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CYCLIC LOADING OF FIBRONECTIN COATED BEADS INDUCES DIFFERENTIATION
IN MOUSE EMBRYONIC STEM CELLS

BY

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THESIS

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ABSTRACT

The role of soluble factors in determining embryonic stem cell fate has been well studied. A growing body of research indicates mechanical cues are equally important to chemical signals in eliciting cellular response, especially during the early developmental stages of embryos. Here, we seek to control substrate stiffness and exposure to mechanical cues in order to gain a better understanding of how mechanotransduction directs mouse embryonic stem cell (mESC) fate. A magnetic twisting cytometer (MTC) was used to apply oscillating force to mES cells through fibronectin (FN) coated magnetic microbeads. The ligand coated magnetic beads were allowed to bind to undifferentiated mES cells plated on 0.6 kPa polyacrylamide gels prior to loading. The cells used were stably transfected with green fluorescent protein (GFP) bound to oct3/4, an important indicator of differentiation. These cells were imaged during and after stress application to record the intensity of GFP expression. Previous research has shown force application through the synthetic ligand RGD results in cell spreading and downregulation of oct3/4. The use of fibronectin, a natural ligand, more closely replicates conditions in vivo and transmits force through different receptors than RGD. As expected, cells stressed with fibronectin-coated beads differentiated on a similar timescale to RGD experiments. Unexpectedly, cells did not spread after force application.

In order to gain a better understanding of GFP downregulation dynamics, mES cells were photobleached and treated with increasing concentrations of retinoic acid (RA), a chemical known to direct pluripotent cells towards a partially differentiated neural fate. The goal was to bleach GFP expression down to a baseline level and then compare the relative magnitude of chemical and mechanical effects on the production or downregulation of oct3/4. Low to medium

concentrations of RA kept oct3/4 expression at or below baseline as expected, with higher concentrations becoming cytotoxic. This RA result could not be compared to an equivalent mechanical test, as the combination of bleaching and stress application was too stressful for cells. However, these data reinforce the idea that chemical and mechanical cues are of similar importance in determining the fate of mESC and that more ligands should be explored for force application.

To my mother and father

Beverly Borduin

Charles Borduin

And to my sister

Lauren Borduin

For their constant love, support, advice and encouragement

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CHAPTER 1

INTRODUCTION

1.1 Mouse Embryonic Stem Cells

Embryonic stem (ES) cells are a promising area of study due to their many potential applications in regenerative medicine and experimental biology [1]. ES cells can rapidly divide an unlimited number of times, allowing for large batches of cells to be grown in vitro for experimentation or medical use. ES cells are also pluripotent, meaning they can differentiate into the mesoderm, endoderm and ectoderm germ layers, eventually forming any type of adult tissue [2]. This ability is extremely desirable as the human body can only grow adult stem cells in small numbers, diminishing its ability to self repair. Although many successful medical treatments utilize adult stem cells, they are limited because adult stem cells have finite division capability in vitro and can only differentiate into a few of cell types. If ES cell differentiation can be controlled, it could lead to cures for many diseases and injuries which are currently untreatable. Eventually, researchers hope to develop a step by step protocol for differentiating pluripotent ES cells into specific cell types with high efficiency and reliability [3]. Efficiency is of great importance, as it saves on research costs in the short term and makes large scale production and treatment viable in the long term.

There are many obstacles to overcome before the potential of ES cells can be fully realized. Ethical concerns, time consuming cell culture and expensive culturing supplies make experimentation with human embryonic stem cells (hESC) challenging. The use of mES cells for research alleviates some of these problems. Mouse and human ES cells share similarities, but

mES cells are easier to culture and allow for experiments that would be unethical to perform on humans, such as implantation to form teratomas or the creation of chimeras [4]. Another major hurdle preventing ES cell use in medical applications is the possibility of cell contamination. It is difficult and expensive to thoroughly test each cell line for viruses or harmful mutations and to ensure it remains uncontaminated once it has been tested. Furthermore, contaminating factors cannot disappear for certain until cells can be cultured with a fully synthetic media free from animal products that can transmit viruses, bacteria, or contain unknown factors that differentiate cells [3, 5].

