permeabilization Buffer is to assure the cells are permeable to the antibodies used for immunostaining. The PB buffer was aspirated, then the primary antibodies were added in PB in a dilution ratio of 1:400 and incubated for 1 hour. The primary antibody used for YAP staining was YAP (D8H1X) XP Rabbit mAb. After 1 hour, the PB was aspirated and the samples were washed with PBS 3 times for 5 minutes per wash. Next, the secondary antibodies were added in 1 mL of PB to the samples in a dilution ratio of 1:500 and were incubated for 45 minutes in the dark. The secondary antibody used was a Goat Anti-Rabbit antibody. The secondary antibody binds to the primary antibody, allowing for the visualization of YAP via immunofluorescence. At this point, it is important to minimize exposure of the samples to light, as the secondary antibodies, as well as Phalloidin and DAPI, are light sensitive, and overexposure of the samples to light can photo bleach the antibodies. Samples should be covered when not in use, and overhead lighting should be dimmed when interacting with the samples. Next, the PB was aspirated, and the samples were washed 3 times with PBS for 5 minutes per wash. Phalloidin was added in 1 mL of PB to the samples in a dilution ratio of 1:1000. Phalloidin is a phallotoxin found in death-cap mushrooms that bind to F-actin in cells. The samples were incubated in the dark for 30 minutes, then were washed with PBS 3 times for 5 minutes per wash. Next, DAPI, a fluorescent stain that binds to DNA, was added in 1 mL of PB to the samples in a dilution ratio of 1:5000 and incubated in the dark for 15 minutes. After incubation, the PB was aspirated and the samples were washed 3 times with PBS for 5 minutes per wash. Next, the coverslips were mounted to microscope slides using mounting media and were allowed to dry overnight. The coverslips were sealed to the slides with clear nail polish along the

outside of the coverslip to help with storage. Upon drying, the samples were stored in the -20 °C freezer for later analysis.

Immunofluorescence Microscopy

After immunostaining, immunofluorescence microscopy and differential interference contrast (DIC) microscopy was used to image the samples. Immunofluorescence microscopy was used to image for YAP, actin, and DNA, while DIC microscopy was used to capture the surface features of the sample, such as fiber size, density, and orientation. LAS X imaging software was used to capture and save the images. Filter cubes were selected to isolate the wavelengths emitted by the immunostained molecules. Imaging parameters, including proteins imaged, filter cubes used, filter cube wavelengths, and channel colors can be found in table 1 below. Large wavelengths are lower in energy then smaller wavelengths. As a result, samples were imaged starting with channels corresponding to large wavelengths and ending with channels corresponding to small wavelengths to minimize the effects of photobleaching. The exposure time of each channel was kept constant for all samples to help with quantification of YAP protein later in the analysis.

| Channel Number | Molecule | Filter Cube | Wavelength (nm) | Channel Color |
|----------------|----------|-------------|-----------------|---------------|
| Channel 1 | YAP | Y5 | 700 | Green |
| Channel 2 | F-actin | N21 | 600 | Red |
| Channel 3 | DNA | А | 450 | Cyan |
| Channel 4 | DIC | DIC | 550 | Gray |

Table 1: Immunofluorescence Microscopy Parameters
Cell Fractionation

In the second experiment, it was necessary to separate the cytoplasmic and nuclear components of the lysed samples. First, the samples were lysed as described in the Sample Lysing section above. Before lysing, protease inhibitors and EDTA were added to the weak lysis buffer help prevent sample degradation. The lysed samples were then transferred to labelled micro-centrifuge tubes. Next, the samples were centrifuged at 3,000 rpm (~900g) at 4 °C for 5 minutes to separate the nuclear component from the cytoplasmic component. Upon completion, the supernatant from each sample was carefully transferred into new pre-labelled micro-centrifuge tubes. This supernatant contained the cytoplasmic fraction of the sample, which is less dense then the nuclear fraction. Next, the nuclear pellet was rinsed with 150 µL of modified lysis buffer and centrifuged at 3,000 rpm at 4 °C for 5 minutes. After completion, the supernatant was again transferred to the cytoplasm centrifuge tubes. The purpose of this second rinse and centrifugation was to assure the vast majority of the cytoplasmic component was separated from the nuclear component. The nuclear pellet was then resuspended in 120 µL of lysis buffer and centrifuged at 13,000 rpm (~3900g) at 4 °C for 15 minutes to separate the nuclear extract from the insoluble nuclear component. Upon completion, the supernatant containing the nuclear fraction was transferred to a new pre-labelled micro-centrifuge tube. All samples were stored in the -80 °C freezer for later use.

Western Blot

Western blotting uses antibodies to identify specific proteins that have been separated by size using gel electrophoresis. First, gel electrophoresis is used to separate the proteins in a sample by their molecular weight. After proteins separation, the gel is placed in a "gel sandwich" with a PVDF membrane. The application of an electrical current through the sandwich transfers the proteins from the gel to the membrane. The membrane is then treated with antibodies that bind to the proteins of interest. The membranes are imaged and used to identify the presence of the target proteins as well as quantify their amounts relative to the other samples. In this study, western blotting is used to identify the presence of YAP and tubulin in the presented samples.



Figure 4: Western blot setup used in all experiments. The proteins are forced through the gel by an electric current, which separates the proteins by size. The Laemmli band can be seen running down the gel. Once the target bands approach the bottom of the gel, the running phase it stopped and the gel is prepared for the transfer of proteins to the membrane.

Gel Preparation

To begin, the western blot glass, ceramic plates, and spacers were cleaned with water and detergent, then were rinsed with deionized (DI) water. From the front to back, a sandwich was made using the glass plate, spacers, and ceramic plate. The sandwich was then inserted into the caster. While pressing down on the sandwich, the screws affixing the sandwich to the caster were carefully tightened. Next, the black screws on the side of the caster were tightened to seal the bottom of the sandwich to the caster. DI water was then added to the cavity formed between the glass and ceramic plate to test for leaks.

Next, the appropriate stacking and running gel solutions were prepared. In all experiments, the membranes are stained for both YAP and tubulin. YAP has a molecular weight of 65-75 kDa, while tubulin has a molecular weight of approximately 50 kDa. As a result, a 10% running gel was selected to obtain adequate band separation between the two proteins. Recipes for the 10% running gel and stacking gel can be found in tables 2-3 below. Ammonium persulfate (APS) and Tetramethylethylenediamine (TEMED) were not added until we were ready for the solution to begin gelling. Next, the caster was checked to see if the water level dropped. If the water level dropped, the caster assembly was taken apart, re-assembled, and re-tested. If the water level did not drop, there were no leaks in the gel cast and we were ready to move forward in the process. The water was emptied from the caster. APS and TEMED were added to the running gel solution, which was then carefully pipetted into the caster until a height approximately twice the length of the comb wells was left unfilled in the cavity. Next, a layer of isopropyl alcohol was added to the top of the gel solution to pop any bubbles and create a flat interface for the running and stacking gels. The running gel was allowed to set for at least 45 minutes, then the isopropyl alcohol was poured off. The combs were then pressed into the sandwich at an angle and secured with red clips and Parafilm. In all experiments, a 10-lane comb was used. Next, APS and TEMED were added to the stacking gel solution, which was then pipetted into the caster between the combs. The stacking Gel was allowed to set for at least 15 minutes before proceeding.

| Components | 5mL | 10mL | 15mL | 20mL | 25mL | 30mL |
|-------------------------|-------|-------|-------|-------|-------|--------|
| H2O | 2.348 | 4.796 | 7.144 | 9.592 | 11.94 | 14.388 |
| 40% Acryl-bisacrylamide | 1.25 | 2.5 | 3.75 | 5 | 6.25 | 7.5 |
| 1.5M Tris (pH 8.8) | 1.3 | 2.5 | 3.8 | 5 | 6.3 | 7.5 |
| 10% SDS | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 |
| 10% Ammonium persulfate | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 |
| TEMED | 0.002 | 0.004 | 0.006 | 0.008 | 0.01 | 0.012 |

Table 2: 10% running gel recipe. All values are in ml.

Table 3: Stacking gel recipe. All values are in ml.

| Components | 1mL | 2mL | 3mL | 4mL | 6mL | 8mL |
|-------------------------|-------|-------|-------|-------|-------|-------|
| H2O | 0.724 | 1.458 | 2.182 | 2.916 | 4.374 | 5.832 |
| 40% Acryl-bisacrylamide | 0.125 | 0.25 | 0.375 | 0.5 | 0.75 | 1 |
| 1.5M Tris (pH 6.8) | 0.13 | 0.25 | 0.38 | 0.5 | 0.75 | 1 |
| 10% SDS | 0.01 | 0.02 | 0.03 | 0.04 | 0.06 | 0.08 |
| 10% Ammonium persulfate | 0.01 | 0.02 | 0.03 | 0.04 | 0.06 | 0.08 |
| TEMED | 0.001 | 0.002 | 0.003 | 0.004 | 0.006 | 0.008 |

Gel Running

First, 500 mL of running buffer was prepared from 8.7 grams of Tris-Glycine Buffer, 500 mL of distilled water, and 10 mL of 10% SDS. Next, the lysed samples were taken out of the freezer and thawed to room temperature. To 30 μ L of sample was added 6 μ L of laemmli. The samples were then vortexed, then boiled at 95 ° C for 10 minutes. Concurrently, the standard ladder was removed from the freezer and allowed to warm to room temperature. After boiling, the samples were centrifuged briefly.

