ABSTRACT

MATHIEU, PATTIE SMITH. Investigating the Role of Cytoskeleton and the Primary Cilia in Osteogenic Differentiation. (Under the direction of Elizabeth G. Loboa).

Human adipose-derived stem cells (hASC) exhibit multilineage differentiation potential with lineage specification that is dictated by both the chemical and mechanical stimuli to which they are exposed. These cells show great potential for use in bone tissue engineering and other regenerative medicine applications.

In the similar cell type of mesenchymal stem cells (MSC), chemically induced differentiation has been shown to change the cytoskeletal properties. The cytoskeleton is known to play a large role in mechanotransduction and a growing number of studies are showing that it can also contribute to MSC differentiation. We analyzed the significant contribution of actin and integrin distribution, and the smaller role of microtubules, in regulating MSC fate. Osteogenic differentiation is more prevalent in MSC with a stiff, spread actin cytoskeleton and greater numbers of focal adhesions. Both adipogenic and chondrogenic differentiation are encouraged when MSC have a spherical morphology associated with a dispersed actin cytoskeleton with few focal adhesions. Different mechanical stimuli can be implemented to alter these cytoskeletal patterns and encourage MSC differentiation to the desired lineage.

Tensile strain has been shown to induce osteogenic differentiation in hASC. Knockdown of primary cilia proteins has been shown to diminish osteogenic differentiation in hASC. Therefore, the first study in this paper focused on a finite element model of a primary cilium undergoing three dimensional tensile strain. This study used finite element analysis (FEA) to determine strains occurring within the ciliary membrane in response to
10% tensile strain applied parallel, or perpendicular, to cilia orientation. To elucidate the mechanical environment experienced by the cilium, several lengths were modeled and evaluated based on cilia lengths measured on hASC grown under varied culture conditions. Principal tensile strains in both hASC and ciliary membranes were calculated using FEA, and the magnitude and location of maximum principal tensile strain determined. We found that maximum principal tensile strain was concentrated at the base of the cilium. In the linear elastic model, applying strain perpendicular to the cilium resulted in maximum strains within the ciliary membrane from 150 to 200%, while applying strain parallel to the cilium resulted in much higher strains, approximately 400%. In the hyperelastic model, applying strain perpendicular to the cilium resulted in maximum strains within the ciliary membrane around 30%, while applying strain parallel to the cilium resulted in much higher strains ranging from 50% to 70%. FEA results indicated that primary cilium length was not directly related to ciliary membrane strain. Rather, it appears that cilium orientation may be more important than cilium length in determining sensitivity of hASC to tensile strain. This is the first study to model the effects of tensile strain on the primary cilium.

The second study focused on the effects of electric fields on osteogenesis and primary cilia related gene expression in hASC. Human ASC were cultured on interdigitated electrodes that produced AC electric fields of 1V/cm at 1 Hz. When cells were exposed to these electric fields for 4 hours a day, they showed upregulation of the osteogenic genes Runx2 and Spp1 at day 3 in Complete Growth Medium (CGM). At day 7 Runx2 was downregulated in response to electrical stimulation in both CGM and Osteogenic Differentiation Medium (ODM). The primary cilia gene Ift88 also showed downregulation at day 7 in both CGM and ODM. Culture in ODM showed increased calcium accretion due to
electrical stimulation at day 10 and 14. Additionally, electrical stimulation showed changes in cell morphology dependent on culture medium. Cells cultured in CGM and exposed to electrical stimulation exhibited morphology aligned perpendicular to the direction of electric fields, while cells cultured in ODM showed a more clustered morphology.
Investigating the Role of Cytoskeleton and the Primary Cilia in Osteogenic Differentiation

by
Pattie Mathieu

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APPROVED BY:

__________________________________________________________
Elizabeth G. Loboa                                        Greg S. McCarty
Committee Chair

__________________________
Jacqueline H. Cole
BIOGRAPHY

Pattie Mathieu was born on July 26 1989 to Rick and Peggy Mathieu. She has one younger brother. She graduated in June of 2006 as valedictorian of Harrisonburg High School. She attended the University of Virginia as a Rodman Scholar where she majored in Biomedical Engineering. She graduated in May 2010 with a B.S. in Biomedical Engineering. She was accepted into the joint graduate program in Biomedical Engineering at North Carolina State University and the University of North Carolina. After Pattie completes her M.S. in Biomedical Engineering, Pattie will be heading to Dublin Ireland to pursue a Ph.D. at Dublin City University.
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CHAPTER 1

Introduction

1.1 Motivation

Tissue engineering has been greatly expanding as a field, paving the way for innovations in regenerative medicine, organ replacement, and treatment of tissue injury or defect. Ultimately, tissue engineering seeks to understand how tissues affect and are affected by stimuli on the cellular level and use this knowledge to create functional full scale tissues or organs for clinical use. When attempting to engineer a functional tissue, there are three components that are essential to developing the correct tissue structure and function. First, the cell source must be considered so that one can be certain that the cells utilized will be able to perform the functions required in the final tissue construct. Second, the scaffold for the cells must be considered. The scaffold must allow for the correct cell and organ structure as well as biocompatibility with the eventual tissue recipient. Finally, extracellular stimuli are important as they can affect differentiation of stem cells as well as mechanical and functional properties of the tissue. Within this body of work, we consider chemical, mechanical, and electrical stimuli. Balancing these three components is essential in creating viable tissue engineered constructs.

The choice of cell type is an important one in the process of tissue engineering. Stem cells are a promising cell source for designing a tissue construct given their multipotent differentiation capability. Although adult stem cells have fewer differentiation pathways they offer other advantages. These cells can also be obtained from the person requiring the tissue engineered construct ensuring the engineered tissue will be an autologous implant. This
avoids the potential for rejection of the engineered tissue. Of the different types of adult stem cells that are currently being investigated, human adipose derived stem cells (hASC) have shown great promise for use in tissue engineering. These cells are easily obtainable and have been shown to have similar differentiation potential to human mesenchymal stem cells (hMSC). Human ASC have shown potential for differentiation into many types of musculoskeletal tissue, including bone tissue.

The type of stimulation chosen to induce stem cell differentiation is also important in tissue engineering. We and others have previously shown that hASC exhibit mechanosensitivity with changes in proliferation and lineage specification in response to many types of mechanical stimuli (1-7). Both tensile strain and electric fields have been shown to induce osteogenic differentiation in ASC. Cyclic tensile strain, whether applied to hASC in monolayer (two-dimensional) or three-dimensional collagen culture, promotes hASC osteogenesis and cell-mediated calcium accretion (4, 5, 8). Electric fields have also been shown to induce osteogenesis in ASC (3, 9, 10).

Understanding the mechanisms for detecting mechanical and electrical stimuli is essential in comprehending the effects of mechanical and electrical stimuli on stem cell differentiation. One mechanism we propose plays a critical role in mechanotransduction of both tensile strain and electric fields is the primary cilium. Primary cilia are non-motile cilia which appear in almost all cell types. Cells typically have one primary cilium at some point during the cell cycle (11). Primary cilia have predominantly been studied for their role in polycystic kidney disease, where they have been shown to be vital in the detection of fluid shear stress (12). However, they have also been shown to play a role in bone formation. In
particular, it has been shown to be important in osteogenic lineage specification of MSC in response to oscillatory fluid flow (13, 14). A recent study from our lab has reported their critical role in lineage specification of human adipose-derived stem cells (hASC) (15).

1.2 Objectives

This body of work has focused on understanding the role of primary cilia in mechanotransduction for hASC in the context of bone tissue engineering. Understanding how a cell senses its environment is essential in being able to use these mechanisms to induce differentiation of these cells into tissue constructs. Primary cilia have been implicated as mechanosensors in fluid shear environments. This body of work aims to understand what role they might play in detecting other types of mechanical, or electrical, stimuli.

The first aim of this project was to understand the role of the primary cilium in detecting tensile strain. While primary cilia are indicated in detecting tensile strain, it is not known if tensile strain could cause primary cilia to bend in the way they have been shown to do under fluid shear. A model was created to measure how a cilium might deform under tensile strain in 3D cell culture. This model allowed us to determine the forces and strains acting on the primary cilium and gave us insight into how these strains in the cilium might be transduced into signals within the cell.

The second aim of this project was to understand the role of primary cilia in detecting electric fields in hASC. We looked at changes in both osteogenic and primary cilia related genes in response to electrical field stimulation. In addition we looked at end product expression as a result of these electric fields. This study allowed us to understand how electric fields would affect hASC on a genetic expression level.
1.3 Dissertation Findings

This body of work has been shared with the scientific community both in publications in peer reviewed journals and at scientific conferences. Published articles and manuscripts in preparation are listed below:

**In Preparation**

Mathieu PS, Amos AN, Hamouda M, Bernacki SH, McCarty GS, Loboa EG. Primary Cilia as Electric Field Sensor: Osteogenic and Primary Cilia Related Gene Expression in Adipose-Derived Stem Cells Exposed to Alternating Current Electric Fields

**Published**

Mathieu PS, Bodle JC, Loboa EG. (2014) Primary cilium mechanotransduction of tensile strain in 3D culture: Finite element analyses of strain amplification caused by tensile strain applied to a primary cilium embedded in a collagen matrix. Journal of Biomechanics. 47(9): 2211-2217

CHAPTER 2
Cytoskeletal and Focal Adhesion Influences on Mesenchymal Stem Cell Shape, Mechanical Properties, and Differentiation Down Osteogenic, Adipogenic, and Chondrogenic Pathways

2.1 Introduction

Mesenchymal stem cells (MSC) show great promise for use in tissue engineering applications because of their potential to regenerate many types of tissue including bone, cartilage, adipose, muscle and possibly nerve tissues (16-21). Since MSC can be isolated from adult patients, this allows for the possibility of using MSC for patient-specific repair of bone and cartilage defects with tissue that will not provoke an immune reaction. Tissues engineered using MSC are known to be sensitive to mechanical stimuli (22-24). Mechanical forces can be used to induce or aid MSC differentiation into various mature cells. Cyclic tensile strain (24, 25) and oscillatory fluid flow (26-28) have both been reported to increase osteogenic differentiation and decrease adipogenic differentiation, while uniaxial, unconfined compression (29) and cyclic hydrostatic pressure (30) increase chondrogenesis (31, 32). How such mechanical forces transmit signals to the cells and thus affect differentiation is currently a topic of great interest and study.