A better understanding of the chemical and mechanical factors that regulate cell behavior is required before the differentiation process can be controlled. One key area of study is cellular interaction with the extracellular matrix (ECM). The ECM is important for mES cell attachment; mES cells, like other tissue cells, are anchorage dependent and will die when left in suspension [6]. Furthermore, the ECM is known to play a role in cell differentiation, spreading, attachment, proliferation, and gene expression [2, 5-9]. For example, cells plated on fibronectin or laminin will spread and differentiate, while cells cultured on collagen-I or collagen-IV remain pluripotent [5]. Cells plated on collagen do not differentiate when left alone because mES cells do not have receptors that bind to collagen I and IV. However, Hayashi has shown that forcing overexpression of “collagen binding integrin subunits” causes cells to differentiate when plated on collagen [5]. Thus, integrins are vital to the interaction between cells and the ECM. Interestingly, ES cells still attach and thrive on gels coated with collagen, despite the lack of a collagen receptor. One possible explanation is that cells plated on collagen bind electrostatically [5].

1.2 Mechanotransduction

Mechanotransduction is the process by which cells convert mechanical cues into a biochemical response [8, 10]. Cellular responses to these cues vary with the length, frequency and method of loading as well as the magnitude of prestress in the cytoskeleton prior to loading [11, 12]. Although mechanotransduction is not fully understood, Ingber suggests it relies on hierarchical systems that arrange cellular components to transmit forces to specific sensory molecules [10]. Some models propose mechanotransduction relies on diffusion triggered by local protein unfolding, but diffusion takes too long for this hypothesis to be the case [13]. Na et al. demonstrated force application in HASM cells activated Src in under .3 seconds, which translates to a force propagation speed of ~ 30 m/s. This is far quicker than activation by soluble factors, which took more than 12 seconds [14]. Other cell models treat the cell as a homogenous object. Through application of Saint-Venant's principle, a locally applied force on a solid, homogenous body would result in a local deformation and response. However, Na et al. have also shown force application can cause Src activation in distant regions of a cell [14]. This behavior can be more accurately predicted by prestressed string and stiff fiber models that account for long distance force transmission. These models embrace the idea that cells contain rigid elements capable of spanning distances on the order of a cell diameter [8, 15].

Further insight into ES cell mechanotransduction can be gained by examining similar processes within the human body. For example, the auditory and balance systems both convert mechanical input into a biochemical response [8, 10]. These large systems are composed of a hierarchy of smaller systems organized to transmit force input. Cells arrange integrins, adhesion complexes and the cytoskeleton to transmit forces to specific integrins, much like the auditory

system is structured to stimulate small signal transducing hairs called cilia [6]. The mechanotransduction process begins with forces generated in the cytoskeleton or carried through the ECM [11]. These forces are sensed by clumps of integrins known as focal adhesions, which form on the surface of the cell and connect to ligands present in the ECM to act as mechanoreceptors [10, 12]. Cells then respond to mechanical stimulation by generating increased tractional forces and restructuring load bearing actin bundles, intermediate filaments, and microtubules [9, 10, 12]. This behavior allows the cells to resist pulling forces, but can only occur in the presence of cytoskeletal prestress and integrins [8, 11].

1.3 Synthetic and Natural Ligands

Integrins allow cellular mechanotransduction to occur by linking the cytoskeleton to the ECM. Therefore, exploring the role of specific integrins in eliciting cellular response is essential to better understanding mechanotransduction [8]. This can be accomplished by using magnetic twisting cytometry to mechanically probe specific integrins, a technique which is described in detail later. In short, magnetic beads are coated with a ligand that binds to cells through specific integrins. An oscillating magnetic field is applied, generating a magnetic moment in the beads and stressing cells through the ligand-integrin connection [16, 29]. One particular molecule of interest for bead coating is Arginine-Glycine-Aspartic acid (RGD), an artificial ligand. Chowdhury et al. found that application of stress through RGD coated magnetic beads caused a decrease in oct3/4 expression and increased cell spreading [16]. This is a significant finding because a change in oct3/4 expression is an indication of differentiation in both human and mouse ES cells [1, 2]. The RGD results prompted us to test the natural ligand fibronectin.

Although it shares a binding site with RGD, fibronectin is a much longer molecule with some interesting mechanical properties not shared by RGD.