Next, the gels were transferred from the caster to the running apparatus. If only one gel was run, a glass plate was secured to the other side of the running apparatus. The running apparatus was then attached to the cooling apparatus, which was turned on to begin cooling the running apparatus. The combs were removed from the gel, and the running buffer was added to the running apparatus. To the first lane was added 4 μ L of standard ladder. Before loading the gel, the order in which the samples were to be loaded was recorded. Next, 30 μ L of each sample was added to their corresponding lanes. Extra lanes were filled with 30 μ L of a laemmli and lysis buffer mixture in a dilution ration of 1 part laemmli to 5 parts lysis buffer. Next, the appropriate leads were connected from the running apparatus to the voltage generator. The gel was run at 120 Volts for 3 to 4 hours until the laemmli was ran off the gel. The protein ladder was used to make sure the target proteins did not run off the gel.

Protein Transfer

Next, 0.5 L of transfer buffer was prepared from 8.7 grams of Tris-Glycine Buffer, 425 mL of DI water, and 75 mL of methanol. An additional liter of transfer buffer was also prepared in a separate container using the same ratios. Once the gel was finished running, the cooling tubes were detached from the running apparatus and connected to the transfer apparatus. This step was completed above the cooling tank to prevent fluid from escaping from the cooling tubes. Detergent was used to lubricate the tubing insertions on the transfer apparatus. 0.5 L of transfer buffer was added to the transfer apparatus, and the cooling apparatus was turned on to begin cooling the transfer buffer. Using the cutting guides, 1 PVDF membrane and 4 filter papers were cut for each gel.

Next, the running buffer was poured out of the running apparatus. To a small plastic tub was added 1 liter of transfer buffer. The gel was removed from the running apparatus and immersed in the tub. The PVDF membrane was activated in methanol for 5 minutes. Care was taken not to touch or mishandle the PVDF membrane, as this would degrade the quality of the scan. Next, the sandwich was carefully disassembled, first removing one of the spacers, then using the spacer to gently pry the glass from the gel. The gel was then carefully separated from the ceramic plate by gently rocking the tub while inserting the glass under the gel, causing it to gradually detach from the ceramic. Upon detachment, the membrane was added into the tub to deactivate the methanol. Upon membrane immersion, a "gel sandwich" was built-in in the follow order, from bottom up: black frame, sponge, 2 pieces of filter paper, gel, PVDF membrane, 2 pieces of filter paper, sponge, and white frame. This process was completed for all gels. The sandwich was then saturated with transfer buffer and placed in the transfer apparatus with the black part of the sandwich facing the back of the apparatus. The transfer buffer in the tub was added to the apparatus until it reached the maximum buffer line. The electric leads were attached to their appropriate connectors, and the transfer was run at 80 volts for 80 minutes. During this time, the proteins

were moved from the gel to the PVDF membrane. A successful transfer is indicated by the visibility of the protein ladder on the PVDF membrane upon completion.

Staining

Upon transfer completion, the membrane was removed from the PVDF sandwich and placed in 5 mL of 5% BSA in TBST for an hour at room temperature or in the refrigerator at 4°C overnight. Next, the primary antibodies were added in 5 ml of 5% BSA in TBST. The YAP primary antibody, D8H1X XP Rabbit mAb, was added at a dilution factor of 1:500. The α -tubulin primary antibody, B-5-1-2 Mouse, was added at a dilution factor of 1:200. The membranes were rocked at room temperature for 1 hour. Next, the primary antibody solutions were poured out, and the membranes were washed 3 times for 5 minutes per wash with TBST on the orbital shaker. Upon completion, the secondary antibodies were added to 5 mL of 5% BSA in TBST. The secondary antibodies used were Anti-Rabbit at a dilution factor of 1:5000 and Anti-Mouse at a dilution factor of 1:5000. The membranes were allowed to rock in the dark at room temperature for one hour, then the secondary antibody solutions were poured out. The membranes were washed 3 times with 5 mL of TBST on the orbital shaker. After the final wash, the membranes were placed in TBST and were imaged using the IR scanner.

IR Analysis

The IR scanner bed was wiped down with lens cleaner before use. Next, the membrane was placed on the scanner bed and imaged. The 700 nm (YAP) and 800 nm (Tubulin) channels were always imaged at the same intensity to allow for comparability. Once complete, the membrane was placed back in the TBST solution and stored in the fridge. The membrane lanes and bands were then analyzed. The intensity of the bands were measured in the program and exported for later analysis.

Experiment 1: Effect of Cell Microenvironment and Cell Density on YAP Expression

In this experiment, the effects of the cell microenvironment as well as cell-cell contact on mouse mesenchymal stem cell (mMSC) YAP expression was investigated. PHEMA cover slips coated with PMMA microfibers were seeded with mMSCs at different cell concentrations and were allowed to incubate for 24 hours. PMMA coated cover slips were also seeded with mMSCs and were used as a control. After the 24-hour incubation period, the samples were analyzed using immunofluorescence microscopy and western blotting to determine YAP expression.

Experimental Setup

To begin, the 2D polymer coated coverslips were prepared. The 2D surfaces were created via spin-coating, using the procedure detailed above. Overall 12 PMMA coated coverslips and 12 PHEMA coated coverslips were utilized in this experiment. Using the settings in table 4, the PHEMA slides were covered in PMMA microfibers via electrospinning.

| PMMA Solution Concentration (%) | 20 |
|---------------------------------|------|
| Tip Gauge | 16 |
| Tip to Plate Distance (cm) | 12.5 |
| Polymer Flow Rate (mL/hr) | 0.8 |
| Voltage (micro-Amps) | 15 |
| Running Time (s) | 25 |

Table 4: PMMA Electrospinning Settings Used to Prepare Fiber Samples in Experiment 1

Upon completion, there were a total of 24 prepared coverslip samples. Half the samples were to be seeded at a concentration of $50,000 \frac{cells}{cm^2}$, and the other half were to be seeded with $5000 \frac{cells}{cm^2}$. Both high and low cell densities were chosen to observe YAP localization in cells experiencing both large and small degrees of cell-cell interactions. Two sets of independent variables gave us a set of 4 samples groups, described below in table 5.

| Group | Extracellular Environment | Cell Density (cells/cm^2) | Number of Samples | |
|-------|---------------------------|---------------------------|-------------------|--|
| 1 | DNANAA Surface | 50000 50000 | | |
| 2 | PIVIIVIA SUITACE | 5000 | 6 | |
| 3 | DNANAA Eibars on DHENAA | 50000 | 6 | |
| 4 | | 5000 | 6 | |

Table 5: This table details a summary of experiment parameters for experiment 1, including samples groups, independent variables, and number of samples for each group.

Each sample was place in the well of a six well plate. The plates were labelled to assure the samples were not mixed up later in the experiment. The plates were then bathed in ultraviolet light, then were sealed with lab tape until they could be seeded with mMSCs.

As stated above, mouse mesenchymal stem cells were (mMSCs) were used in this experiment. All samples were seeded with passage 16 mMSCs from the same population. The mMSCs were grown to confluency in four large dishes to assure there were enough cells to seed the samples at the specified concentrations. 12 coverslips were to be seeded at $50,000 \frac{cells}{cm^2}$, and the other 12 were to be seeded with $5000 \frac{cells}{cm^2}$. Using this calculation, a total of 6,270,000 cells were needed for the 24 samples. Upon reaching a large enough quantity of cells in the dishes, the cells were passaged and spun down into a pellet, then resuspended in 10 mL of α MEM media. The concentration of the cell solution was determined using a hemocytometer to be 2,007,500 $\frac{cells}{ml}$. The required amount of cell solution to obtain the desired cell density for each sample was calculated, then added to the corresponding well. Next, α MEM equal to the amount of 2 mL minus the amount of added cell solution was added to each sample to bring the total amount of solution up to 2 mL per well. Using the tip of a metal syringe, the corners of each coverslip sample were carefully pressed down to assure the samples were completely submerged in the solution. The samples were then covered, rocked back and forth to encourage uniform cell coverage, and placed in the incubator overnight.

Quantification

To analyze the data, 2 of the 6 samples from each group were immunostained using the protocol in the procedures section above. The immunostained samples were then imaged using immunofluorescence microscopy and DIC microscopy to determine YAP localization as well as examine the behavior of the cells in their microenvironment. The samples were imaged for YAP, Actin, and DNA, and the exposure time for each channel was kept constant between samples. To characterize cell behavior, Cell Profiler was used to determine the area and eccentricity of each cell. Cell Profiler was also used to overlay the boundaries of the cytoplasm and nucleus onto the corresponding YAP channel image. These overlaid images were then used to determine the degree of nuclear localization in each sample. This was carried out by looking at each cell in an image, then determining whether or not YAP was localized in the nucleus. YAP was determined to be localized in the nucleus if the YAP intensity in the nucleus was greater than or equal to the YAP intensity of the cytoplasm.

The remaining 4 of the 6 samples from each group were lysed and utilized in Western Blot. The purpose of this analysis was to determine the relative concentrations of YAP protein in each sample and normalize the result using the concentration of tubulin, which should be proportional to the number of cells present before sample lysing.

Statistical Analysis

In this experiment, there were two independent variables; surface morphology and cell density. There were two surface type selections and two cell density selections, providing us with a total of 4 sampling groups.

To determine whether the surface type and cell density had a significant effect on the population mean of cell area and cell eccentricity, a standard linear model two-way ANOVA test with a confidence of 0.95 was carried out for each of these dependent variables. Furthermore, if the ANOVA tests stated that area or eccentricity differed significantly between sample groups, a Tukey Pairwise Comparison analysis was carried out to determine how surface morphology and cell confluence effected the particular dependent variable.

To determine whether surface type and cell density had a significant effect on the fraction of cells with significant nuclear YAP concentrations, a number of Chi-Squared tests for independence were carried out. Because there were two independent categorical variables (surface type, cell density) and one dependent categorical variable (YAP localization location), multiple Chi Square tests were carried out to examine all combinations of the variables. For all tests, the null hypothesis states that the two categorical variables are independent, while the alternative hypothesis states that the two categorical variables are dependent.