The cytoskeleton is known to play a role in mechanosensing and mechanotransduction (33-36). The interactions between cytoskeletal proteins, integrins and mechanical forces can influence cells to change shape, proliferate and even differentiate (33). Because of this, there has been increasing interest in the interaction between the cytoskeleton and the differentiation of mesenchymal stem cells. There appear to be important links
between the mechanosensing role of the cytoskeleton and MSC fate. However, this is a burgeoning field of research and much knowledge is yet to be acquired. This chapter provides information on what has been published in this relatively new field with specific emphasis on cytoskeletal and focal adhesion influence on MSC shape, mechanical properties, and differentiation.

2.2 Cytoskeletal Properties of MSC

As MSC differentiate, their mechanical properties change according to their lineage specification. Young’s modulus is a measurement of how much a material will deform in response to a stress placed on it. Materials with a higher Young’s modulus are stiffer and don’t deform as easily. Prior to differentiation, human MSC (hMSC) have a Young’s modulus of approximately 3.2kPa (37, 38). However, sample temperature can change the cell stiffness. Human MSC measured at 20°C have been reported to have lower viscosity and higher stiffness than hMSC measured at 37°C (39). Cytoskeletal structure has a large impact on hMSC mechanical properties. Disrupting the actin cytoskeleton with cytochalasin significantly decreases the stiffness and increases the viscosity of hMSC (38, 39). However, disrupting the microtubule structure with nocodazole does not significantly change the stiffness (38). This implies that much of the stiffness of hMSC is dependent on the actin cytoskeleton as opposed to the microtubules.

Two non-mechanical factors have been reported to influence the structure of the actin cytoskeleton in hMSC. If the perinuclear actin cap is inhibited, the formation of actin stress fibers in hMSC is prevented (40). Disruption of actin configuration can also be influenced by population doubling of the cells. Human MSC at passage 6 are more susceptible to actin
cytoskeleton disruption by jasplakinolide than cells at passage 2 (41). Therefore, hMSC should be used at a low passage to ensure cytoskeletal integrity.

2.3 Mechanical Properties

As MSC differentiate, they exhibit changes in their mechanical properties (38, 42). These changes in mechanical properties can be indicative of underlying cytoskeletal changes, especially in the actin cytoskeleton (38, 39). Actin stress fibers are one of the stiffest structures within hMSC (43) and disrupting the actin cytoskeleton decreases cell stiffness (38, 39). The mechanical properties of MSC depend on actin fiber structure, which varies based on the differentiation pathway.

Studies have differed in their findings of the value of the elastic modulus of hMSC. Yu et al. reported that hMSC have an instantaneous Young’s modulus of about 0.5 kPa and an equilibrium Young’s modulus of about 0.1 kPa (42). The viscoelastic properties of the cells were measured using micropipette aspiration of suspended cells. The instantaneous Young’s modulus was measured over the initial aspiration of the cell and the equilibrium Young’s modulus was measured after an adequate amount of time to measure cell equilibrium response (42). On the other hand, Titushkin et al. reported a much larger Young’s modulus of about 3.2 kPa (38). They measured the Young’s modulus by atomic force microscopy (AFM) indentation at a velocity of 2µm/s (38). This value was corroborated by Darling et al. who reported a Young’s modulus of approximately 3.2kPa for hMSC attached to a surface, and 2.5kPa for spherical cells using AFM at an indentation rate of 6.25 µm/s (37). However, the differing protocols for cell loading make it difficult to compare the Young’s moduli between studies.
2.3.1 Osteogenesis

Using AFM, Titushkin et al. reported that osteoblasts have a Young’s modulus of approximately 1.7 kPa (38). That study also found that hMSC exposed to osteogenic media for 10 days exhibited a significant decrease in elastic modulus to approximately 2 kPa from 3.2 kPa (38). Yu et al., using the same procedure used for hMSC, showed an increase in both instantaneous and equilibrium Young’s moduli when hMSC were cultured in osteogenic differentiation medium for 21 days (42). The instantaneous Young’s modulus increased from 0.5 kPa to 0.9 kPa while the equilibrium Young’s modulus increased from 0.1 kPa to about 0.2 kPa (42). As MSC differentiate into osteoblasts, their mechanical properties become similar to those of osteoblasts, however, whether or not this equates to a significant increase or decrease in their stiffness is debatable.

Darling et al. reported that the elastic modulus of osteoblasts was dependent upon cell spreading and morphology. They reported that spherical osteoblasts had an elastic modulus of 2.0 kPa while spread osteoblasts had an elastic modulus of 5.8 kPa (37). These findings contradict the findings of Yu et al. whose measurements showed that osteoblasts were less stiff than hMSC, whereas Darling et al. determined that osteoblasts were stiffer than hMSC. However, differing methods used for measuring the stiffness could be causing the disparate responses. Because cell shape affects mechanical properties, we would also expect differing cytoskeletal arrangements between cell shapes.

2.3.2 Adipogenesis

Adipocytes have been shown to have a much lower stiffness than hMSC and osteoblasts. Yu et al. showed a decrease in Young’s modulus of hMSC after 21 days of
exposure to adipogenic differentiation media (42). The instantaneous Young’s modulus was measured to be 0.42 kPa and the equilibrium Young’s modulus was measured to be 0.09 kPa (42). Darling et al. reported the elastic modulus of adipocytes to be approximately 0.61 kPa for both spread and spherical cells (37). These relatively low moduli, which do not change with cell shape, imply that adipocytes do not have a very dense cytoskeleton (44).

2.3.3 Chondrogenesis

Darling et al. reported that chondrocytes have an stiffness that is midway between MSC and adipocytes at approximately 1.2 kPa (37). As with adipocytes, the modulus did not change significantly relative to cell spreading (37). This might indicate that the actin cytoskeleton is not as important for mechanotransduction in chondrocytes as it is in osteoblasts.

2.4 Cell Shape

2.4.1 Osteogenesis

Cell shape, and the cytoskeletal changes related to cell shape, highlight how important the cytoskeleton may be in regulating MSC differentiation. As MSC differentiate into osteoblasts, they become more elongated and spread (45). When MSC are plated at a low density, where they have the ability to spread, they have a greater osteogenic potential than cells plated at a high density (44). Even if these cells are replated at a high density after 48 hours, they still exhibit increased osteogenic potential (44). Confining cells on micropatterns designed to prevent them from spreading inhibits osteogenic differentiation (44). Therefore, cell shape plays an important role in early differentiation, and this effect is not due to cell-cell interactions (44). Cell shape has been shown to be related to actin
cytoskeleton regulation. Mesenchymal stem cells plated on micropatterns which allowed them to spread expressed more RhoA, which is responsible for actin organization, than cells that were not allowed to spread (44). Human MSC that are more spread have actin and focal adhesion arrangements more similar to osteoblasts than hMSC that are less spread (45). This exemplifies that the shape of MSC, their osteogenic potential, and the actin cytoskeleton are likely related.

Figure 2.1: (A) Flower shape used in Kilian et al (46). (B) Star shape used in Kilian et al (46). Mesenchymal stem cells (MSC) confined to the flower shape showed increased adipogenesis. MSC confined to the star shape showed increased osteogenesis. Adapted from Kilian et al (46).

Cell spreading is not the only component of cell shape that influences cytoskeletal configuration and osteogenic differentiation. When MSC are confined to micropatterned rectangles, rectangles with increased aspect ratios increase MSC osteogenic differentiation, indicating that more elongated cells are more likely to undergo osteogenesis (46). When MSC are constrained to micropatterned “flowers” with convex edges, as shown in figure 2.1, osteogenesis decreases (46). Constraining the cells to micropatterned “stars”, with concave edges, as shown in figure 2.1, increases osteogenesis (46). The authors of that study analyzed F-actin in the flower patterns and found that it was mostly disperse within the cells, while
star-patterned cells had more prominent stress fibers along the outer edges with more
disperse fibers in the interior (46). Focal adhesion distribution was also found to change
between the two shapes. In flower-patterned cells, vinculin was dispersed throughout the cell;
while in star-patterned cells, vinculin was concentrated in the points of the stars (46).
Disrupting microtubules with nocodazole caused both patterns to become equally osteogenic,
while disrupting the actin cytoskeleton with cytochalasin-D, and Rho-kinase with Y-27632
caused both patterns to become equally non-osteogenic (46). This implies that the ability of
cell shape to determine MSC differentiation is dependent on cytoskeletal cues from both the
actin cytoskeleton and the microtubule skeleton.

2.4.2 Adipogenesis

Cytoskeleton configuration can also affect the ability of MSC to undergo
adipogenesis. Like osteogenesis, adipogenesis has been shown to be dependent on cell shape
and, by extension, the underlying cytoskeleton. MSC are more adipogenic when the cells are
rounded. When MSC are geometrically constrained by micropatterns so that they are unable
to spread, they become more adipogenic (44). Similarly, when cells are plated at a high
density, such that they cannot spread, they show a greater adipogenic potential (44). Even
when cells are later replated at a low density, high-density plating in the first 48 hours of
differentiation encourages adipogenesis (44). As with osteogenic differentiation, cell shape
within the first 48 hours appears to be essential to regulating the lineage specification to
which MSC commit.