Fibronectin undergoes a series of conformational changes when placed in tension. These changes are specific to mechanical loading, and cannot be induced by thermal or chemical methods [17]. As increasing tension is applied to fibronectin, its FNIII domains unfold in series causing the molecule to increase in length. Importantly, cells can generate adequate tension to cause these conformational changes and removing tension causes fibronectin to quickly return to its original state [17]. The ability of fibronectin to fold and unfold plays a role in ECM assembly and elasticity [17].

As a result of these tension-dependent states, Friedland hypothesizes that two types of $\alpha V\beta 1$ integrin connections exist, one formed while the molecule is in tension and one formed while the molecule is relaxed [18]. Similarly, Danen has shown that fibronectin unfolding due to tension exposes previously hidden binding sites [19]. Integrin connections are known to regulate soluble factors, for example, fibronectin's presence in the ECM plays an important role in triggering the G1 stage of the cell cycle [19]. Thus, applying an oscillating force through a fibronectin molecule could cause fibronectin to change conformation as it is tensed and relaxed, exposing the cell to different binding sites. While relaxed, the fibronectin molecule binds at the RGD site, which has been shown to differentiate mES cells [16]. When in tension, fibronectin exposes the R1374 and R1379 synergy sites for binding [18]. Friedland has demonstrated that the tension dependent fibronectin configuration can also be controlled by substrate stiffness. A higher stiffness resulted in a higher number of bonds in tension without changing the total number of bonds [18]. The use of magnetic twisting cytometry allows for rapid switching between

tensioned and untensioned fibronectin conformations independent of substrate stiffness by controlling the rate and magnitude of loading.

1.4 Polyacrylamide Gels

In addition to sensing mechanical forces, cells can also sense the mechanical properties of their environment [7]. The effects of substrate stiffness on cell morphology and differentiation have been well documented [6, 9]. For example, naïve mesenchymal stem cells (MSC) differentiate into neurogenic cell types on soft substrates and osteogenic cell types on more rigid substrates [20]. Inhibiting myosin II blocked differentiation, which supports the idea that cells sense substrate stiffness based on resistance to tractional forces generated at focal adhesions [7, 20]. Friedland found that myosin II generates tension in the cytoskeleton in response to substrate stiffness, which can trigger the $\alpha V\beta 1$ integrin switch to change conformation. For this reason, it is important to control substrate stiffness when conducting experiments on mES cells.

Polyacrylamide gels are an ideal substrate for culturing ES cells. ES cells are generally cultured on glass or plastic, but these rigid substrates cause cells to spread by strongly resisting traction forces generated at focal adhesions [9, 21]. Soft polyacrylamide gels provide an environment much closer to the natural surroundings of a cell while retaining the desirable optical clarity of glass and plastic substrates [22]. Gel stiffness can be adjusted to meet culturing needs by changing acrylamide concentration, and the gels deform linearly in response to force application over a wide range of stiffnesses [9, 22]. Unlike smooth glass or plastic, gels are porous which allows ES media to envelop more of the cell's surface area [21, 22]. Another benefit of polyacrylamide gels is that cells cannot bind to them directly so chemical and

mechanical cues can be better isolated during experiments [9, 22]. Receptors and proteins in serum cannot attach to polyacrylamide gels either, which further reduces unknown factors that could potentially differentiate cells [9].

1.5 Retinoic Acid

Chemicals and cell-conditioned medium are soluble factors often used in differentiation assays [20]. Retinoic acid is a soluble factor known to terminate proliferation and induce differentiation in mES cells [3]. Specifically, RA is an oxidized form of vitamin A that binds to nuclear retinoic acid receptors and retinoid X receptors [23]. RA is effective at inducing neuronal type differentiation in pluripotent mES cells [24-26]. In fact, almost 90% of cells in a population can be converted to neural cells through RA treatment [26]. RA has also been used to differentiate ES cells into pancreatic endoderm cells when applied on day 4 of culturing [27]. However, in both the neural and pancreatic cases the cells were only partially differentiated. Adding retinoic acid as a soluble factor in medium is a potential first step in a multistep protocol for creating large batches of neural or pancreatic cells for biological study or medicinal purposes [26]. Cancer cells share similarities with ES cells so it is of little surprise that RA has also been explored as a method to limit the spread of cancerous cells [23]. Interestingly, RA has the opposite effect in pig preadipocytes, where it has been shown to prevent differentiation [28].