To determine whether the concentration of YAP normalized to tubulin varies significantly between surface types, a two-sample t-test with a confidence of 0.95 was carried out. The null hypothesis for this analysis states that the difference between normalized YAP concentrations is zero for the surface types, while the alternative hypothesis states that the difference in normalized YAP concentrations is not zero. In this experiment, the effect of the fiber microenvironment and cell-cell interactions on mMSC YAP localization and expression was further investigated. Specifically, both the diameter of the PMMA fibers and cell density were altered between sample groups. PHEMA coverslips were covered in PMMA microfibers via electrospinning, then seeded with mMSCs at a high and low cell concentrations and allowed to incubate for 24 hours. PMMA coated coverslips were also seeded with cells and were used as a control. After the incubation period, the groups were analyzed using immunofluorescence and DIC microscopy. Additionally, cellular fractionation and western blotting were also used to quantify YAP expression in the cytoplasm and nucleus for the different surface samples.

Experimental Setup

To begin, the 2D polymer coated coverslips were prepared. The 2D surfaces were created via spin-coating using the procedure detailed above. Overall 5 PMMA coated coverslips and 20 PHEMA coated coverslips were utilized in this experiment. Next, electrospinning was used to deposit PMMA fibers on the PHEMA coverslips. The settings used to create the large, medium, small, and smallest microfibers are described in table 6. Generally, polymer concentration was used to control fiber diameter, while the voltage and tip gauge were tuned to improve the quality of the fibers.

| Parameters | Large | Medium | Small | Smallest |
|---------------------------------|-------|--------|-------|----------|
| PMMA Solution Concentration (%) | 22.5 | 20 | 17.5 | 15 |
| Tip (Gauge) | 16 | 16 | 16 | 20 |
| Tip to Plate Distance (cm) | 12.5 | 12.5 | 12.5 | 12.5 |
| Polymer Flow Rate (mL/hr) | 0.8 | 0.8 | 0.8 | 0.8 |
| Voltage (micro-Amps) | 15 | 15 | 15 | 15 |
| Running Time (s) | 90 | 80 | 60 | 80 |

Table 6: Electrospinning Parameters for Large, Medium, Small, and Smallest PMMA fibers for Experiment 2

Upon completion, there were a total of 25 prepared cover slip samples. Each extracellular environment type had a total of 5 prepared cover slips. All cover slips were placed into pre-labelled 6-well plates and bathed in ultraviolet light for 15 minutes to remove potential contamination. 4 of the coverslips from each surface type were seeded at a cell density of 50000 cells/cm², while the remaining sample was seeded at a cell density of 5000 cells/cm². All samples were seeded with passage 13 mMSCs. The groups are summarized in table 7 below.

 Table 7: This table details a summary of experiment parameters for experiment 2, including samples groups, independent variables, and number of samples for each group.

| Group | Extracellular Environment | Cell Density (cells/cm^2) | Number of Samples |
|-------|---------------------------|---------------------------|-------------------|
| 1 | DNANAA Surface | 50000 | 4 |
| 2 | PIVIIVIA SUITACE | 5000 | 1 |
| 3 | Large PMMA Fibers on | 50000 | 4 |
| 4 | PHEMA | 5000 | 1 |
| 5 | Medium PMMA Fibers on | 50000 | 4 |
| 6 | PHEMA | 5000 | 1 |
| 7 | Small PMMA Fibers on | 50000 | 4 |
| 8 | PHEMA | 5000 | 1 |
| 9 | Smallest PMMA Fibers on | 50000 | 4 |
| 10 | PHEMA | 5000 | 1 |

Quantification

To quantify the data, one of the samples from each group was immunostained using the protocol detailed in the procedures section. The immunostained samples were then imaged using immunofluorescence and DIC microscopy to examine YAP localization as well as the physical behavior of the cells in their microenvironment. To characterize cell behavior, Cell Profiler was used to determine the area and eccentricity of each cell. Cell Profiler was also used to overlay the boundaries of the cytoplasm and nucleus onto the corresponding YAP channel image. These overlaid images were then used to determine the degree of nuclear localization in each sample. This was carried out by looking at

each cell in an image, then determining whether or not YAP was localized in the nucleus. YAP was determined to be localized in the nucleus if the YAP intensity in the nucleus was greater than or equal to the YAP intensity of the cytoplasm. DIC microscopy was also used to measure the diameter of the fibers from each of the fiber groups. This helps to determine the overall distribution of fiber sizes between each group.

The remaining samples from each of the high cell density groups were lysed, then were separated into nuclear and cytoplasmic components via cell fractionation. The separated extracts were then analyzed using western blot, where they were stained and imaged for YAP and tubulin. The purpose of the western blot was to quantify the concentration of YAP and tubulin found in the cytoplasmic and nuclear components of the mMSCs. Low cell density samples were not analyzed using western blot, as the low cell concentrations would not produce target protein concentrations large enough to show up on the membrane when scanned.

Statistical Analysis

In this experiment, there were two independent variables; surface morphology and cell density. There were five different surface types and two cell density selections, providing us with a total of ten sampling groups.

To determine if the fiber diameters of each fiber group were significantly different, a standard one-way ANOVA test with a confidence of 0.95 was utilized. In this analysis, the null hypothesis stated that the mean fiber diameter of each group would be the same, while the alternative hypothesis stated that the not all means would be equal. If the ANOVA test stated that the fiber diameter varied significantly between groups, a Tukey Pairwise analysis was carried out to determine which fiber groups had significantly different mean diameters. To determine whether surface morphology and cell density had a significant effect on the population mean of cell area, a standard linear model two-way ANOVA test was utilized. In this analysis, the null hypothesis stated that the population means of all sample groups would not be significantly different, while the alternative hypothesis stated that not all population means are the same. A similar ANOVA test with the same confidence was carried out to determine whether surface morphology and cell density had a significant effect on the population mean of cell eccentricity. For both dependent variables, if the ANOVA test stated that surface morphology and cell density had a significant effect on the population mean of cell density had a significant effect on the morphology and cell density had a significant effect on the morphology and cell density had a significant effect on the morphology and cell density had a significant effect on the morphology and cell density had a significant effect on the morphology and cell density had a significant effect on the morphology and cell density had a significant effect on the morphology and cell density had a significant effect on the dependent variable, a Tukey Pairwise analysis was carried out to determine how changes in surface morphology and cell density effected the specific dependent variable.

To determine whether surface type and cell density had a significant effect on the fraction of cells with significant nuclear YAP concentrations, a number of Chi-Square tests for independence with a confidence of 0.95 were utilized. Because there were two independent categorical variables (surface type, cell density) and one dependent categorical variable (YAP localization location), multiple Chi Square tests were carried out to examine all combinations of the variables. For all tests, the null hypothesis states that the two categorical variables are independent, while the alternative hypothesis states that the two categorical variables are dependent.

Next, YAP and tubulin intensities from the western blot analysis were compared to determine if the intensities varied significantly between each sample population. In this analysis, we were attempting to verify that the nuclear separation was successful, as well as determine how nuclear and cytoplasmic YAP concentrations change as a function of surface morphology at high cell densities. To verify that the cell fractionation process was successful, all tubulin measurements were grouped into cytoplasmic and nuclear measurements. A two-sample t test with a confidence of 0.95 was used to determine whether the mean tubulin intensities deferred significantly between the nuclear and cytoplasmic extracts.

Finally, a 1-way ANOVA test with a confidence of 0.95 was utilized to determine if normalized YAP intensity differed significantly between the surface types for the cytoplasmic extracts. In this

analysis, the null hypothesis stated that the mean normalized intensity of each surface group would be the same, while the alternative hypothesis stated that not all means would be equal. If the ANOVA test stated that surface morphology had a significant effect on the normalized YAP intensity, a Tukey Pairwise analysis was carried out to determine how changes in surface morphology effected normalized cytoplasmic YAP intensity.

Chapter 3

Results

Experiment 1: Effect of Cell Microenvironment and Cell Density on YAP Expression

After the incubation period, samples from each experimental group were immunostained and imaged using immunofluorescence microscopy to determine how surface morphology and cell density effected cell geometry and YAP localization. The samples were imaged for actin, DNA, and YAP protein. Images from each sample group are displayed in figures 5 and 6. Once the cells were imaged, Cell Profiler was used to measure the area and eccentricity of the cells to help characterize their behavior. Additionally, Cell Profiler was also used to aid in the determination of YAP localization for each cell.



Figure 5: mMSCs seeded on PMMA fibers imaged for DNA (blue), actin (red) and YAP protein (green). Images in the top row display cells seeded at a high density, while images on the bottom row display cells seeded at a low density. Images in the second and third columns are the YAP and DNA channels, respectively.



Figure 6: mMSCs seeded on flat PMMA surfaces imaged for DNA (blue), actin (red) and YAP protein (green). Images in the top row display cells seeded at a high density, while images on the bottom row display cells seeded at a low density. Images in the second and third columns are the YAP and DNA channels, respectively.

Area

To begin, the area of the cells from each sample group were measured using Cell Profiler and recorded. The results are summarized in table 8 and figure 6 below.

| | Cell | Number of | Average Area | Standard | Standard Error |
|---------|---------|--------------|--------------|------------------|----------------|
| Surface | Density | Measurements | (um^2) | Deviation (um^2) | (um^2) |
| Fibors | High | 120 | 355 | 228 | 21 |
| Fibers | Low | 17 | 475 | 389 | 94 |
| Flat | High | 97 | 362 | 246 | 25 |
| Flat | Low | 19 | 1941 | 1286 | 295 |

Table 8: Summary of mMSC area data obtained from the Cell Profiler analysis.



Figure 7: Area interval plots displaying the samples means for fiber and flat samples seeded at both high and low cell densities. The bars are one standard error from the mean.