Adipogenic differentiation is not only affected by cell spreading but also by cell
geometry. When MSC are confined to micropatterned squares, they are more adipogenic than
when confined to rectangles (46). This again exemplifies that when MSC are maintained in a more rounded morphology, it increases the likelihood that they will undergo adipogenesis as opposed to osteogenesis. Furthermore, in direct contrast to osteogenic differentiation, when MSC are constrained to micropatterned “flowers”, adipogenesis increases, while constraint to micropatterned “stars” decreases adipogenesis (46). As noted previously in this review, the differences between these two patterns are dependent both on the actin and microtubule cytoskeleton; the trend found for osteogenic differentiation is opposite to adipogenesis (46).

2.5 Actin Cytoskeleton and RhoA/ROCK Signaling

2.5.1 Osteogenesis

The actin cytoskeleton plays an important role in the osteogenic differentiation of MSC. Changes in the actin cytoskeleton occur as MSC differentiate. As suggested by the changing stiffness of MSC, the arrangement of the cytoskeleton significantly changes as MSC differentiate into osteoblasts. Human MSC have thicker actin stress fibers, while osteoblasts exhibit a much more disperse actin cytoskeleton (38). As MSC undergo osteogenic differentiation, the actin cytoskeleton becomes more disperse and begins looking more similar to that of osteoblasts (47). When the actin cytoskeleton is disrupted with Cytochalasin-D, MSC become rounded (47). Disrupting the actin cytoskeletal structure within the first 48 hours of exposure to osteogenic media prevents hMSC from reaching the same alkaline phosphatase activity as cells with no cytoskeletal disruption (47). This again exemplifies that the structure of MSC in the first 48 hours of differentiation is extremely important for osteogenesis. Disrupting the actin cytoskeleton decreases osteogenesis in favor of adipogenesis (44). This result has been shown to be dependent on ROCK and RhoA.
Inhibiting ROCK with Y-27632 causes decreased osteogenesis \((44, 48)\). Expressing dominant-negative RhoA decreases osteogenesis, while constitutively active RhoA increases osteogenesis \((44)\). These findings indicate that actin cytoskeletal tension is necessary for MSC osteogenic differentiation. Because actin configuration is important in osteogenesis, the actin cytoskeleton can be used to predict if a cell is differentiating down an osteogenic pathway within 48 hours of culture in osteogenic differentiation medium \((49)\). By analyzing the organizational characteristics of the actin cytoskeleton, Trieser \textit{et al.} have developed an algorithm that could accurately determine the osteogenic potential of various surfaces in only 48 hours, a test that normally takes two weeks \((49)\). A follow up to that study determined that genetic changes could be detected within the first 24 hours \((50)\). These findings exemplify how interrelated the actin cytoskeleton is with osteogenic differentiation and again demonstrates that cytoskeletal activity in the first 48 hours of differentiation determines the osteogenic potential of MSC.

\subsection*{2.5.2 Adipogenesis}

In the one article published to date investigating a link between the actin cytoskeleton and adipogenesis, disrupting the actin cytoskeleton with cytochalasin increased the adipogenic potential of MSC \((44)\). This suggests that having a stable actin cytoskeleton inhibits adipogenesis. Inhibiting both ROCK and RhoA also increases the adipogenic potential of MSC \((44)\). Therefore, it appears that increased actin polymerization in MSC decreases adipogenic differentiation. Overall, cells with a less organized and less stiff actin cytoskeleton are more likely to differentiate into adipocytes.
2.5.3 Chondrogenesis

Actin also plays a role in chondrogenesis. In chick wing-bud MSC, disrupting the actin cytoskeleton with cytochalasin-D encouraged chondrogenesis (51). Various factors known to regulate the actin cytoskeleton play an important role in regulating chondrogenesis. As MSC undergo chondrogenesis, they exhibit a decrease in RhoA activity (52). This decrease in RhoA is at least partially responsible for chondrogenic differentiation of MSC as MSC made to overexpress RhoA exhibited decreased chondrogenesis (52). This shows that the cell causes the actin cytoskeleton to become more diffuse through decreased RhoA activity in order to undergo chondrogenesis.

Manipulating the RhoA/ROCK pathway can also affect the chondrogenic potential of MSC. In mouse limb bud MSC, treatment with Y27632 to inhibit ROCK increased GAG production of the cells and caused cortical actin organization (53). This treatment also reduced the number of actin fibers and caused cell rounding (53). Conversely, RhoA overexpression inhibited GAG synthesis and Sox9 (53). Therefore, the RhoA/ROCK pathway plays a role in regulating the markers of chondrogenic differentiation. Cytochalasin also has been shown to increase Sox9 (26). This strongly indicates that the mechanism for this regulation is likely related to the actin cytoskeleton. Therefore, decreased actin cytoskeletal organization appears to increase chondrogenesis in MSC.

2.6 Microtubules

Despite the fact that they seem to play a negligible role in the mechanical properties of hMSC, there is evidence that the microtubule structure plays at least some role in MSC differentiation. In order to evaluate the effects of microtubules in MSC, various treatments
can be used to disrupt the microtubule structure. Normally MSC show a spindle-like microtubule morphology (54). Taxol, which targets and stabilizes β-tubulin, and nocodazole, which prevents microtubule polymerization, cause significant changes to the microtubule structure of MSC (54). When exposed to taxol, hMSC exhibit a stabilized microtubule structure, and when exposed to nocodazole, hMSC have a depolymerized microtubule structure (54). Of the two treatments, only taxol changes overall tubulin production within cells, increasing the amount of tubulin nine-fold (54). Since these chemicals can disrupt the microtubule structure, one could use this to assess the role microtubules play in the differentiation of hMSC. However, the limited effects of microtubule disruption mean very few studies have been done to investigate their role in differentiation. Those that have been published to date are reviewed here.

2.6.1 Osteogenesis

Microtubules have been shown to play a minor role in osteogenic differentiation. As MSC differentiate, the structure of microtubules does not change significantly (47). However, cells in which the cytoskeleton has been disrupted show a quicker change in the actin cytoskeleton from the undifferentiated to the differentiated morphology (47). Even if the actin cytoskeleton plays the primary cytoskeletal role in differentiation, microtubules might play a small role in the ability of cells to differentiate quickly.

2.7 Focal Adhesions

2.7.1 Osteogenesis

The relationship between focal adhesions and the cytoskeleton also changes as MSC undergo osteogenic differentiation. Osteoblasts have more ERM (ezrin, radixin, moesin
family) proteins than MSC, indicating that there are more focal adhesions in osteoblasts (38). When these ERM proteins are knocked down with siRNA, it decreases alkaline phosphatase production and mRNA expression of osteogenic markers (55). Therefore focal adhesions appear to play an important role in the ability of MSC to undergo osteogenic differentiation. It has been shown that the cytoskeletal changes necessary for osteogenesis are integrin dependent (48, 49, 56). When cells are cultured on glass with no extracellular matrix (ECM) then exposed to osteogenic differentiation media, cytoskeletal changes observable in the first 24 hours in MSC plated on fibronectin, do not occur until 72 hours after plating (49). Since by 72 hours the cells are capable of depositing their own ECM, integrins appear to require a place to bind before differentiation can occur. Osteogenic differentiation of hMSC has been shown to be focal adhesion kinase (FAK) dependent when cells are plated on collagen-1, but not when they are plated on fibronectin or vitronectin (56). FAK knockdown decreased alkaline phosphate activity in hMSC plated on collagen-1 and vitronectin but not fibronectin (56). Another study, which plated hMSC on polyacrylamide gels showed that FAK inhibition and alpha 2 integrin inhibition decrease osteogenesis (48). Focal adhesions appear to be vital for osteogenic differentiation when cells are binding to collagen-1.

2.7.2 Adipogenesis

There has been only one study reported on the role of focal adhesions in MSC adipogenic differentiation. That study found that heparin promotes adipogenic differentiation by disrupting focal adhesions in immortalized and normal mouse MSC by upregulating adipogenic genes (57). This increase in adipogenesis was consistent for multiple types of
adipogenic induction (57). They concluded that having fewer focal adhesions increases the adipogenic potential of MSC (57).

2.7.3 Chondrogenesis

Cytoskeletal configuration and regulation has also been shown to play an important role in chondrogenesis. Like adipogenesis, chondrogenesis is encouraged by having a rounded cell shape. MSC plated on surfaces modified with RGD (arginine, glycine, aspartic acid) peptides spread out, while MSC on RGE (arginine, glycine, glutamic acid) peptide modified surfaces, which prevent focal adhesion attachment, remain rounded (58). MSC on the RGD surfaces showed decreased chondrogenesis as evidenced by lower levels of mRNA for collagen II and aggrecan (58). These surfaces also exemplified the importance of interactions between integrins and the actin cytoskeleton in chondrogenesis. While cells seeded on RGD surfaces had high levels of localized vinculin expression, MSC on RGE surfaces expressed only low levels of vinculin that were not localized (58). This implies that strong focal adhesion attachments are not necessary or beneficial to chondrogenesis.

2.8 Mechanical Interventions

Once we understand the cytoskeletal arrangements that encourage various types of MSC differentiation, we next need to understand how we can encourage MSC to configure their cytoskeletons in a manner conducive to the type of differentiation that is desired. Micro or nano-scale patterns can be used to control the cytoskeletal configuration of MSC. Nanoscale ridges made of polydimethylsiloxane (PDMS) or polystyrene have been used to cause hMSC to align in the direction of the ridges (59, 60). Not only is this alignment translated into an alignment of the actin cytoskeleton, but it also causes a deformation of the
nucleus (59). It has been shown that FAK is decreased in MSC by nanopatterned ridges (61). On polystyrene, but not PDMS, hMSC produce higher levels of vinculin than non-patterned cells (61). Nanopatterns also increase both microtubule expression and the stiffness of MSC (61).