1.6 Objective

Application of oscillating forces through beads coated with the artificial ligand RGD has been shown to cause differentiation and spreading in mES cells [16]. This study seeks to expand upon those findings by applying force through fibronectin, a natural ligand. The goal is to see if stress application through fibronectin differentiates cells in the same way as RGD. Very similar experimental results would suggest that the dissimilar properties in fibronectin and RGD are less important for differentiation than their shared RGD binding site. Some of these properties include molecule length, ability to fold and unfold when stretched and the exposure of extra binding sites not found in RGD. If force application through fibronectin produces significantly different results than RGD it may reveal new insights about mechanotransduction in mES cells and warrant testing of more natural ligands. The secondary objective of this paper is to determine how different concentrations of RA affect oct3/4 expression in mES cells, and to better understand the rate at which oct3/4 is expressed or downregulated in mES cells after bleaching and RA application.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Culture

Undifferentiated mES cells (W4, 129/SvEv) were thawed and cultured in 6 well TC-treated polystyrene plates (Corning) coated with .1% gelatin in phosphate buffered saline (PBS). The cells were incubated at 37° C, 5% CO₂ in humid conditions. Medium consisted of these supplies purchased from Invitrogen: Dulbecco's Modified Eagle's Medium (DMEM), Glutamax I, penicillin-streptomycin, Modified Eagle's Medium non-essential amino acid, sodium pyruvate, 15% fetal bovine serum (FBS), 2-mercaptoethanol and leukemia inhibitory factor (LIF). Cells reached 70-80% confluency every two days and were passed at a ratio of 1:4. During passaging, cells were gently washed with PBS (Sigma-Aldrich) and then detached with TrypLE (Invitrogen) for one minute. Medium was then added to neutralize the TrypLE. Suspended cells were fine tip transfer pipetted to break up clumps before being plated into a new well. Prior to experiments, ~10,000 cells were plated onto 0.6 kPa gels (~14 mm diameter) coated with 200 µg/ml collagen-I. The cells were allowed to sit 12 hours before stress application or addition of retinoic acid to ensure they were well attached to the substrate.

2.2 Bead Coating

Ferromagnetic microbeads (4.5 µm diameter) were suspended in 95% ethanol and aliquoted into 0.2 mg clumps. The ethanol was allowed to evaporate and the remaining dry beads were then suspended in 200 µl of carbonate buffer. The bead-carbonate buffer solution was sonicated for 4 seconds to eliminate clumping and ensure even coating of the bead surface. Next,

5 μ l of 1mg/ml fibronectin from bovine plasma (Sigma-Aldrich) was added to the solution, bringing the ligand coating concentration to 25 μ g/ml. A concentration of 50 μ g/ml was used for bead coating in RGD stress application protocols [29]. However, coating beads with 50 μ g/ml of fibronectin resulted in large, adhesive clumps of beads. The clumps could not be broken apart with pipetting or sonication and had a tendency to stick to pipettes, resulting in a high percentage of the beads being lost. Lowering the coating concentration to 25 μ g/ml greatly reduced bead clumping and still allowed for strong cell-bead attachment. Once fibronectin was added to the bead-carbonate buffer solution, the mixture was turned at slow speed in a tube rotator for 12 hours so fibronectin could fully coat each bead. The beads were then spun down in a centrifuge and the supernatant was carefully pipetted away, leaving only the fibronectin coated beads. Finally, 200 μ l of DMEM was added to the beads which were then sonicated for 1 second immediately before use in stress application experiments.

2.3 Polyacrylamide Gel Making

Polyacrylamide gels were made based on earlier protocols developed by Pelham and Wang [7, 22]. Uncoated 35 mm gridded dishes (MatTek) were obtained for gel making. First, the glass surface was treated with .1M NaOH and left to dry overnight. Next, the crystals formed by NaOH treatment were dissolved with 3-aminopropyltrimethoxysilane and washed with distilled H₂O. After drying, dishes were treated with .5% glutaraldehyde in PBS for 30 minutes and washed again with distilled H₂O. BIS and acrylamide were mixed with distilled H₂O to 3% BIS by volume and 0.06% acrylamide by volume, which produced gels with a stiffness of 0.6 kPa. Yeung et al. have generated a plot of the acrylamide to BIS ratios needed to obtain a variety of gel stiffnesses [21]. TEMED and 10% APS were then added at 1/2000 and 1/200 volume,

respectively, to start polymerization. The solution was well mixed and 15 μ l drops were quickly pipetted onto each dish to form 75 μ m thick gels. The gels were covered with glass coverslips, flipped upside down and incubated at 37° C for 30 minutes. Upon removal from incubation, the gels were flooded with 100 mM HEPES and the glass coverslips were gently pried off with a razor blade. Next, the gels were coated with 1 mM sulfo-SANPAH photoreactive crosslinker (Thermo Scientific) dissolved in 100 mM HEPES and exposed to UV light for 6 minutes to crosslink the gel surface. The SANPAH coating process was repeated, then gels were coated with collagen-I and incubated for 48 hours at 20° C then sterilized and plated with cells.