From figure 6, we see that at low cell densities, mMSCs seeded on flat PMMA surfaces generally had a larger area then mMSCs seeded on PMMA fibers. In contrast, cells seeded on flat surfaces at high cell densities appeared to have areas that were similar to those of the high-density cells seeded on PMMA fibers. Overall, the results suggest that cells seeded at low densities tended to have larger areas then cells seeded at higher densities.

To determine whether cell density and extracellular environment had significant effects on the population mean of cell area, a standard linear model two-way ANOVA test with a confidence of 0.95 was employed. In this analysis, the null hypothesis stated that population means of all sample groups would not be significantly different, while the alternative hypothesis stated that at least one population mean would be significantly different from the others. The analysis resulted in a P value of 0.001 for the surface variable, indicating with 95% confidence that the morphology of the extracellular environment has a significant effect on cell area. Furthermore, the analysis resulted in a P value of 0.000 for the cell density variable, indicating with 95% confidence that cell density (confluence) also has a statistically significant impact on cell area.

To further examine the effects of surface morphology and cell density on mMSC area, a Tukey Pairwise Comparison analysis was carried out. In this analysis, the measured response was area, while the independent terms were surface type and cell density. The effects of the independent variables were analyzed independently to obtain a clearer image of their individual effects on cell area. The results of the Tukey tests are summarized in tables 9 and 10.

| Surface | Ν | Mean (µm^2) | Grouping |
|---------|-----|-------------|----------|
| Fibers | 137 | 698 | А |
| Flat | 116 | 914 | В |

Table 9: Tukey Pairwise Comparisons: Response = Area (μm²), Term = Surface. Means that do not share a letter are significantly different.

| Table 10: | Tukey | Pairwise | Comparisons: | Response = | = Area (µm′ | ^2), Term = | Cell I | Density. Mean | s that | do not | share |
|-----------|-------|----------|---------------------|-------------------|---------------|-------------|--------|---------------|--------|--------|-------|
| | | | a | letter are | significantly | different. | | | | | |

| Cell Density | Ν | Mean (µm^2) | Grouping |
|--------------|-----|-------------|----------|
| Low | 36 | 1242 | А |
| High | 217 | 370 | В |

From the Tukey test, we see that mMSCs seeded on PMMA Fibers tend to have statistically smaller areas then mMSCs seeded on flat surfaces. Furthermore, samples seeded at low cell densities

were observed to have statistically greater areas then cells seeded at higher cell densities.

Eccentricity

Next, the eccentricity of the cells from each sample group was measured using Cell Profiler and recorded. The results of the analysis are summarized in table 11 and figure 8 below.

| | Cell | Number of | Average | Standard | Standard |
|---------|---------|--------------|--------------|-----------|----------|
| Surface | Density | Measurements | Eccentricity | Deviation | Error |
| Fibore | High | 120 | 0.844 | 0.132 | 0.012 |
| Fibers | Low | 17 | 0.862 | 0.156 | 0.038 |
| Flat | High | 97 | 0.776 | 0.158 | 0.016 |
| Flat | Low | 19 | 0.899 | 0.097 | 0.022 |

Table 11: Summary of eccentricity data from the cell imaging



Figure 8: Eccentricity Interval Plots displaying the samples means for each test group. The bars are one standard error from the mean.

From figure 7, we can see that eccentricity appears to decrease as cell density increases. Furthermore, the eccentricity of the cells seeded on the fiber surfaces vary to a lesser extent between low and high cell densities then the cells seeded on the flat surface, which display a more extreme change in average eccentricity with changing cell density. To determine whether cell density or surface morphology have a significant effect on cell eccentricity, a standard linear model two-way ANOVA test with a confidence of 0.95 was used. In this analysis, the null hypothesis stated that population means of all sample groups would not be significantly different, while the alternative hypothesis stated that at least one population mean would be significantly different from the others. The two parameters analyzed for variance in this analysis were cell density and surface morphology. The test resulted in a P value of 0.004 for the surface variable, indicating with 95 percent confidence that surface parameters have a significant effect on the population mean of cell eccentricity. The confluency variable resulted in a P value of 0.005, indicating with 95 percent confidence that cell density also has a significant effect on the population mean of cell eccentricity.

To further examine the effects of surface morphology and cell density on mMSC eccentricity, a Tukey Pairwise Comparison analysis was completed. In this analysis, the measured response was eccentricity, while the independent terms were surface type and cell density. The effects of the variables were analyzed independently to obtain a clearer image of their individual effects on cell eccentricity. The results of the Tukey tests are summarized in tables 12 and 13.

| Surface | Ν | Mean | Grouping |
|---------|-----|-------|----------|
| Fibers | 137 | 0.874 | А |
| Flat | 116 | 0.820 | В |

 Table 12: Tukey Pairwise Comparisons: Response = Eccentricity, Term = Surface. Means that do not share a letter are significantly different.

 Table 13: Tukey Pairwise Comparisons: Response = Eccentricity, Term = Cell Density. Means that do not share a letter are significantly different.

| Cell Density | Ν | Mean | Grouping |
|--------------|-----|-------|----------|
| Low | 36 | 0.883 | А |
| High | 217 | 0.811 | В |

From table 12, we see that population means of all fibers measurements is significantly different from the population mean for all flat surface measurements in regards to cell eccentricity. This further supports our findings that surface characteristics have a significant impact on cell eccentricity.

Furthermore, we see that fibers results in an overall higher cell eccentricity then flat surfaces, meaning fibers tend to make cells elongate more.

From table 13, we see that the population mean eccentricity of all low cell density sample groups is significantly different from the population mean eccentricity of all high cell density sample groups. Furthermore, we see that cells seeded at low cell densities have a higher average eccentricity then cells seeded at high cell densities. This suggests that cells are more circular in high cell density samples.

YAP

Next, the localization of YAP protein in the nucleus of the cells was analyzed for the different sample groups. Using Cell Profiler, the nuclear and cytoplasmic regions of each cell were identified. The nuclear and cytoplasmic boundaries were then overlaid on top of the images displaying YAP intensity, as shown in figure 9.



Figure 9: Outlines of the Nucleus and Cell Membrane overlaid onto the YAP channel of a high confluency fiber sample. The YAP intensity was adjusted in ImageJ to aid in the identification of YAP nucleus localization.

Image J was then used to adjust the image contrast and intensity to determine whether YAP had localized in the nucleus of each cell in the sample. YAP was determined to be localized in the cell nucleus when the YAP intensity within the nucleus was at least as bright as the corresponding YAP intensity in the cytoplasm of the same cell. The results were categorized simply as localized or not localized (binary "on" or "off") The results of the analysis are displayed below in table 14 and figure 9.

Surface Cell Density Samples Nuclear **Nuclear Fraction** 102 35 High 34% Fibers 9 53% Low 17 91 33 36% High Flat 15 5 33% Low



 Table 14: This table shows the number of cells with significant nuclear localized YAP concentrations in each sample group.

Figure 10: Fraction of mMSCs with significicant nucleus YAP intensities for each sample group.

From our fiber samples, we see that nucleus YAP localization decreases with increasing cell density. This coincides with expected trends found in literature. In the flat samples, the nuclear fraction between high and low cell densities was relatively similar, which was different from our expected outcome. This may be attributed to the low sample size for the flat low cell density data and will be

further discussed in the discussion section below. Fiber samples at low cell densities also displayed higher nuclear YAP fractions then the corresponding flat samples at similar cell densities. This matches our expected result.

To determine whether surface type or cell density had a significant effect on the percent of cells with nuclear localized YAP, Chi-Square Tests for independence with a confidence of 0.95 were utilized. Because there were two independent categorical variables (cell density, surface type) and one dependent categorical variable (YAP localization location), 4 Chi Square tests were carried out to examine all combinations of the variables. For all tests, the null hypothesis stated that the two categorical variables are independent, while the alternative hypothesis stated that the two categorical variables are dependent.

The first Chi-Square test compared high density cells seeded on fibers with low density cells seeded on fibers. This test resulted in a P value of 0.141, meaning we cannot conclude from this analysis that seeding cells on fibers at different densities significantly effects their YAP localization.

The second Chi-Square test compared high density cells seeded on a flat surface with low density cells seeded on a flat surface. This test resulted in a P value of 0.826, meaning we cannot conclude from this analysis that the seeding cells on flat surface at different densities significantly effects their YAP localization.

The third Chi-Square test compared high density cells seeded on fibers to high density cells seeded on a flat surface. The test resulted in a P value of 0.777, meaning we cannot conclude from this analysis that the seeding cells on different surfaces at high densities significantly effects their YAP localization.

The fourth Chi-Square test compared low density cells seeded on fibers to low density cells seeded on a flat surface. The test resulted in a P value of 0.265, which is still significantly higher than 0.05. As a result, we cannot conclude from our data set that seeding cells at low densities on different surfaces has a significant effect on YAP localization.

Next, western blotting was used to examine how the overall YAP concentration changed as a function of cell density and cell microenvironment. To account for the differences in cell number between samples, the YAP concentrations were normalized by dividing the YAP concentrations by the concentration of tubulin for each sample. In total 16 samples were lysed and used in Western Blotting. The blots are displayed in figure 11 below.







From figure 11, we can clearly see intense tubulin bands as well as more faint YAP bands for both the high cell density fiber samples and high cell density flat samples. Unfortunately, the low confluency samples did not have enough protein to show up on the blots due to their low cell densities. As a result, the western blot was used primarily to quantify protein concentrations for the high cell density sample groups. The intensity of the YAP and tubulin bands were measured for each sample and recorded. Next, the YAP concentration of each sample was normalized with respect to its corresponding tubulin concentration and plotted in figure 12 to help visualize the effect of the cell microenvironment on normalized YAP intensity.



Figure 12: This interval plot shows the YAP intensity of each sample when normalized to its corresponding tubulin intensity. The error bars are one standard error from the mean. The figure illustrates the fiber samples had a Normalized YAP concentration greater than those of the PMMA surface samples.