Active mechanical interventions can also cause actin-cytoskeletal alignment in MSC. In 3D culture, MSC show actin-cytoskeletal alignment parallel to the compression direction both in cyclic and static unconfined compression (62). Because the pellets are unconfined, there is likely an element of tensile strain in the direction perpendicular to applied compression. Therefore, it is not surprising that cyclic uniaxial stretch can also be used to cause actin cytoskeletal alignment in MSC (63). We have shown that actin fiber alignment in the direction of applied strain occurs in hMSC exposed to uniaxial cyclic tensile strain (63). However, the alignment is more consistent in 10% strain versus 12% strain, which is likely due to cellular damage caused by the higher 12% strain (63). Shear stress can also cause cytoskeletal changes consistent with osteogenic differentiation. Oscillatory fluid flow at 1 Hz increases RhoA and ROCK II and creates a denser actin cytoskeleton (26). All of these methods could be used to increase cytoskeletal organization and thus osteogenic differentiation.

Other mechanical interventions can be used to promote a more disperse actin cytoskeleton. Microgravity causes the actin cytoskeleton to rearrange in as little as 30 minutes (64). The low gravity environment causes MSC to change from having prominent stress fibers to having a more amorphous actin distribution with actin redistributed from the edges to the center of the cells (64, 65). However, by 120 hours in microgravity, the cells
regain the actin structure of cells not subjected to a microgravity environment (64). Cells cultured in microgravity, then returned to regular gravity also show a return to normal actin organization (64). As expected from a treatment that reduces actin cytoskeleton organization, microgravity can also reduce RhoA activity (65). In response to microgravity, it has been shown that vinculin redistributes in a manner co-localized with actin (64). These changes were not noticeable until 6 hours, much longer than the 30 minutes it took to notice changes in actin (64). Microtubule organization was not affected by microgravity conditions (64). Given the quick return to normal actin organization, microgravity might not be an effective technique for differentiation.

Electric fields can also disrupt the actin cytoskeleton of MSC. Exposure to a direct current (DC) electric field of 2V/cm for one hour has been shown to decrease stiffness and F-actin content of MSC when cells were cultured in Hanks’ balanced salt solution (HBBS) but not in complete growth media (66). This treatment also depolymerized the actin cytoskeletal structure (66). However, the structure could be recovered after an hour in complete growth media with no electric field (66). These treatments that decrease the actin cytoskeletal structure would be useful in encouraging MSC adipogenic or chondrogenic differentiation.

Integrin configuration can also be affected by mechanical interventions. Stiffer substrates cause increased alpha 2 integrin expression (48). Therefore stiffer substrates should be more suitable for MSC osteogenic differentiation, and this has been shown to be the case (48, 67). It has also been shown that focal adhesions adapt differently to various forces. Using ligand coated magnetic microbeads, focal adhesions were shown to adapt within 15 seconds to multiple exposures to three seconds of 130 pN of force (68). This
reaction could be suppressed using Y27632, 2,3-butanedione 2-monoxime (BDM), or cell cooling to 4°C (68). A stronger adaptation occurred after 15 seconds of a sustained 130 pN force, however this reaction could not be inhibited by inhibiting myosin, stretch activated channels or Src tyrosine kinases (68). Only cooling the cells to 4°C prevented this cellular reaction (68). When cells were exposed to more than 60 seconds of strain on the beads, the cells began actively reacting to the strain by retracting against the movement of the beads (68).

2.9 Conclusion

An important cytoskeletal factor in osteogenic, adipogenic and chondrogenic differentiation of MSC appears to be the actin cytoskeleton. Osteogenic differentiation appears to require a stiff, spread actin cytoskeleton. This is not surprising because, in vivo, osteoblasts are generally found as flat cells on the bone surface. Both disrupting actin and limiting the ability of MSC to spread decreases the osteogenic potential of MSC. These conditions are especially important within the first 48 hours of differentiation. Disrupting the actin cytoskeleton or not allowing cells to spread within those first two vital days appears to prevent MSC from ever reaching their full differentiation potential. Adipogenesis requires a cytoskeleton that does not have much organization or tension. Since adipocytes are normally spherical cells with little structural function, they do not need an organized cytoskeleton. Cells that are less spread have a greater potential to undergo adipogenesis. Disrupting the actin cytoskeleton will increase adipogenesis, especially if this is done in the first 48 hours of exposure to an adipogenic environment. Chondrogenesis seems to share the same cytoskeletal configuration as adipogenesis. However, chondrocytes show a larger range of
shapes *in vivo* than adipocytes. In articular cartilage, chondrocytes exhibit a spherical morphology in the intermediate zone, an elongated shape in the superficial tangential zone, and an oblong shape in the deep zone. However these shape differences are not apparent in cytoskeletal configuration leading to chondrogenic differentiation. The factors that can be modified to distinguish chondrogenic differentiation of MSC from adipogenic differentiation of MSC may be chemical and not cytoskeletal. Both adipogenesis and chondrogenesis can be induced by addition of chemical factors to cell growth media. Focal adhesions are also important in differentiation. Osteogenesis requires larger numbers of focal adhesions, while adipogenesis and chondrogenesis are encouraged by preventing focal adhesion attachment.

**Figure 2.2:** Mechanical interventions have been shown to alter the cytoskeletal arrangement of MSC. This knowledge can be used to determine which mechanical interventions to use to encourage various types of differentiation.
The usefulness of understanding cytoskeletal properties of stem cells comes in the ability to use this knowledge to influence stem cell differentiation. Figure 2.2 shows our proposed routes for manipulating differentiation based on studies of cytoskeletal changes under various mechanical manipulations. Nanopatterned ridges, uniaxial compression, cyclic uniaxial tensile strain, oscillatory fluid flow and stiff substrates should all encourage osteogenesis based on cytoskeletal responses to these treatments. Microgravity and DC electric fields cause MSC to assume a rounded shape with little cytoskeletal structure, conducive to both adipogenesis and chondrogenesis. However, physical interventions that have not been tested for cytoskeletal configuration, such as unconfined uniaxial compression and hydrostatic pressure and have also been shown to be conducive to chondrogenic differentiation in MSC and should be a future subject of cytoskeletal studies (29-32). These cytoskeletal changes have been previously shown to translate into the predicted differentiation pathway. Increased stiffness in scaffold matrices has been shown to increase the expression of osteogenic proteins (48, 67). Microgravity has been shown to increase intracellular lipid accumulation, thus showing that microgravity does increase adipogenic differentiation (65). However, cytoskeletal changes might not always predict differentiation. Oscillatory fluid flow, which has been shown to increase cytoskeletal organization, has also been reported to increase Sox9 production in MSC when delivered at 1Hz with a peak force of 1Pa (26). In this instance, it appears that a force that causes cytoskeletal alignment also increased MSC propensity for chondrogenesis.

The results for MSC can also be used to predict behavior in other stem cell types. Many studies have shown that adipose derived stem cells (ASC) have similar differentiation
capabilities as MSC (69-71). Although they do differentiate similarly, MSC and ASC are not exactly alike. MSC have been shown to be more osteogenic under certain conditions (72, 73) and more chondrogenic under others (72, 74, 75). Like MSC, ASC are known to have increased osteogenesis when they are exposed to uniaxial cyclic tensile strain (4, 8, 76, 77). Therefore, we would expect that cyclic tensile strain causes ASC to develop a stiff, spread actin cytoskeleton like that found in MSC exposed to cyclic tensile strain.

In conclusion, actin cytoskeleton and focal adhesion configuration can likely be used to determine how MSC are going to differentiate based on external cues. Cytoskeletal structure is very important to understanding MSC differentiation and how we can regulate it for functional tissue engineering and regenerative medicine applications.
CHAPTER 3

Primary Cilium Mechanotransduction of Tensile Strain in 3D Culture: Finite Element Analyses of Strain Amplification Caused by 10% Tensile Strain Applied to a Primary Cilium Embedded in a Collagen Matrix

In the prior chapter we introduced how the cytoskeletal structure of MSC changes as it undergoes osteogenic, adipogenic, and chondrogenic differentiation. Much like the cytoskeleton undergoes structural changes in response to differentiation stimuli in MSC, primary cilia undergo conformational changes in hASC when exposed to osteogenic and adipogenic stimuli. In this chapter, we present a finite element model of a primary cilium undergoing tensile strain in a three-dimensional strain environment. The goal of this model is to understand how primary cilia might be able to transduce tensile strain.
3.1 Introduction

Primary cilia are organelles found in nearly every cell in the body. They are non-motile cilia structures and cells typically have one primary cilium at some point during the cell cycle (11). Primary cilia have predominantly been studied for their role in polycystic kidney disease, where they have been shown to be vital in the detection of fluid shear stress (12). However, they have also been shown to play a role in bone formation. It has been reported that they are critical for embryonic skeletal development in mice and zebrafish (78). Mutations in PKD1, the gene that codes for the primary cilium protein polycystin 1, have been shown to cause decreased skeletogenesis in embryonic mice (79). Further, primary cilia have been proposed as a mechanism for detection of fluid shear by osteocytes, although this was not empirically confirmed (80). The primary cilium is also considered to be important in osteogenic lineage specification of mesenchymal stems cells (MSC) in response to oscillatory fluid flow (13, 14). Therefore, primary cilia comprise a growing area of investigation in bone tissue engineering.