2.4 Magnetic Twisting Cytometry

Magnetic twisting cytometry is a technique that uses oscillating magnetic fields to apply stress to cells through ligand coated ferromagnetic microbeads. The MTC apparatus, which can be seen in Figs. 1 and 2, allows for observation and image capture of cells during loading. For this experiment, 50 μ l of 1 mg/ml fibronectin coated beads in DMEM were added to a dish of mES cells. The dish was incubated for 15 minutes at 37° C to allow beads to form strong connections with cells. Medium was added to the dish and warm air was fed through the MTC to keep cells at 37° C during stress application. A diagram of the experimental setup can be seen in Fig. 3.

Cells with a single well-attached magnetic bead were located at 20X magnification using a phase contrast 0 (Ph0) filter. Although images were recorded at 40X magnification in brightfield, Ph0 at 20X magnification allowed for quicker scanning of the dish and reduced eye strain. The entire gridded area covered by the gel was searched and cells with attached beads

were closely examined. As many cells as possible were located per dish, since only a few usually end up being suitable for imaging. For each potential bead-cell pair found, the microscope stage was lightly tapped to ensure the bead was well attached to the cell. Next, each potential cell was checked to ensure high levels of GFP expression by briefly exposing it to fluorescent light through the 475 nm filter. Carefully screening cells before choosing them for imaging is important, because stress application experiments are time consuming and can only be conducted once per dish.