Figure 11 suggests that seeding the mMSCs on PMMA fibers increases their expression of YAP. To further validate this conclusion, a two sample T-Test with a confidence of 0.95 was carried out to determine whether the population means of the two groups were statistically different. The null hypothesis for this analysis stated that the difference between the population means of the two groups was zero, while the alternative hypothesis stated that the difference between the population means was not zero. The resulting P-value for this analysis was 0.042, which is less than 0.05, indicating with 95% confidence that the population means were statistically different. This further supports the conclusion that PMMA fibers result in higher YAP concentrations in mMSCs.

Experiment 2: Effect of Fiber Morphology and Cell Density on YAP Expression and Localization

After the incubation period, a sample from each experiment group was immunostained for actin, DNA, and YAP, then imaged using immunofluorescence and DIC microscopy to determine how surface morphology and cell density effected cell geometry and YAP localization. In total, there were 5 surface types and 2 cell densities, resulting in a total of 10 sample groups. Images from each sample group are displayed in figures 13-17. Cell Profiler was then used to measure the area and eccentricity of the imaged cells to help characterize their behavior. Additionally, Cell Profiler as well as Image J were also used to determine the fraction of cells with significant nuclear YAP concentrations in each sample. Image J was also used to measure the diameter of the fibers from each fiber type to quantify their size.



Figure 13: mMSCs seeded on flat samples imaged for DNA (blue), actin filaments (red), and YAP protein (green). The top row is seeded at a high cell density, while the bottom row is seeded at a low cell density.



Figure 14: mMSCs seeded on large diameter fibers imaged for DNA (blue), actin filaments (red), and YAP protein (green). The top row is seeded at a high cell density, while the bottom row is seeded at a low cell density.



Figure 15: mMSCs seeded on medium diameter fibers imaged for DNA (blue), actin filaments (red), and YAP protein (green). The top row is seeded at a high cell density, while the bottom row is seeded at a low cell density.



Figure 16: mMSCs seeded on small diameter fibers imaged for DNA (blue), actin filaments (red), and YAP protein (green). The top row is seeded at a high cell density, while the bottom row is seeded at a low cell density.



Figure 17: mMSCs seeded on the smallest diameter fibers imaged for DNA (blue), actin filaments (red), and YAP protein (green). The top row is seeded at a high cell density, while the bottom row is seeded at a low cell density.

To begin, Image J was used to measure the diameter of 21 random fibers from the images of each fiber type. The images obtained from DIC microscopy were used in this analysis, as they provided the clearest representation of the fibers. The results of this analysis are summarized in table 15 and figure 17 below.

 Table 15: Summary of the fiber diameter measurements obtained from the measurements taken from each fiber type.

| Fiber Type | Large | Medium | Small | Smallest |
|-------------------------|-------|--------|-------|----------|
| Average Diameter (µm) | 1.95 | 1.29 | 1.06 | 0.81 |
| Standard Deviation (µm) | 0.30 | 0.19 | 0.24 | 0.12 |
| Standard Error (µm) | 0.07 | 0.04 | 0.05 | 0.03 |



Figure 18: Fiber diameter interval plots displaying the sample means for all fiber groups used in this experiment. The error bars are one standard error from the mean.

From figure 18, it appears that the mean fiber diameter of each group corresponds well to the group name. To further verify that the population mean fiber diameter of each sample group is significantly different from one another, a general linear model 1-way ANOVA test with a confidence of 0.95 was utilized. In this analysis, the null hypothesis states that all means are equal, while the alternative

hypothesis states that not all means are equal. From the ANOVA test, a P value of 0.000 was obtained, indicating with 95 percent confidence that mean diameters from each fiber group are not all the same.

To determine how the mean fiber diameters differ between groups, a Tukey Pairwise Comparison analysis was completed on the dataset. The results of the Tukey test are summarized in table 16.

| Surface | Ν | Mean (µm) | Grouping |
|----------|----|-----------|----------|
| Large | 21 | 1.95 | А |
| Medium | 21 | 1.29 | В |
| Small | 21 | 1.06 | С |
| Smallest | 21 | 0.808 | D |

 Table 16: Tukey Pairwise Comparison: Response =Diameter, Term=Surface. Means that do not share a letter are significantly different

From table 16, we see that none of the fiber groups share a grouping letter. As a result, we can conclude that the mean diameter of each group is significantly different from all other groups. This conclusion will be useful when comparing other characteristics, such as YAP activation, cell area, and cell eccentricity, between sample groups.

Area

To begin, the area of the cells from each sample group was measured using Cell Profiler and recorded. The results are summarized in table 17 and figure 19 below.

| Surface | Cell | Number of | Average | Standard Deviation | Standard Error |
|----------|---------|--------------|---------|--------------------|----------------|
| Surface | Density | Measurements | (µm^2) | (µm^2) | (µm^2) |
| Flat | High | 283 | 749 | 802 | 48 |
| Fidt | Low | 83 | 1516 | 1261 | 138 |
| Largo | High | 210 | 607 | 536 | 37 |
| Large | Low | 56 | 670 | 390 | 52 |
| Modium | High | 101 | 948 | 702 | 70 |
| weulum | Low | 39 | 880 | 454 | 73 |
| Small | High | 146 | 626 | 402 | 33 |
| SIIIdii | Low | 29 | 861 | 587 | 109 |
| Smallast | High | 156 | 576 | 417 | 33 |
| Smallest | Low | 27 | 520 | 278 | 54 |

Table 17: Summary of mMSC area data obtained from the Cell Profiler analysis for experiment 2



Figure 19: Area interval plots displaying the sample means for all surface types seeded at both high and low cell densities. The bars are one standard error from the mean.

From figure 18, we see that for flat samples, the area of the cells are generally greater at lower cell densities then at higher cell densities. This was also true, although to a lesser extent, for the large and small fiber samples. The medium and smallest fiber samples displayed the opposite trend. However, with

the exception of the small fiber samples, the high cell density and low cell density mean areas for each fiber size have error bars that overlap significantly, meaning the differences may not be significant. Overall, it appears that fibers decrease the effect of cell density on area. Additionally, it appears that fiber samples at each cell density tended to have lower mean areas then corresponding flat samples of the same cell density.

To determine whether cell density and extracellular environment had significant effects on the population mean of cell area, a standard linear model two-way ANOVA test with a confidence of 0.95 was employed. In this analysis, the null hypothesis stated that population means of all sample groups would not be significantly different, while the alternative hypothesis stated that at least one population mean would be significantly different from the others. The analysis resulted in a P value of 0.000 for the surface variable, indicating with 95 percent confidence that the morphology of the extracellular environment has a significant effect on the mean area of mMSCs. Furthermore, the analysis also resulted in a P value of 0.000 for the cell density variable, indicating with 95 percent confidence that cell density has a significant effect on cell area.

To determine how mMSC area is affected by surface morphology and cell density, a Tukey Pairwise Comparison analysis was carried out. In this analysis, the measured response was cell area, while the independent terms were surface type and cell density. The results of the Tukey Tests are summarized in tables 18 and 19 below.

| Surface | Ν | Mean (µm^2) | Grouping |
|----------|-----|-------------|----------|
| Flat | 367 | 1003 | А |
| Large | 266 | 706 | В |
| Medium | 140 | 995 | А |
| Small | 175 | 765 | В |
| Smallest | 183 | 672 | В |

Table 18: Tukey Pairwise Comparisons: Response = Area (μm^2), Term = Surface. Means that do not share a letter are significantly different

| Cell Density | Ν | Mean (µm^2) | Grouping |
|--------------|-----|-------------|----------|
| High | 897 | 679 | А |
| Low | 234 | 977 | В |

Table 19: Tukey Pairwise Comparisons: Response = Area (μm^2), Term = Cell Density. Means that do not share a letter are significantly different

From the Tukey Test, we see that mMSCs seeded on flat samples tend to have significantly greater areas then cells seeded on fiber samples, with the exception of cells seeded on medium fibers. Cells seeded on the large, small, and smallest fibers had areas that were not statistically different from one another. In contrast, the area of cells seeded on medium fibers was statistically different from all of the other fiber groups. This will be discussed further in the discussion section. Furthermore, mMSCs seeded at low cell densities had statistically greater areas then cells seeded at higher cell densities. This is likely due to cells having more area to spread out in the low-density samples due to lack of competition with other cells.

Eccentricity

Next, the eccentricity of the cells from each sample group were measure using Cell Profiler and recorded. The results are summarized in table 20 and figure 20 below.

| Fable 20: Summary | y of mMSC eccentricity | data obtained from the | Cell Profiler analysis fo | or experiment 2. |
|--------------------------|------------------------|------------------------|----------------------------------|------------------|
|--------------------------|------------------------|------------------------|----------------------------------|------------------|

| | Cell | Number of | | Standard | Standard |
|----------|---------|--------------|---------|-----------|----------|
| Surface | Density | Measurements | Average | Deviation | Error |
| Flat | High | 284 | 0.778 | 0.148 | 0.009 |
| Fidt | Low | 83 | 0.852 | 0.125 | 0.014 |
| Lorgo | High | 210 | 0.832 | 0.148 | 0.010 |
| Large | Low | 56 | 0.863 | 0.137 | 0.018 |
| Madium | High | 101 | 0.862 | 0.102 | 0.010 |
| Medium | Low | 39 | 0.906 | 0.109 | 0.017 |
| Small | High | 146 | 0.854 | 0.115 | 0.010 |
| Small | Low | 29 | 0.941 | 0.096 | 0.018 |
| Smallast | High | 156 | 0.859 | 0.127 | 0.010 |
| Smallest | Low | 27 | 0.916 | 0.054 | 0.010 |



Figure 20: Eccentricity interval plots displaying the samples means for all surface types seeded at both high and low cell densities. The bars are one standard error from the mean.