Primary cilia have also been studied in other cell types. We recently reported their critical role in lineage specification of human adipose-derived stem cells (hASC) (15) which can differentiate into musculoskeletal tissue similarly to MSC (69-71). We and others have previously shown that hASC exhibit mechanosensitivity with changes in proliferation and lineage specification in response to many types of mechanical stimuli (1-7). In particular, cyclic tensile strain, whether applied to hASC in monolayer (two-dimensional) or three-dimensional collagen culture, promotes hASC osteogenesis and cell-mediated calcium accretion (4, 5, 8). Given the role of primary cilia in detecting fluid shear stress, we
hypothesize that primary cilia are a potential mechanotransduction mechanism in hASC since cilia extending from the cell body have the potential to be deflected by the collagen gel of 3D culture. One potential mechanism for primary cilia mechanotransduction of tensile strain is through stretch activated calcium channels (81). However, it has been reported that cilia mechanotransduction occurs independently of calcium ion flux (82). We have recently shown that expression of cilia-associated proteins PC1 and IFT88 affects hASC osteogenesis and moreover, changes in lineage specification of hASC result in length and conformation changes of the primary cilia (15). Additionally we have observed that hASC exposed to 10% cyclic tensile strain will exhibit primary cilia length changes (unpublished data), consistent with work performed in tendon explants (83). We hypothesize that these length changes occur to allow hASC, and other cells exposed to tensile strain, to modulate their sensitivity to strains in the surrounding mechanical environment.

Several previous studies have modeled primary cilia (84-86), although only one has used finite element analysis (84). Rydholm et al. (2010) developed a mechanical model of the primary cilium exposed to shear stress using Comsol Multiphysics (84). The model was comprised of two different layers, one representing the underlying structure of the cilia, and the other the cellular membrane (84). They reported that application of fluid shear stress resulted in a stress concentration in the cell membrane near the base of the cilium (84). Since this region is also the location of stretch activated calcium channels, the authors concluded that primary cilia mechanotransduction is likely related to these channels. Other cilia models have modeled primary cilia as they deflect under fluid shear (85). However, to our knowledge no previous models have modeled the primary cilia in a tensile strain culture.
environment. We hypothesized that primary cilia length changes would yield differential stresses and strains within the cilium structure that are thus translated to the hASC while under tensile strain; comprising a mechanosensory role for the hASC primary cilium and proposing a mechanical mechanism for the cilia length change phenomenon observed in culture.

3.2 Methods

Three idealized finite element models of a primary cilium extending from the cell membrane of an hASC were created using 3D structural mechanics in Comsol Multiphysics (Version 3.4a, Burlington, MA). Each model was generated using different cilium lengths acquired from empirical data of cilium lengths derived from immunofluorescently stained images of hASC. Standard fixation and immunofluorescent staining methods were used to generate the images. Briefly, hASC were fixed in 10% formalin for 20 minutes and then were permeabilized in 0.2% Triton-X-100 and 0.5% BSA in PBS. A primary mouse antibody against α-acetylated tubulin (Sigma) with a chicken anti-mouse secondary and IFT88 with a donkey anti-goat (Alexafluor 594, Invitrogen) were used to visualize the cilium structure in 3D collagen I culture (Figure 3.1.A). In both 2D and 3D culture images, DAPI was used to identify the cell nucleus. In 2D culture images, phalloidin 594 was used to visualize the actin cytoskeleton to establish cilium orientation with respect to the long axis of the cell body and the axis of strain (Figure 3.1.B).
Figure 3.1: Primary cilia on hASC visualized by acetylated α-tubulin (green), in 3D collagen I culture with further staining for IFT88 (red) (a) and in 2D culture with further staining for actin (red) (b). DAPI Nuclei (blue). Scale bar represents 25 µm.

Length data were derived from hASC cultured on collagen I-coated silicone membranes in 2D culture exposed to 10% cyclic tensile strain at 1Hz for 4 hours/day. The cells were cultured in complete growth medium (CGM), containing Eagle's Minimum Essential Medium, alpha-modified supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin; osteogenic differentiation medium (ODM), made of CGM and 50 µM ascorbic acid, 0.1 µM dexamethasone, and 10 mM β–glycerolphosphate; or adipogenic differentiation medium (ADM), CGM supplemented with 1 mM dexamethasone, 5mg/mL insulin, 100 mM indomethacin, and 500 mM isobutylmethylxanthine; for up to 72 hours to acquire representative lineage-specific
cilia parameters (Table 3.1). ImageJ was used to measure the length and orientation of primary cilia.

Table 3.1: Primary cilium lengths used in finite element models. Three different primary cilium lengths were based on empirical measurement of primary cilium lengths on human adipose-derived stem cells (hASC) cultured in either complete growth medium (CGM), osteogenic differentiation medium (ODM), or adipogenic differentiation medium (ADM) in the presence of 10% cyclic tensile strain.

<table>
<thead>
<tr>
<th></th>
<th>CGM</th>
<th>ODM</th>
<th>ADM</th>
</tr>
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<tbody>
<tr>
<td>Length</td>
<td>3.05</td>
<td>2.72</td>
<td>3.90</td>
</tr>
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Over 400 cells were measured for each condition to generate average length values. Further, to inform our idealized 3D model, observations of primary cilia orientation and length on hASC cilia in 3D collagen I culture cultured with CGM and ODM under 10% cyclic tensile strain were also incorporated into the model. The hASC cultured in 3D were similarly stained and visualized using immunofluorescence.

3.2.1 Linear Elastic Model

Combining both 2D and 3D empirical observations, an idealized model comprised of a primary cilium and ciliary membrane attached to a 5 µm² segment of cell membrane where the cilium extends into a 5µm³ block of collagen gel (scaffold used for 3D cell culture) was created (Figure 3.2.A) using Comsol Version 3.4a Structural Mechanics Module.
Figure 3.2: Boundary conditions and geometry for linear elastic model. (A) Primary cilium model shown with cell and ciliary membrane in purple. Collagen gel is shown in pink. (B) Perpendicular strain is applied along the x-axis. (C) Parallel strain is applied along the z-axis.

The cilium was modeled as a continuous elastic beam extending 0.5 μm into the cell body and extending out of the cell body at each of the following lengths dependent on culture medium conditions: (1) 3.05 μm (complete growth medium (CGM) culture), (2) 2.72 μm
(osteogenic differentiation medium (ODM) culture) or (3) 3.90 µm (adipogenic differentiation medium (ADM) culture). These numbers were calculated from the average cilia length for each culture condition and do not account for the variance in cilia length within each culture condition. The primary cilium was prescribed a radius of 100 nm (87) and a Young’s modulus of 178 kPa (88). It was modeled as a solid cylinder of radius 95 nm coated with a 5nm thick ciliary membrane. This ciliary membrane was also connected to the cell membrane that extended in a 5µm by 5 µm by 5nm thick square orthogonal to the cilium. The ciliary and cell membrane was prescribed a Young’s Modulus of 1 kPa (89). These two membranes were contiguous and modeled as a linear elastic material. A 5 µm³ cube of collagen gel was placed over the primary cilium and the gel modeled as a homogeneous, linear elastic material with a Young’s modulus of 17 kPa (90). The collagen gel was modeled as fully bonded to both the cellular membrane and the ciliary membrane. Each model was evaluated under two strain conditions, 10% strain perpendicular to the primary cilium and 10% strain parallel to the primary cilium (Figure 3.2.B, 3.2 C). To apply 10% uniaxial tensile strain in a parallel direction, a 1780 N/m² load was applied to the two faces of the collagen cube orthogonal to the x-direction in the x and –x directions. To apply 10% uniaxial tensile strain in the perpendicular direction, a 1780 N/m² load was applied to the two faces of the collagen cube orthogonal to the z-direction in the z and –z direction. 3D graphs were generated of the first principal tensile strain in the membrane in each modeled condition. Both the magnitude and location of maximum strain of the cell membrane was determined for each condition.
3.2.2 Elastoplastic Model

A second set of three models were then created using the Structural Mechanics Module to include an elastoplastic material for the cell and ciliary membrane. The models were identical to the linear elastic model described above except that the cell membrane was modeled as a Neo-Hookean elastoplastic material with Young’s Modulus of 1 kPa (89), shear modulus of 30 MPa and bulk modulus of 16.7 MPa (91).

![Figure 3.3: Boundary conditions and applied forces for the hyperelastic model.](image)

(A) Perpendicular strain is applied along the x-axis by holding one face of the cube and displacing the opposite face. (B) Parallel strain is applied along the x-axis by holding one face of the cube and displacing the opposite face.

In this method, the 10% tensile strain applied perpendicular to the primary cilium was applied by holding the left face of the cube orthogonal to the x-direction fixed while applying a 0.5 µm displacement to the other face orthogonal to the x-direction (Figure 3.3.A) 10%
strain parallel to the primary cilium was applied by holding the top face of the cube orthogonal to the z-direction fixed while applying a -0.5 µm displacement to the bottom face orthogonal to the z-direction (Figure 3.3.B). 3D graphs were generated of the first principal tensile strain in the membrane in each modeled condition. Both the magnitude and location of maximum principal tensile strain of the cell membrane were determined for each condition.

### 3.3 Results

To determine how tensile strain was transferred from the 3D collagen culture environment to the primary cilia and cells embedded within the collagen, principal tensile strains in the cell and ciliary membranes in response to an applied 10% strain of the collagen gel were calculated using FEA. Magnitudes and locations of highest principal tensile strain for both strain conditions (parallel or perpendicular to cilium) of each of three different primary cilium lengths were determined. All linear elastic models exhibited greatest magnitude of principal tensile strain in the ciliary membrane at the base of the cilium (Figure 3.4). All maximum principal tensile strains were within 50 nm of the base of the cilium. For the linear elastic ODM model with a cilium length of 2.72 µm, the maximum principal tensile strain was 380% for strain parallel to the cilium and 160% for strain perpendicular to the cilium (Figure 3.5). The linear elastic CGM model (3.05 µm) exhibited maximum principal tensile strains of 350% and 150% for parallel and perpendicular strain conditions, respectively. The linear elastic ADM model (3.90 µm) had maximum principal tensile strains of 340% and 170% for parallel and perpendicular strain conditions, respectively.
Interestingly, primary cillum length did not directly correlate to membrane strain, nor did there appear to be a trend of increasing strain with increasing ciliary length.