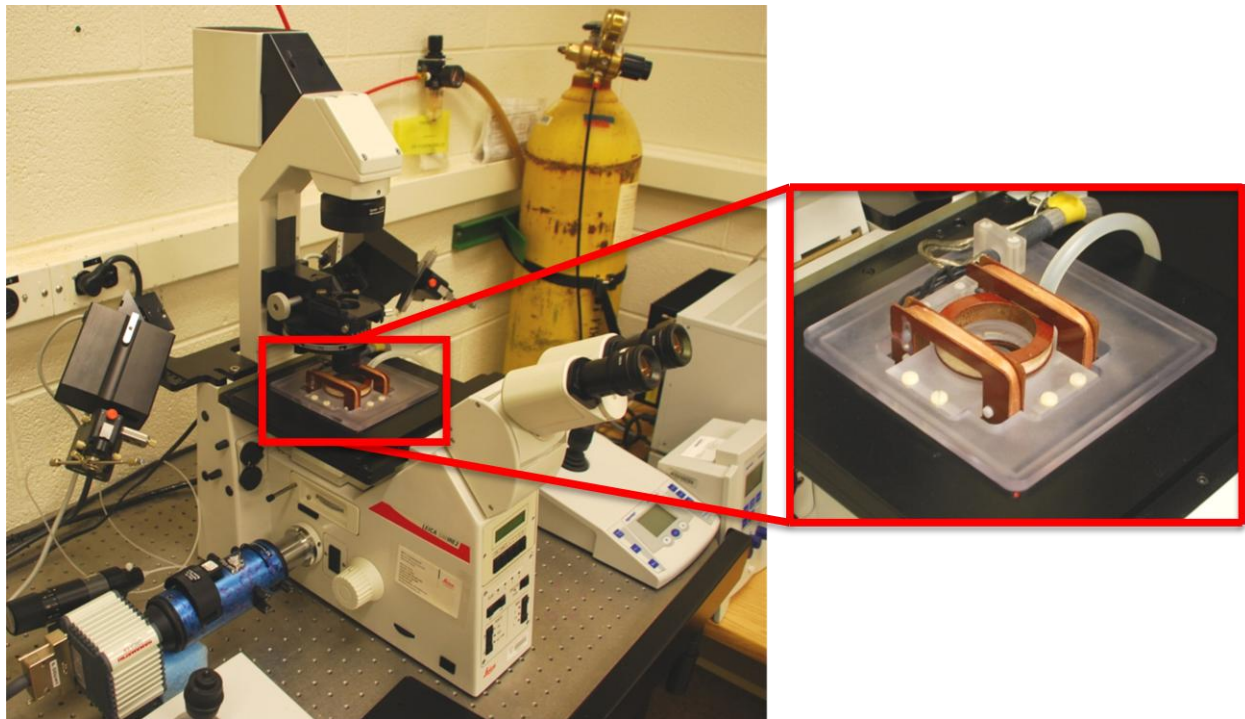


Figure 1. *Leica inverted microscope fitted with magnetic twisting cytometer. On the right is an enlarged view of the MTC seated in the microscope stage. Current is applied through the wire coils, generating an oscillating magnetic field across the opening in the middle, where the 35 mm dish is placed. Hot air flows into the MTC through the clear plastic tube on the right to keep the cells at 37° C during loading.*



Figure 2. Image of the MTC control unit. This unit is linked to a computer where parameters such as magnetic field strength, oscillation frequency and waveform (sinusoidal or step function) are set. Once the parameters are set, the MTC control unit sends current to the MTC. Bead magnetization is done by pressing the charge button and then pressing a button corresponding to the desired orientation. The unit also controls the temperature of air running into the MTC.

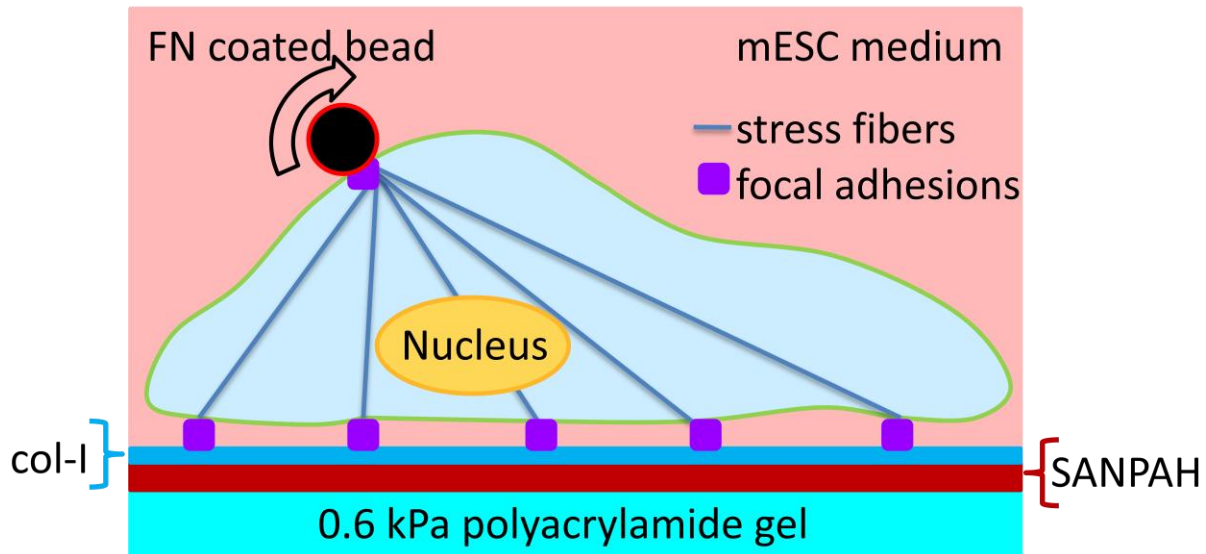


Figure 3. Schematic of stress application. A rounded, undifferentiated mES cell sits on a 0.6 kPa polyacrylamide gel crosslinked with SANPAH and coated in collagen-I. A fibronectin coated

bead binds to the cell surface through integrins which clump to form focal adhesions. Oscillating stress is applied by the magnetic bead and propagated along the stress fibers, causing conformational change in sensory molecules and triggering cellular response.