From figure 20, we see a clear trend across all surface groups, where the low-density samples were more eccentric then the high-density samples. In other words, the high-density samples were more circular then the low-density samples. This was our expected outcome, as the high-density samples would be pressed together, forcing them into a more circular geometry, whereas the low-density sample are free to spread out, allowing them to adopt a more amorphous geometry. Furthermore, it appears that the fiber samples tended to be more eccentric then the flat samples.

To determine whether cell density or surface morphology had a significant effect on cell eccentricity, a standard linear model two-way ANOVA test with a confidence of 0.95 was used. In this analysis, the null hypothesis stated that population means of all sample groups would not be significantly different, while the alternative hypothesis stated that at least one population mean would be significantly different from the others. For the surface variable, a P value of 0.000 was obtained, indicating with 95 percent confidence that the extracellular environment has a significant effect on cell eccentricity. Furthermore, for the cell density variable, a P value of 0.000 was obtained, indicating with 95 percent confidence that cell density variable, a P value of 0.000 was obtained, indicating with 95 percent confidence that cell density variable, a P value of 0.000 was obtained, indicating with 95 percent confidence that cell density variable, a P value of 0.000 was obtained, indicating with 95 percent confidence that cell density variable, a P value of 0.000 was obtained, indicating with 95 percent confidence that cell density variable, a P value of 0.000 was obtained, indicating with 95 percent confidence that cell density also has a significant effect on cell eccentricity.

To determine how changes in surface morphology and cell density effect mMSC eccentricity, a Tukey Pairwise analysis was completed. In this analysis, the measured response was eccentricity, while the independent terms were surface type and cell eccentricity. The results of the Tukey Tests are summarized in tables 21 and 22 below.

| Surface | N | Mean | Grouping |
|----------|-----|-------|----------|
| Flat | 367 | 0.811 | А |
| Large | 266 | 0.855 | В |
| Medium | 140 | 0.887 | В |
| Small | 175 | 0.888 | В |
| Smallest | 183 | 0.888 | В |

 Table 21: Tukey Pairwise Comparisons: Response = Eccentricity, Term = Surface. Means that do not share a letter are significantly different

 Table 22: Tukey Pairwise Comparisons: Response = Eccentricity, Term = Cell Density. Means that do not share a letter are significantly different

| Cell Density | Ν | Mean | Grouping |
|--------------|-----|-------|----------|
| High | 897 | 0.837 | А |
| Low | 234 | 0.895 | В |

From table 21, we see that the population eccentricity of the flat samples is statistically less than the population mean eccentricity of each fiber sample. Furthermore, we see that mean cell eccentricity is not statistically different between the fiber groups. As a result, our data suggests that introducing fibers increases the eccentricity of cells. However, the diameter of the fibers does not appear to play a significant role in influencing eccentricity.

From table 22, we see that the mean eccentricity of all low-density samples is significantly greater than the mean eccentricity of all high-density samples. This supports our previous observation that cell eccentricity tends to decrease with increasing cell density.
Next, the localization of YAP protein in the nucleus of the cells was analyzed for the different sample groups. As in the previous experiment, Cell Profiler was used to identify the nuclear and cytoplasmic regions of each cell. The nuclear and cytoplasmic regions were overlaid on top of the image displaying YAP intensity, then Image J was used adjust the image intensity to determine whether YAP was localized in the nucleus of each cell in the image. Like the previous experiment, a cell was determined to have a significant nuclear YAP concentration when the YAP intensity in the nucleus region was at least as bright as the YAP intensity in the cell's cytoplasm region. For each cell, the result was recorded categorically as either nuclear localized or cytoplasm localized. This was completed for multiple images for each sample group to obtain a sizable sample size for both high and low cell density groups. It is important to note that the flat samples were prepared and imaged at a later date then the fiber samples. However, the cells used were from the same mMSC population. Additionally, the cells were of a similar passage number when they were seeded. As a result, they have been deemed comparable for this experiment. The results of the analysis are displayed in table 23 and figure 21 below.

| Surface | Cell Density | Samples | Activated Nucleus | Nuclear Fraction |
|----------|--------------|---------|-------------------|------------------|
| Flat | High | 264 | 161 | 61% |
| | Low | 81 | 56 | 69% |
| Large | High | 168 | 117 | 70% |
| | Low | 57 | 39 | 68% |
| Medium | High | 102 | 58 | 57% |
| | Low | 39 | 23 | 59% |
| Small | High | 143 | 77 | 54% |
| | Low | 26 | 21 | 81% |
| Smallest | High | 151 | 89 | 59% |
| | Low | 27 | 19 | 70% |

 Table 23: This table shows the number of cells with significant nuclear localized YAP concentrations in each sample group for experiment 2





From figure 21, it appears that with the exception of the large fiber samples, mMSCs seeded on all surfaces displayed higher degrees of nuclear YAP localization at low cell densities then at higher cell densities. This observation agrees with trends observed in literature [24]. For the fiber samples, the small fibers displayed the largest difference in nuclear localized YAP between the surface type's low and high cell density samples. In contrast, the large and medium fiber groups both displayed low cell density nuclear YAP fractions that were similar to their corresponding high cell density nuclear YAP fractions. The small fiber samples possess both the lowest nuclear YAP concentrations for their high cell density samples, as well as the highest nuclear YAP fractions for their low cell density samples.

To determine whether surface type or cell density has a significant effect on the percent of cells with nuclear localized YAP, chi-squared tests for independence with a confidence of 0.95 were utilized. Because there were two independent categorical variables (surface type, cell density) and one dependent categorical variable (YAP localization location), multiple Chi Square tests were carried out to examine all combinations of the variables. For all tests, the null hypothesis states that the two categorical variables are independent, while the alternative hypothesis states that the two categorical variables are dependent. To begin, a Chi Square test including all sample groups was carried out. Because Chi Square tests can only compare two variables, the surface and cell density variables were grouped together for this analysis, giving us a total of ten independent variables. The Pearson Chi Square Test resulted in a P value of 0.039, indicating with 95% confidence that either surface morphology, cell density, or both have a significant effect on YAP localization.

Next, a Chi Square test including only surfaces seeded at high cell densities was carried out. The purpose of this analysis was to determine if surfaces have a significant effect on YAP localization at high cell densities. The Pearson Chi Square test resulted in a P value of 0.097, which is greater than 0.05. As a result, we fail to reject the null hypothesis and cannot conclude that the tested surface morphologies have a significant effect on the YAP localization of cells seeded at high densities. However, the relatively low P value suggests that the data may become significant at a large enough sample size.

Next, a Chi Square test including only surfaces seeded at low cell densities was carried out. The purpose of this analysis was to determine if surfaces have a significant effect on YAP localization at low cell densities. The Pearson Chi Square test resulted in a P value of 0.475, which is greater than 0.05. As a result, we fail to reject the null hypothesis and cannot conclude that the tested surface morphologies have a significant effect on the YAP localization of cells seeded at low densities.

Finally, all samples were grouped by their cell density into two groups. A Chi Square test was performed on the resulting data to determine whether YAP localization was dependent on cell confluency. The Pearson Chi-Square test resulted in a P value of 0.025, which is less than 0.05. As a result, we can state with 95 percent confidence that YAP localization is dependent on cell confluence.

Western Blot

Next, a Western Blot was carried out on the cytoplasmic and nuclear extract samples to determine how cytoplasmic and nuclear YAP concentrations changed as a function of surface morphology. In an attempt to obtain measurable signals, only high cell density samples were used in this portion of the experiment. Additionally, due to limitations in the number of lanes, only 2 nuclear extracts and 2 cytoplasmic extracts from each surface type were used, except for the smallest fiber type, which only had one nuclear extract and one cytoplasmic extract. The samples were stained for tubulin to evaluate the success of the cell fractionation. Additionally, the samples were stained for YAP to quantify the relative amount of YAP protein in each sample. Due to differences in both cell volumes and sample dilutions from the fractionation process, directly comparing cytoplasmic protein intensities to nuclear intensities would provide misleading information. As a result, cytoplasmic extracts were only compared to other cytoplasmic extracts and nuclear extract were only compared to other nuclear extracts. The blots are displayed in figure 22 below.



Figure 22: Membranes stained for YAP and tubulin in experiment 2. Green bands are tubulin, while red bands are YAP. For both membranes, the first 8 lanes are two flat samples, two large fiber samples, two medium fiber samples, and two small fiber samples. The samples alternate between nuclear and cytoplasmic extracts. In membrane 1, the last lane is the smallest fiber nuclear extract, while in membrane 2, the last lane is the smallest fiber cytoplasmic extract.

From figure 22, we see that the cytoplasmic extracts had more intense tubulin bands then the

nuclear extracts. This is as expected, as tubulin is primarily localized in the cytoplasm. YAP bands can

also be easily seen for all cytoplasmic extracts with the exception of the cytoplasmic extracts

corresponding to the flat sample. After imaging the membranes, the tubulin intensities, as well as the

YAP intensities normalized to cytoplasmic tubulin were recorded and plotted in figures 23 and 24 below.



Figure 23: This interval plot displays the tubulin intensity of each sample group for experiment 2. The error bars are one standard error from the mean.



Figure 24: This interval plot displays the YAP intensity normalized to cytoplasmic tubulin intensity of each sample group for experiment 2. The error bars are one standard error from the mean.

From figure 23, we see that all nuclear extracts with the exception of the small fiber samples had tubulin intensities of almost zero. The small fiber nuclear tubulin intensity was likely the result of a contamination of one of the nuclear wells with a cytoplasmic component during the Western Blotting

process. However, the cytoplasmic extracts all had tubulin intensities much greater than their corresponding nuclear extracts, which supports the claim that the cell fractionation was a success.