Figure 3.4: Representative images showing strain amplification at the cillum base in the linear elastic model. Principal tensile strain is shown in the cell and ciliary membrane for a primary cillum 3.05 mm in length. Cillum strained perpendicular to cillum orientation (A and B) exhibited less membrane strain than cillum strained parallel to cillum orientation (C and D). Both directions of applied tensile strain result in strain concentrations at the cillum base.
Figure 3.5: Maximum principal tensile strain in the ciliary membrane in the linear elastic model for cilium with 10% tensile strain applied to surrounding collagen gel either parallel or perpendicular to the cilium. Perpendicular strain induces maximum tensile strain in the range of 150–200% while parallel strain shows maximum strains between 300% and 400%. There is no apparent trend between cilium length and magnitude of maximum principal tensile strain.

Because these strains were too high to be accurately represented by a linear elastic model, three additional models were created incorporating a hyperelastic constitutive model for the cell membrane. These models also showed that the greatest principal tensile strain was exhibited at the base of the cilium (Figure 3.6). All maximum tensile strains were within 1 nm of the base of the cilium.
Figure 3.6: Representative images showing strain amplification at the cillum base in the hyperelastic model. Principal tensile strain is shown in the cell and ciliary membrane for a primary cillum 3.05 mm in length. Cilia strained perpendicular to cillum orientation (A and B) exhibited less membrane strain than cilia strained parallel to cillum orientation (C and D). Both directions of applied tensile strain result in strain concentrations at the cillum base.
Figure 3.7: Maximum principal tensile strain in the ciliary membrane in the hyperelastic model for cilium with 10% tensile strain applied to surrounding collagen gel either parallel or perpendicular to the cilium. Perpendicular strain induces maximum principal tensile strains within the ciliary membrane around 30% while parallel strain induces maximum principal tensile strains within the ciliary membrane between 50% and 70%. There is no apparent trend between cilium length and magnitude of maximum principal tensile strain.

As expected, the magnitude of principal tensile strains was much lower for the hyperelastic membrane model. For the ODM model, the maximum principal tensile strain was 68% for strain parallel to the cilium, and 30% for strain perpendicular to the primary cilium (Figure 3.7). The CGM model exhibited maximum principal tensile strains of 49% and 29% for parallel and perpendicular strain, respectively. For the ADM model, maximum principal tensile strains of 60% and 34% were shown for parallel and perpendicular strain,
respectively. However, consistent with the linear elastic model, results using the hyperelastic model indicated no correlation between membrane strain and cillum length.

3.4 Discussion

Results of our finite element analyses indicated that tensile strain amplification was concentrated around the base of the primary cilium. These findings are consistent with previous investigations of primary cilium response to fluid shear concluding that fluid shear-induced strains are primarily concentrated at the base of the cilium (84). However, unlike the findings from those previous fluid shear models, our results indicate that primary cilium length does not appear to affect the amount of strain transmitted to the ciliary membrane for a primary cilium exposed to tensile strain as opposed to fluid shear. Based on our experimental observations that primary cilia exhibit length changes in response to culture under 10% cyclic tensile strain (Table 1), we expected that changes in primary cilium length might play a role in the ability of the primary cilium to detect these tensile strains. However, findings from this study indicate that the orientation of the primary cilium with respect to the tensile strain appear to be more important in detecting tensile strains than the length of the primary cilium. In our calculations of tensile strain, strain parallel to cilium orientation resulted in a greater strain amplification in the ciliary membrane, leading us to conclude that the primary cilium will have greater sensitivity to tensile strain applied parallel to cilium orientation than to that applied perpendicular to cilium orientation. Interestingly, a study by Lavagnino et al. that imaged and quantified cilia on tendon explants demonstrated a correlation between cilium deflection angle and tensile strain (92). Taken together with our model’s predictions, cilia orientation is likely a key mechanism of modulating the mechanosensitivity of the
cilium structure. Further, their empirical observation in tendon, combined with our computational data suggests that orientation likely modulates the molecular mechanisms localized in the cilium. Although it does not disprove our theory that cilium length changes during differentiation to allow differences in mechanosensitivity between cell types, it does indicate that orientation, rather than length may be more directly involved in modulating cilium mechanosensitivity.

Given our findings of tensile strain amplification at the base of the cilium, future studies and computational models should evaluate refinement of the current model in the region around the cilium base. Primary cilia on many cell types have a feature at the base of the cilium known as the ciliary pocket. This pocket is a remnant of cellular membrane from ciliary growth, but is also the location for a number of endocytotic processes (93) and serves as an interface between the cilium and the actin cytoskeleton (94). The presence and structure of this ciliary pocket may be of importance in strain amplification in the primary cilium. Inclusion of cilia pocket architecture should be incorporated in future models.

Findings from this study lead to other exciting questions that could be addressed in future work. We have recently shown that primary cilia on hASC not only exhibit changes in length when hASC undergo differentiation, but also exhibit changes in conformation. Both primary cilium length and conformation alterations have also been observed on tenocytes in response to the mechanical environment (83, 92). Future computational studies should expand upon these empirical findings to include analyses of primary cilia with varying shapes to determine the role of primary cilium shape in detecting strains in the surrounding
environment. It is possible and indeed probable that primary cilia that exhibit significant changes in conformation might be better able to detect strains in multiple directions.

Such computational investigations could be performed concurrently with *in vitro* investigations of ciliary changes to validate mechanically-induced molecular mechanisms. Since we suspect that hASC might be adapting ciliary length to better sense their mechanical environments, but have now found that cilia length might not be as sensitive to changes in applied tensile strain as cilia orientation, it is possible that cilium orientation is being modified in response to applied tensile strain. Studies of tenocytes have shown that primary cilia typically align in the direction of migration ([83](#)). Measurements of primary cilia orientation with respect to the direction of applied strain could potentially be a method to determine if hASC are using the cilia to sense and respond to changes in direction of tensile strain.

In conclusion, this is the first study to computationally predict the principal tensile strains and localized strain profiles on primary cilia exposed to strain applied either perpendicular or parallel to the ciliary axoneme. The findings of this study suggest that the orientation of the cilium structure within a tensile strain environment has a large impact on strain distribution within the structure and thus may be a mode of modulating mechanically-induced cell processes. In the case of hASC, this may be involved in mechanically-enhanced differentiation and/or may have implications for lineage-specific mechanosensitivity. These new insights provide great advances to our understanding of the role of primary cilia in the detection of mechanical forces at the cellular level. Further, it indicates that primary cilia can play a key mechanosensory role in tissues that are not exposed to fluid shear.
CHAPTER 4

Primary Cilia as Electric Field Sensors: Osteogenic and Primary Cilia Related Gene Expression in Adipose-Derived Stem Cells Exposed to Alternating Current Electric Fields

In the previous chapter, we created and evaluated findings from a finite element model explaining how a primary cilium might mechanotransduce three-dimensional tensile strain. We hypothesize the primary cilium is also a transducer for electrical fields. In this chapter, we explore how electrical fields can influence osteogenic differentiation as well as primary cilia related genes.
4.1 Introduction

4.1.1 Electric Fields and Osteogenesis

When considering how to tissue engineer bone, it is important to consider many different properties of this biomaterial. Since bone is a material that has electric properties, these properties also should be considered when developing bone tissue. Bone is a piezoelectric material that produces electrical fields when it is deformed (95). Some investigators have hypothesized that these electrical fields are essential for bone growth and remodeling (96, 97). As early as the 1840’s direct current electrical stimulation was used clinically to help heal bone fractures (98, 99). Stimulation of bone by electrical fields has been shown to aid in the healing of bone fractures (100-102). As a result, many studies on the effects of electrical fields on osteogenesis have been performed (3, 9, 10, 103-106).

Most of the studies done of the effects of osteogenesis as a result of electric fields have been performed using pulsed direct current (DC) electric fields. Pulsed DC electric fields have been studied in several different cell types including osteoblasts (104), mesenchymal stem cells (MSC) (9, 103, 105, 106) and adipose-derived stem cells (ASC) (9, 10). Osteoblasts showed changes in both proliferation and ALP activity due to pulsed DC electric fields. Osteoblasts seeded on PLGA scaffolds and exposed to pulsed electric fields at a field strength of .13mT for 2 hours a day for 6 and 12 days exhibited increased proliferation but returned to levels of unstimulated controls by day 18 (104). Greater field strength or length of exposure per day resulted in decreased proliferation by day 18 relative to unstimulated controls (104). These same experiments showed increased ALP activity in osteoblasts on days 6, 12, and 18 (104).
Other studies have shown that pulsed DC electric fields have been shown to affect osteogenic differentiation in MSC (9, 103, 105, 106). MSC cultured in osteogenic medium and seeded at an initial cell density of 1,500 cells/cm² showed increased ALP and Runx2 mRNA expression at 7 days and decreased ALP and Runx2 mRNA expression at day 10 in response to DC electric fields (103). However, when the MSC had an initial seeding density of 3,000 cells/cm² the only difference in mRNA expression was an increase in ALP expression at day 7 due to pulsed electric fields (103). In addition to cell seeding density, frequency has also been shown to be important in experiments with MSC (106). In MSC exposed to pulsed DC electric fields of 1.1mT for 30 minutes a day and various frequencies, 50 Hz electric fields showed the highest levels of ALP activity at 3-15 days and showed highest osteocalcin concentration after 21 days (106). MSC have also shown changes in calcium accretion and proliferation due to pulsed electric fields (105). MSC exposed to 1Gauss pulsed electric fields at 15 Hz showed increased calcium accretion at days 9 and 14 and decreased proliferation at day 14 (105).