To further determine the success of the cell fractionation, a two-sample t test with a confidence of 0.95 was used to determine whether the concentration of tubulin in the nuclear samples was statistically different from the concentration of tubulin in the cytoplasmic samples. All samples were grouped together based on their fractionation type and the analysis was carried out. The analysis resulted in a P value of 0.000, indicating with 95 percent confidence that the tubulin concentrations of the cytoplasmic components were significantly greater than the tubulin concentrations of the nuclear components. This result supports the claim that the samples were successfully separated into cytoplasmic and nuclear components.

From figure 24, we see that with the exception of the flat samples, cytoplasmic components had greater normalized YAP concentrations then their corresponding normalized nuclear YAP concentration. The normalized nuclear YAP intensities for all samples were very close to zero. This likely means that the amount of YAP protein in the nuclear samples was too small to be easily detected by western blot. Comparing the cytoplasmic YAP concentrations, we see that the small and smallest fiber samples appear to have the greatest normalized YAP intensities.

Next, a one-way ANOVA test with a confidence of 0.95 was carried out to determine whether the normalized cytoplasmic YAP intensity differed significantly between the surface types. In this analysis, the null hypothesis stated that all means are equal, while the alternative hypothesis stated that all means are not equal. The test resulted in a P value of 0.017, which is less than 0.05, indicating with 95 percent confidence that not all normalized YAP intensities are equal for the samples.

To determine how normalized cytoplasmic YAP intensity is affected by surface morphology, a Tukey Pairwise Comparison analysis was carried out. In this analysis, the measured response was normalized YAP intensity, while the independent term was surface type. The results of the Tukey Test are summarized in table 24 below.

| Surface | N | Mean | Grouping |
|----------|---|---------|----------|
| Flat | 2 | -0.0062 | А |
| Large | 2 | 0.0169 | А, В |
| Medium | 2 | 0.0227 | А, В |
| Small | 2 | 0.0592 | В |
| Smallest | 1 | 0.0628 | В |

 Table 24: Pairwise Comparisons: Response = Normalized Cytoplasmic YAP Intensity, Term = Surface. Means that do not share a letter are significantly different

From table 24, we see that only the small and smallest fiber groups possess normalized cytoplasmic YAP intensities that are significantly greater than the flat samples. The test shows that the mean normalized YAP intensities of the flat, large fiber, and medium fiber samples are not significantly different. Furthermore, none of the fiber groups possess normalized YAP intensities that are significantly different from one another. This is most likely due to the low samples size of each surface. Increasing the number of samples would likely results in more significant differences between the groups.

Chapter 4

Discussion

In this study, we attempted to determine whether cell-cell contact and the morphology of the cell microenvironment have a significant effect on cell morphology, YAP localization and overall YAP expression. In this chapter, the results from both experiments are analyzed and compared to draw conclusions from the data. Later, sources of error and proposed follow up experiments are discussed.

Experiment 1

In our first experiment, we seeded cells at high and low cell densities on both PMMA fibers and PMMA surfaces and observed the response. From our data, we observed that the cells seeded on flat samples had a statistically greater area then those seeded on fiber samples. This result agrees with our expected findings, as seeding cells on fibers encourages the cells to spread out along the fibers, limiting the directions to which they are spreading. In contrast, cells seeded on the flat samples receive mechanical signals from their entire surroundings, encouraging them to spread out in all directions. We also observed that cells seeded on samples at high densities had a smaller average area then cells seeded at lower cell densities. This also agrees with our expected results, as cells seeded at high densities have a high likelihood of interacting with other cells. These cells are competing with other cells for surface area on the same substrate, which restricts the area each individual cell can cover. Additionally, we observed that cells seeded on flat samples had a statistically lower eccentricity then cells seeded on the fiber samples. This also agrees with our expected results. Cells seeded on fibers tend to migrate and elongate along the fibers, causing them to be more eccentric. In contrast, cells seeded on flat substrates receive signals from all portions of their surface, encouraging them to adopt a less guided and more circular geometry. Furthermore, we observed that cells seeded at high densities were statistically less eccentric then cells seeded at low cell densities. We believe the reason for this observation is again due to the competitive

environment of high cell density samples. Due to the presence of other cells, high density cells have less room to spread out then their low-density counterparts. This forces them to remain in a relatively small and circular area in contrast to cells seeded at low densities, which have more room to spread out and elongate in all directions.

From the YAP localization analysis, we observed that on fibers, cells seeded at high densities had a lower fraction of cells with nuclear localized YAP concentrations then cells seeded at high cell densities. This agrees with our expected results, as contact with other cells results in activation of the Hippo Signaling Pathway, which in turn results in the transport of YAP out of the nucleus into the cytoplasm, where it is degraded. The same trend was not observed in flat samples, where the fraction of cells with nuclear YAP concentrations was comparable between the high and low cell density samples. Reasons for this deviation from expected results may be due to the low number of cells analyzed for YAP localization in the low-density samples. A statistical analysis via Chi-Square test for independence failed to elucidate an association of either surface type or cell density with the categorical localization of YAP in either the nucleus or cytoplasm. In the follow up experiment, a greater number of low cell density samples were observed to obtain low density cell fractions that were more representative of the cells seeded on each surface type.

Analysis of the relative YAP concentrations in the different samples did however result in some interesting findings. When normalized to tubulin intensity, which was assumed to be proportional to the number of cells originally on the samples before they were lysed, we observed that the cells seeded on PMMA fibers displayed a significantly higher normalized YAP concentration then the PMMA surface cells. Possible explanations for this observation may be due to the tendency of flat PMMA samples to support a higher cell density then PMMA fibers seeded on a PHEMA surface. This is due to the tendency of PHEMA to inhibit cell adhesion, resulting in less surface area for the cells to adhere to on the PMMA fiber samples. This statement is supported by lower intensity tubulin bands from the fiber samples when compared to the flat samples. As a result, the cells on the PMMA fibers likely have less interactions with

other cells, resulting in a less active Hippo signaling pathway, which in turn results in decreased cytoplasmic YAP degradation. Other possible explanations for the increased YAP expression in the fiber samples can include mechanical signals originating from the cells interacting with the fibers. However, this is only a suggestion and requires further investigation.

Experiment 2

In the second experiment, we investigated the effect of varying microfiber diameter as well as cell density on mMSC morphology, YAP localization, and overall YAP expression. Cells were seeded on different diameter PMMA fibers and flat PMMA surfaces at different cell densities. Using DIC microscopy, it was confirmed that the fiber sizes of each group were statistically different from one another. From our results, we saw that cells seeded on the large, medium, and smallest fiber groups resulted in similar mean cell areas. In contrast, cells seeded on the flat surface and medium fiber samples also displayed statistically similar mean cell areas. The flat and medium fiber cells were on average larger than the cells seeded on the large, small, and smallest fibers. Though we expected the cells seeded on the flat surface to be larger than the cells seeded on the fibers, it was interesting to find that the medium fiber cell areas were also significantly different from cells seeded on the other fiber types. If this difference persists through repeated experiments and larger sample sizes, it would indicate that changing microfiber diameter does indeed have an effect on cell area. Cells seeded at high cell densities were statistically smaller than cells seeded at low cell densities. This result agrees with both our expected outcome as well as the results in experiment one. Additionally, we observed that cells seeded on flat samples were statistically less eccentric then cells seeded on fibers. This is in agreement with our results from experiment one. Furthermore, cell eccentricity was not statistically different between fiber types. These results suggest that merely the presence of fibers results in more elongated cells. The diameter of the fibers does not appear play a large role in modulating cell eccentricity for the fiber diameters tested. Cells

seeded at high cell densities were statistically less eccentric then cells seeded at low cell densities. This agrees with both our expected outcome as well as our results from experiment one.

From the YAP localization analysis, we observed that for our flat samples, as well as our small and smallest fiber samples, the low cell density samples displayed a higher fraction of cells with YAP activated nuclei that their corresponding high cell density samples. In contrast, for the large fiber and medium fiber samples, the fraction of cells with significant nuclear YAP localizations did not appear to vary based on cell density. A Chi-Square test for independence confirmed with 95 percent confidence that YAP localization is dependent on cell density, suggesting that cell-cell interactions plays a role in regulating cellular YAP localization. This result is in agreement with our expected results, as well as our intuition gained from experiment one. However, our data did not show from Chi-Square analysis that surface type and cell nuclear localization fraction are related at either high or low cell densities. The highdensity sample Chi Square analysis returned a P value of 0.097, which is close to 0.05. As a result, a significant effect may be observed if the number of analyzed cells was significantly increased. However, the low-density sample Chi-Square analysis resulted in a P value of 0.475, which is much greater than 0.05. As a result, our data suggests that at low cell densities, surface type and cell nuclear localization fraction are likely not related.

From the western blot analysis, we first attempted to verify that the cell fractionation procedure was a success. Using a two sample t-test, we saw that the tubulin intensity of the cytoplasmic samples was statistically greater than the tubulin intensity in the nuclear samples. This was expected, as tubulin is localized primarily in the cytoplasm, where it is the main constituent of the microtubules of the cytoskeleton. To compare the relative YAP concentrations between the samples, both the cytoplasmic and nuclear YAP intensities were normalized with respect to the corresponding cytoplasmic tubulin intensity. We observed that with the exception of the flat samples, cytoplasmic normalized YAP concentrations were much greater than the corresponding nuclear normalized YAP concentrations. For the flat samples, both the normalized cytoplasmic and nuclear YAP intensities were close to zero. This result deviated

from our expected results. In experiment one, though the normalized YAP concentration was lower for the flat samples then the fiber samples, it was still greater than zero. Possible reasons for this deviation are discussed in the error sections below. Furthermore, in all samples, the normalized nuclear YAP intensities were very close to zero. This likely indicates that the nuclear extracts were too dilute to detect YAP protein. Though less likely, it could also indicate that the nuclear and cytoplasmic components were not successfully separated.

Still, the normalized cytoplasmic YAP concentrations were compared to obtain insightful information. A one-way ANOVA test demonstrated with 95 percent confidence that the mean normalized YAP intensities for each sample group were not all equal. Furthermore, a follow up Tukey Pairwise comparison analysis demonstrated that the normalized YAP intensities of the flat samples were significantly different from the normalized YAP intensities of the small fiber and smallest fibers. The large fiber and medium fiber normalized YAP intensities were not significantly different from any of the other surface types. However, this may be attributed to the low sample sizes used in the western blot analysis. Observing figure 24, it appears that for the fiber samples, normalized cytoplasmic YAP concentration tended to be largest for the small and smallest fiber groups. This suggests that PMMA fiber with an average diameter approximately between 0.8 and 1.1 micrometers result in the highest level of cytoplasmic YAP expression for our samples.

Sources of Variance and Error

As stated in the methods section, cells were categorically labelled as either having a cytoplasmic or nuclear localized YAP concentration. A cell's YAP concentration was categorized as nuclear localized if the YAP intensity in the nucleus region was at least as bright as the YAP intensity in the cytoplasm region of the same cell. Though this method worked well as a general method of analysis used to estimate the percentage of cells with nuclear YAP concentrations for comparison purposes, and though methods were implemented to ensure categorization methods were kept consistent for all samples, it presented a number of obstacles that likely introduced significant variance in the data set.



Figure 25: This figure depicts 4 cells with differing levels of nuclear YAP localization

The first obstacle was that not all cells in a sample would have the same cytoplasmic intensity. This would cause variance, as it would be hard to determine whether a cell with a lower overall YAP intensity is activated. For example, in figure 25, cell (a) clearly has a nuclear YAP intensity that is greater in the nucleus then the cytoplasm and is therefore considered to be nuclear localized. Cell (b) has a nuclear YAP intensity that is clearly less than its cytoplasmic YAP intensity and is therefore considered to be cytoplasm localized. Cells (c) and (d) are more difficult to categorize, as their nuclear YAP intensity is much lower than in cell (a) but is still greater than their respective cytoplasmic intensities. To stay consistent between samples with varying YAP intensities, the categorization method based on comparing nuclear to cytoplasmic intensity was developed. However, such a categorization method likely resulted in a significant number of misclassifications, though the misclassifications were most likely consistent for all samples. To minimize the degree to which the data was skewed, all sample images were analyzed in one session. Furthermore, the first ten images analyzed at the beginning of the session were also analyzed

at the end. The results from both counts were then compared to assure the number of cells with nuclear localized YAP did not change significantly.

The second obstacle was that some cells had YAP concentrations that were not uniform in the cytoplasm. For example, some cells had large YAP intensities directly outside the nucleus, but lower intensities farther away. These cells were usually classified as cytoplasm localized, as their YAP intensity in the nucleus appeared to be less than that in the cytoplasm. However, the bordering of a bright YAP intensity around the nuclear region may have created the illusion of a nucleus without significant YAP localization, when in reality, it was greater than the average intensity found in the rest of the cytoplasm. These phenomenon, as well as other cells that had nonuniform YAP intensities in their nucleus, likely resulted in a number of false classifications that resulted in increased variance in our data.

The third obstacle was due to the need to repeat the analysis on the immunostained flat samples. In the first trial, the flat samples' polymer surfaces became detached from the coverslips during the immunostaining process, resulting in unusable samples. The trial was repeated with new flat surfaces using mMSCs from the same population and similar passage number. However, the fact that the flat samples were not seeded at the same time as the fiber samples likely introduced variance to our data. Furthermore, during the immunostaining process, there was insufficient YAP antibody available to use the same amount as the previous samples. This was partially compensated for by increasing the imaging time during immunofluorescence microscopy. Nonetheless, the flat images displayed much larger amounts of background noise due to the weak YAP signal and long exposure time. Fortunately, the adaptive categorization method described above likely helped limit the amount of variance this event added to our data.

There were a number of sources of error that could have affected the results from the western blotting process. In one instance there was a small spill over of a well containing a cytoplasmic fraction into a well containing a nuclear fraction. The nuclear fraction ended up displaying a tubulin and YAP intensity that was somewhat larger than the other nuclear extracts. Additionally, in both experiments, due to the large number of total samples, two gels were used. Inconsistencies in the gels, running, transfer, and staining processes could have resulted in variances in YAP and tubulin intensities for the samples imaged on different gels. To help compensate for this, YAP was normalized with respect to the sample's corresponding tubulin intensity.

Finally, a number of sources of variance could had developed during the surface preparation process. Most significantly would have been those arising from fiber formation through electrospinning. Fiber creation via electrospinning depends on a number of controllable variables, including voltage, polymer type, polymer concentration, flow rate, needle gauge, and distance. In addition, there are a number of variables that are difficult to control that also effect fiber formation, including humidity and temperature. To minimize variance due to uncontrollable variables, most fiber samples of a certain type were prepared in one session.

Improvements and Future Works

In future iterations of this experiment, steps should be taken to assure that equal amounts of YAP primary and secondary antibody are applied to each surface sample during the immunostaining process. This can be accomplished by preparing an antibody solution of the correct dilution in a separate container, then adding the pre-prepared solution to each of the sample wells, rather than adding the antibody directly into the sample wells, which likely introduces variance due to pipetting inconsistencies. Using consistent antibody concentrations and imaging all samples at the same exposure time will make it easier to pick a static threshold for YAP activation that can be used for all samples. Having a static threshold will allow for the more precise categorization of cells as having nuclear or cytoplasmic YAP localizations. Software, such as ImageJ, can then be used to determine if the nucleus of each cell meets this threshold.

Furthermore, to obtain more conclusive data from the western blot analysis, more samples of the same type should be utilized. This can be accomplished either by increasing the number of wells in each

gel, increasing the number of gels, or decreasing the number of surface groups. I would recommend running the western blot analysis with just the flat, large fiber, and smallest fiber sample extracts to allow for more lysed samples from each of the groups to be used. More data points for each group better categorizes the YAP intensities of the cell cytoplasm and nucleus extracts on each surface and allows for more significant comparisons.

Finally, the effect of microfiber orientation on mMSC morphology, YAP localization, and overall YAP expression can be analyzed. This can be accomplished through the use of a 3D printer modified to extrude polymers in a fashion similar to an electro spinner. Using G-code, fibers can be deposited onto a substrate at different spacings as well as alignments. PMMA fiber samples would be prepared with fiber diameter and density remaining constant between groups. For all groups, half of the fibers will be aligned. Half of the other fibers will then be deposited at various degrees of alignment (0°, 30°, 45°, 60°, 90°) for each sample type. The samples would then be seeded with mMSCs, then analyzed using methods similar to those of experiment 2.

Chapter 5

Conclusion

Mesenchymal stem cells provide a strong foundation on which a new era of autologous regenerative therapies can be built. Yes-associated protein, previously known for its role in the Hippo Signaling Pathway, is now a known nuclear transducer of mechanical information in MSCs and will likely continue to be a key protein interest as researchers work to predict and control stem cell fate. In this study, we aimed to help decipher the role of this protein in transmitting signals relating to MSC environment. Specifically, we examined whether changes in the morphology of a cell's microenvironment, as well as cell density, had a significant effect on cell morphology, intracellular YAP localization, as well as overall YAP expression. Our results confirm assertions in literature that the cellular YAP localization is affected by cell density, indicating that YAP plays a role in the signaling pathways regarding the sensing of other cells. Furthermore, we observed that the fraction of mMSCs with significant nuclear YAP concentrations varies based on the characteristics of the cell's microenvironment, such as the presence of a three-dimensional PMMA microfiber matrix versus a flat PMMA surface. Our results also suggested that varying microfiber properties, specifically diameter, also has an effect on intracellular YAP concentrations, indicating that YAP plays a significant role in the sensing of the threedimensional characteristics of a MSC's microenvironment. Future experiments examining the role of YAP in sensing microfiber alignment and fiber density may also prove to elucidate additional insights regarding YAP's role in MSC mechanotransduction. Uncovering the full role of Yes associated protein in transducing mechanical signals into cell responses, including phenotypic expression and differentiation, will likely lead to the manipulation and control of these pathways, paving the way for one of the next breakthroughs in guided regenerative therapies.

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Education

The Pennsylvania State University, University Park, Pennsylvania Penn State Schreyer Honors College Scholar May 2018

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Masters of Science in Bioengineering Bachelors of Science in Biomedical Engineering Bachelors of Science in Mechanical Engineering

Technical Skills

Software: SolidWorks, MATLAB, ANSYS, COMSOL, Mathematica, R, Minitab, Microsoft Office, Windows, Linux
 Laboratory: Experiment Design, 3D Printing, Fluid Flow, Machining, Finite Element Analysis, Simulation, Biological Safety Cabinet experience, Cell Culture, Immunostaining, Western Blot, Circuitry, Server Maintenance

Internship and Research Experience

Dr. Justin Brown, University Park, Pennsylvania- Thesis Research 08/2014-Present

o Utilized immunofluorescence and western blots to examine the effects of cell density and extracellular

- environments on YAP signaling protein expression and localization in human mesenchymal stem cells.
- Employed the scientific and iterative methods to design and modify research experiments.

Janssen Pharmaceuticals, Titusville, New Jersey- Cardiovascular Associate 05/2016-08/2016

- Evaluated and cataloged patient severe adverse events to track long term drug viability.
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Center for Disease Control, Cincinnati, Ohio- Research and Lab Assistant 06/2015-08/2015

- \circ Aided in innovative engineering control design to protect workers from silicosis.
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- Monitored lab contamination and completed diversified lab functions.

Orthocell, Murdoch University, Perth, Australia- Lab Assistant

- \circ Gained proficiency in the standard operating procedures of an industrial lab.
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Work Experience

Lion Tutors, State College, Pennsylvania- Calculus Tutor 01/2014-Present

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