ASCs have been shown to be slightly less responsive to pulsed DC electric fields than MSC. (9). MSC and ASC exposed to 2mT fields at 75Hz for 21 days showed increases in mRNA expression of multiple bone related genes (9). While the responses were less in ASC than MSC, ASC still showed significant changes (9). Other studies of mouse ASC (mASC) have also shown this increase in osteogenic mRNA in ASC at day 21 as well as increases in ALP activity and mRNA expression at day 7 (10). These studies all show that exposing ASC and related cells to electric fields should promote osteogenesis.
Previous studies in our lab have tested the effects of alternating current (AC) electric fields on ASC (3). That study tested several magnitudes of AC electric fields at 1 Hz. A field strength of 1V/cm resulted in significantly increased cell-mediated calcium accretion after 14 days when the ASC were cultured in osteogenic differentiation medium (ODM) (3). Because these AC electric fields cause an increase in calcium deposition in ASC, we expect that they will also affect osteogenic genes.

4.1.2 Primary Cilia as Mechanosensors

Once it has been established that ASC can detect electric fields, there still remains the question of how these electric fields are detected. A previous study in ASC in pulsed electric fields showed that osteogenic gene upregulation due to electric fields was not attenuated by the rho-kinase protein inhibitor, Y27632 (10). This means that the detection of the electric fields is not dependent on the cytoskeletal stiffness of the cell.

One proposed mechanism for the detection of electric fields is the primary cilium. This organelle is a non-motile cilium and has been shown to play an important role in chemosensing and mechanosensing (107). Primary cilia have been shown to be mechanotransducers of both fluid shear forces and tensile strain (79, 80, 108, 109). Primary cilia have been shown to deflect under fluid shear stress which provides a mechanism for these extracellular forces to be converted into intracellular signals (107). Polycystin-1 is one of the proteins important in this mechanotransduction reaction (110). Polycystin-1 and Polycystin-2 form a complex at the base of primary cilia and when they are mechanically stimulated they cause an influx of calcium ions (110). Since primary cilia are charged structures, we expect that primary cilia deflect under AC electric fields in a similar way to
the way that they deflect under oscillatory shear stress. In this manner, primary cilia may play a role in ASC detection of electrical fields.

4.1.3 Primary Cilia in Osteogenesis

The primary cilia have been shown to play an important role in bone development. Primary cilia have been shown to be important in skeletal growth, development and maintenance (78, 79, 111). Primary cilia related proteins have also been shown to play an important role in osteogenesis. IFT proteins have been shown to be important in skeletal development and mechanotransduction (112).

Primary cilia genes have also been shown to play an important role in osteogenic differentiation. In osteoblasts/osteocytes, deletion of Pkd1 in mice resulted in a lower bone density (113). Knockdown of PC1 and IFT88 in ASCs show changes to osteogenic differentiation of ASC (15). Primary cilia have also been implicated in mechanically induced osteogenesis. Mechanical stimulation of polycystin-1 has been shown to up regulate osteogenic genes in primary human osteoblast-like cells (114).

Because of the link between primary cilia-related genes and osteogenesis in ASC, we expect that stimuli that effect osteogenesis will affect primary cilia-related genes. In our experiment, we expect to see an upregulation of the osteogenic genes Runx2 and Osteopontin and a change in the expression ciliary related genes PC1, PC2 and IFT88, especially PC1 and IFT88 in ASCs in response to AC electric fields.
4.2 Materials and Methods

4.2.1 Cell Culture

Cells were isolated from human adipose tissue derived from anonymous patients undergoing elective abdominoplasty surgeries (IRB exemption protocol #10-0201), at University of North Carolina hospitals (Chapel Hill, NC). The isolation protocols were derived from previously reported methods (115). The cells were then allowed to expand in complete growth medium (CGM) until they reached approximately 80% confluency. Cells were then trypsinized and frozen at passage 0. Superlots were made as previously described (116). In brief, five ASC lines were combined from five premenopausal female donors ranging in age from 24 to 36. The cells were combined at passage 3 and used at passage 4, one passage after superlot creation. The cells were cultured in CGM containing Eagle's Minimum Essential Medium, alpha-modified supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Later osteogenic differentiation was induced with osteogenic differentiation medium (ODM) composed of CGM and 50 µM ascorbic acid, 0.1 µM dexamethasone, and 10 mM β-glycerolphosphate.

4.2.2 IDE Fabrication

Interdigitated electrodes (IDEs) described here contain two contact pads, each connected to 25 “digits” which have a width of 100 µm in width, and a separation between digits of 100 µm. The IDEs were produced using UV-lithography and wet etching as follows. Glass slides of 1mm thickness (VWR International, Radnor, PA) were cleaned in a pirhana solution containing 3 parts deionized water, 3 parts 96% sulfuric acid (Sigma Aldrich, St. Louis, MO), and 1 part 34-37% hydrogen peroxide (Fisher Scientific, Pittsburg,
PA). After cleaning, glass slides were coated with 15 nm Cr and 60 nm Au, using an e-beam evaporator. The slides were then spin coated with HMDS (Microchem, Newton, MA) and AZ5214-IR photoresist (Microchem, Newton, MA) using a spin coater (Laurell Technologies Corp., North Wales, PA), followed by a bake at 90°C for 60 sec on a hot plate. The slides were exposed to UV light for 15 seconds (Optical Associates, Inc., San Jose, CA) using a chrome mask with the desired IDE pattern, and hard baked at 115°C for 45 sec. The slides were then flood exposed to UV light for 40 sec. without the mask, and developed in MIF CD-26 (Microchem, Newton, MA). The excess metals were removed using standard chrome and gold etchants (Sigma Aldrich, St. Louis, MO), and the remaining photoresist was removed in acetone. The electrodes were connected in parallel using conductive silver epoxy (Ted Pella, Redding, CA) and 250 µm diameter silver wire (Goodfellow, Coraopolis, PA) insulated with 10 µm Parylene-C (Specialty Coating Systems, Indianapolis, IN). The silver wires were attached to the contact pads on the IDE with silver epoxy and soldered to gold pin connectors (Newark Electronics, Richfield, OH). Matching gold pin connectors were soldered to BNC connectors inside the tissue culture flask to link the electrodes to the external electrical stimulation.

4.2.3 Electrical Stimulation

Human ASC superlots were plated on IDEs at 20,000 cells/cm² in 100ul of CGM. The cells were allowed to adhere for two hours before the dish with IDEs was flooded. The IDEs were then allowed to sit overnight for 12 hours in CGM. Medium was then changed, to fresh CGM or ODM. Human ASC were exposed to a 1 V/cm² AC electric field for 4 hours/day for 3, 7, 10 or 14 days.
4.2.4 RNA Analysis

Human ASCs were harvested from each individual IDE directly after completion of electric stimulation. Each IDE was covered in 350µl of RNA lysis buffer (Qiagen buffer RLT) and scraped using a cell scraper, before the buffer was pipetted into a microfuge tube. Samples were stored at -20°C until they were ready for RNA isolation. Messenger RNA isolation and quantitative PCR was performed on these samples as previously described (2, 15). In brief, each lysate sample was run through a Qiashredder column (Qiagen, Valencia, CA) at 15,000 rcf for 2 minutes to homogenize samples. Total RNA was isolated using the RNeasy Micro Kit (Qiagen). This was then reverse transcribed into cDNA using the Superscript III First-Strand Synthesis System (Invitrogen) and the Mastercycler ®Nexus (Eppendorf) thermocycler. Real time PCR was performed using Taqman® Gene Expression Assays (Invitrogen) and ABI Prism 7000 Sequence Detection System (Applied Biosciences). Samples were analyzed for RUNX2 (Invitrogen, Hs00231692_m1), SPP1 (Osteopontin) (Invitrogen, Hs00959010_m1), IFT88 (Polaris) (Invitrogen, Hs00197926_m1), PKD1 (Polycystin 1) (Invitrogen, Hs00947377_m1), and PKD2 (Polycystin 2) (Invitrogen, Hs00165517_m1) and were normalized to GAPDH (Invitrogen, Hs02758991_g1).

4.2.5 End Product Analysis

End product analysis was performed on cells at days 7, 10 and 14. Electrodes were washed twice in PBS, and then each electrode was covered in 500µl of 0.5N HCl and scraped using a cell scraper. The cell suspension was pipetted into a microfuge tube. The electrode was then rinsed with an additional 500µl of 0.5N HCl and combined with the first 500µl in the tube. Samples were stored at -20°C until analysis. Samples were rocked on an orbital
shaker for 12 hours at 4°C. They were then centrifuged for 2 minutes at 15,000 rcf and the solution was transferred to a new tube. This solution was then analyzed for calcium concentration using the Calcium Liquicolor Assay (Stanbio) and for protein concentration using Micro BCA Assay (Pierce) Calcium production on IDEs was reported as calcium/protein.

4.2.6 Live/Dead Stain

At day 14 ASC undergoing electrical stimulation were stained to determine viability. Using the Live/Dead Assay Viability Cytotoxicity for Mammalian Cells (Invitrogen) cells were imaged after 30 minutes of incubation in the Live/Dead stain.

4.3 Results

4.3.1 PCR

RUNX2 and SPP1 mRNA expression was upregulated at day 3, significantly for SPP1, in hASC cultured in CGM and exposed to electrical stimulation relative to unstimulated controls (Figure 4.1). RUNX2 showed a significant down regulation due to electrical stimulation at day 7 in both CGM and ODM. Consistent with RUNX2 expression, primary cilia gene IFT88 was upregulated in response to electrical stimulation at day 3 in CGM and significantly downregulated at day 7 (Figure 4.2) in hASC cultured in CGM and ODM. PKD1 showed an upregulation in response to electrical stimulation at day 3 in CGM and a downregulation in response to electrical stimulation at day 7 in both CGM and ODM. PKD2 did not show significant changes or patterns in mRNA expression due to electrical stimulation.
Figure 4.1: RT PCR results for osteogenic genes RUNX2 and SPP1 (Osteopontin) at days 3 and 7 on electrodes with or without 1Hz AC electric fields of 1 V/cm in complete growth medium (CGM) or osteogenic differentiation medium (ODM). A) Day 3 B) Day 3. On day 3 both RUNX2 is upregulated and SPP1 is significantly upregulated by electric stimulation in CGM. Day 7 RUNX2 is significantly downregulated by electric stimulation in both CGM and ODM.

Figure 4.2: RT PCR results for the primary cilia related genes IFT88, PKD2 and PKD1 at days 3 and 7 on electrodes with or without 1Hz AC electric fields of 1 V/cm in complete growth medium(CGM) or osteogenic differentiation medium (ODM). A) Day 3 B) Day 7. On day 3 IFT88 and PC1 both show upregulation due to electrical stimulation in CGM and IFT88 also shows upregulation in ODM. On day 7 IFT88 and PKD1 show significant downregulation due to electric stimulation in both CGM and ODM with the IFT88 showing significant downregulation.
Calcium Accretion

Electrical stimulation caused an increase in calcium that increased at each time point for cells cultured in ODM (Figure 4.3). ASC cultured in CGM did not show large amounts of calcium accretion, nor did they show a consistent response to electrical stimulation.

![Calcium ODM](image)

**Figure 4.3:** Calcium for ASC cultured in ODM either with or without 1Hz AC electric fields of 1 V/cm. Electrical stimulation shows significant upregulation by day 10.

Total protein was also measured for all culture conditions. Both CGM and ODM showed increased protein from electric stimulation on day 7 and 10 and decreased protein from electrical stimulation at day 14 (Figure 4). Normalizing calcium for protein at day 14
shows significantly increased calcium per protein due to electrical stimulation at day 14 in ODM, but not in CGM, as expected (Figure 4.5).

Figure 4.4: Protein in ASC cultured in A) CGM and B) ODM either with or without 1Hz AC electric fields of 1 V/cm. Day 7 and 10 show increased protein due to electrical stimulation, while day 14 shows decreased protein due to electrical stimulation.

Figure 4.5: Calcium per protein day 14 in ASC cultured in A) CGM and B) ODM either with or without 1Hz AC electric fields of 1 V/cm. ODM showed significant increase in calcium per protein.
4.3.3 Live/Dead

Because of the decreased protein results, at day 14 due to electrical stimulation, we assessed cell viability at day 14 to ensure that electrical stimulation was not causing cell death. After 14 days of electrical stimulation, a live dead assay was performed (Figure 4.6) which showed that the majority of cells were viable under stimulation.

Figure 4.6: ASC after 14 days of 4 hours of 1V/cm 1Hz AC electric fields in A) CGM and B) ODM. CGM shows minimal cell death, while ODM shows a small amount of cell death with many cells still living. Cells in CGM show an aligned morphology perpendicular to the electrode digits in response to electric stimulation. Cells in ODM show a more clustered morphology.

There was slightly increased cell death in ODM compared to CGM under stimulation. Cells in CGM also showed a more aligned morphology while cells in ODM showed a more clustered morphology.

4.4 Conclusion

From these experiments we have shown that 1V/cm AC electrical fields at 1 Hz affect both osteogenic and primary cilia related genes. The osteogenic genes were shown to be up regulated more significantly in CGM. For RUNX2 we saw an upregulation in gene activity
for this early marker of bone at day 3 that was significant in CGM, and then a decrease at day 7 that was significant in both CGM and ODM. SSP1 (Osteopontin) showed an upregulation in CGM at day 3. Tsai et al. (2009) show a similar pattern of gene upregulation then down regulation in hMSC for both ALP and Runx2 in response to pulsed electromagnetic fields (103). However, these patterns occurred at a different time period, with the upregulation happening at day 7 and the downregulation at day 10 (103). This change in timing could be due to many different factors including different cell type, different cell seeding density, or different type of electric field. These upregulations of osteogenic genes are also consistent with increased calcium production in the presence of electrical stimulation. Previous studies in our lab have shown that these electric fields cause increased calcium production in ASCs grown in ODM (3). MSC have also shown similar calcium accretion results. Jansen et al. showed increased calcium at day 9 and 14 in MSC in response to pulsed electromagnetic fields (105). Similar to this study, MSC showed decreased proliferation at day 14 from these electromagnetic fields, though this was measured using DNA instead of protein (105). In our study, this decrease in proliferation due to electric fields does not appear to result from cell death caused by the electric fields.

This is the first study to show that electric fields affect the expression of the primary cilia proteins IFT88 and PC1. Both of these genes also exhibited upregulation at day 3 and downregulation at day 7 consistent with Runx2 expression, although only IFT88 showed these changes significantly. Previous studies in our lab have shown that IFT88 and PC1 are important in the ability of ASC to osteogenically differentiate (15) and to detect tensile strain. This study also showed that PC2 did not significantly affect osteogenic
differentiation\((15)\), which seems to correlate with the fact that PC2 mRNA did not show any consistent changes in expression in response to electric fields. Changes in IFT88 and PC1 due to electrical stimulation seem to imply that the primary cilia may also play a role in the detection of electrical fields. In order to determine if primary cilia play a role in detecting 1V/cm AC electric fields at 1Hz and directing the osteogenic differentiation process, our next step would be to knock down the expression of IFT88 and PC1 in ASC to determine if cells lacking these proteins still show an osteogenic response to electrical fields. These knockdowns could be done transiently, as previously done in our lab\((15)\), or with a stable knockdown depending on the time points to be measured. Another possible way to confirm cilia involvement in the detection of electric fields would be to obtain an image of the cilium deflecting in response to electric stimulation. Similar images have been obtained of primary cilium deformation in response to fluid shear \((82)\).

Another notable finding from these experiments is the conformational changes of the ASC exposed to electric fields. Those in CGM showed alignment perpendicular to the electric fields. Those cultured in ODM exhibited a clumped morphology. These findings are consistent with Hammrick et al., which showed that in cell growth medium mASC aligned perpendicular to pulsed electric fields for six hours a day and this alignment could be observed as early as 1 day\((10)\). Further studies should be done to assess cell alignment in ASC exposed to electric field stimulation. These studies might also allow further insight into the role of primary cilia in the detection of electric fields. Primary cilia have been shown to align parallel to the orientation of tenocytes \((117)\). It is possible that other cell types may show a similar association between cell and primary cilia alignment.
Overall, this study leads to a better understanding of how electric fields influence osteogenesis. It also sheds light on a possible mechanism for electric field detection, the primary cilium.
CHAPTER 5

Conclusions

5.1 Summary of Findings

Results from the finite element model of a primary cilium undergoing 10% tensile strain in 3D culture show that the deformation of the ciliary membrane is concentrated around the base of the primary cilium, the location of many of the stretch based calcium channels. This study also showed that magnitude of strain is not correlated to cilium length, but to cilium orientation. Strain parallel to cilium orientation resulted in a greater strain amplification in the ciliary membrane, leading us to conclude that the primary cilium will have greater sensitivity to tensile strain applied parallel to cilium orientation than to that applied perpendicular to cilium orientation.

Experiments with interdigitated electrodes showed changes in both osteogenic and primary cilia related genes due to stimulations with AC electric fields of 1 V/cm at 1Hz for 4 hours a day. In CGM at day 3, Runx2 and Spp1 (Osteopontin) show upregulation at day 3 due to electric stimulation. Runx2 shows downregulation at day 7 in both CGM and ODM. Ift88 also shows downregulation in both CGM and ODM at day 7. Electrical stimulation was also shown to increase calcium production in hASC cultured in ODM after 10 and 14 days of stimulation.

Additionally, electrical stimulation on interdigitated electrodes showed changes in hASC morphology. Human ASC cultured for 14 days under stimulated conditions in CGM showed alignment perpendicular to the direction of the electric fields. Stimulated hASC grown in ODM showed a more clustered morphology.
5.2 Recommendations for Future Research

This research on the role of primary cilia in ASC differentiation poses several interesting questions for further research. Understanding of the role that the primary cilium plays in detecting the osteogenic stimuli of cyclic tensile strain and electric fields will allow us to better understand how to induce osteogenic differentiation.

Work on modeling the primary cilia in 3D tensile strain has shown that the orientation of the primary cilia to the direction of strain might be more important than cilia length in the ability to detect tensile strain. An analysis of cilia orientation during cyclic tensile strain would allow us to determine how cilia adapt to better sense tensile strain. Additionally, the primary cilium model can be further refined. Since the strain is focused primarily around the base of the cilium, it would be advantageous to make a model that better specifies the true geometry around the base of the cilium. Therefore, a model that includes the ciliary pocket would be helpful to understand the strains incurred on the ciliary membrane when a cell is exposed to strain in 3D.

Exploring the genetic reactions of hASC exposed to electric fields shows that primary cilia related genes are affected under electrical stimulation. To see what effects these genetic changes have on primary cilia, further studies need to be conducted measuring changes in primary cilia length, shape, and number in response to electric fields. In addition, in order to determine if the primary cilium is necessary for detecting electric fields, osteogenic differentiation in hASC needs to be tested while knocking down the primary cilia genes IFT88 and PKD1. Additionally, imaging a primary cilia moving in response to electric fields would show that mechanotransduction happens due to primary cilia bending similar to the
way that they detect fluid flow. The morphological changes observed in hASC due to
electrical stimulation, also generate questions for further study. Being able to use electric
fields to induce alignment in ASC has many potential applications in tissue engineering,
especially to engineer tissues that show cell alignment.

Overall, this body of research has generated many new questions and avenues of
research that should be pursued. A greater understanding of the role of the primary cilia in
ASC differentiation will allow for better bone tissue engineering. This body of work paves
the way for new techniques in tissue engineering which take advantage of the primary cilia as
a mechanism for inducing bone formation. These techniques will ultimately allow for better
treatment of bone injuries and malformations which allow for a patient to use their own cells
tissues to replace damaged bone.
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