The magnetic fields are generated by coils of copper wire orthogonally positioned around a central opening where the 35 mm dish containing culture dish sits. Beads were magnetized by applying a 1000 G magnetic field for 10 μ s [29]. After the beads have been magnetized, the MTC can generate oscillating magnetic fields at frequencies up to 1 kHz and magnitudes up to 75 G to create a twisting moment in the bead, as seen in Fig. 4 [30]. For the fibronectin stress application experiments, magnetic field strengths of 25 G and 50 G were applied to cells at a loading rate of 0.3 Hz. The stress applied to cells by the beads depends on c , the bead constant. For this these experiments, beads with a c value of 0.35 Pa/G were used, which translates to 8.8 Pa and 17.5 Pa of stress, for 25 G and 50 G magnetic fields, respectively.

The following equations show the relation between the oscillating magnetic fields and the stress applied by magnetic beads. They were developed by Poh and based on the work of Valberg and Wang [31-33]. According to Valberg, torque can be expressed as

$$T = \mu_0 (\vec{M} \times \vec{H}), \quad (1)$$

where $\mu_0 = 4\pi \times 10^{-7} \frac{N}{A^2}$ = magnetic permeability in a vacuum, $\vec{M}$ = magnetic moment of bead, $\vec{H}$ = oscillating magnetic field generated by the MTC. The cross product in Equation 1 can be substituted for the magnitude of each vector times the sine of the angle between them

$$T = \mu_0 |M| |H| \sin(\theta), \quad (2)$$

where $\theta = \frac{\pi}{2} - \alpha$, and α is the angle between the magnetizing pulse $\vec{P}$ and the magnetic bead moment $\vec{M}$ as seen in Fig. 4. Thus, (2) can be simplified as

$$T = \mu_0 MH \cos \alpha. \quad (3)$$

Specific torque (T_{sp}) is simply torque per unit volume, so dividing (3) by volume gives

$$T_{sp} = \frac{T}{V} = \frac{\mu_0 M}{V} H \cos \alpha. \quad (4)$$

Alternately, torque can be expressed as force multiplied by distance

$$T = FD = T_{sp}V. \quad (5)$$

In this case, the force applied is equivalent to stress applied by the bead times bead area, resulting in

$$T = (\sigma 4\pi r^2)D, \quad (6)$$

where σ = stress, r = bead radius and D = bead diameter. Substituting $2r$ for D allows for simplification of $\frac{4}{3}\pi r^3$ into V (bead volume), giving

$$\sigma = \frac{T}{6V}. \quad (7)$$

Substituting (3) into (7) results in

$$\sigma = \frac{T_{sp}}{6} = \frac{T}{6V} = \frac{\mu_0 M}{6V} H \cos \alpha, \quad (8)$$

which can be further simplified when $\frac{\mu_0 M}{6V}$ is substituted for c , the bead constant

$$\sigma = cH \cos \alpha. \quad (9)$$

Equation 9 can be further simplified when referring to a bead attached to a cell. As mentioned in Fig. 4, α becomes an extremely small angle when the bead's motion is resisted by the cell, so cosine is assumed to be 1.

$$\sigma \approx cH \quad (10)$$

Equation 10 tells us that stress applied by the magnetic beads is a product of the bead constant and the magnetic field generated by the MTC, which is the relation that was used above to determine stresses of 8.8 Pa and 17.5 Pa were applied.

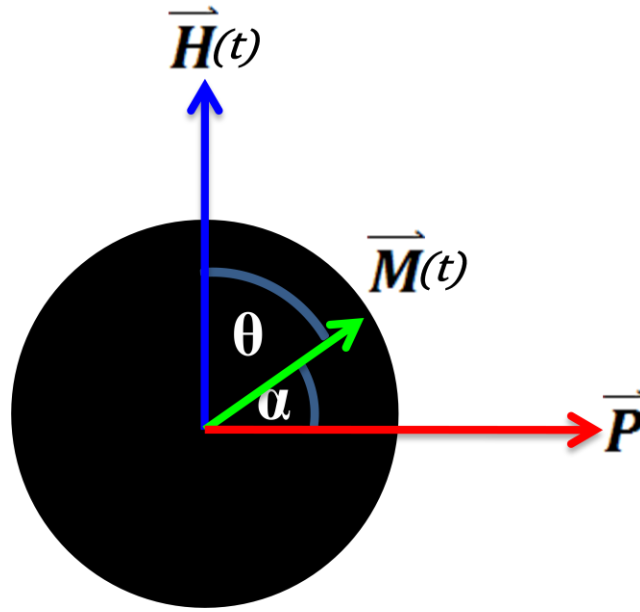


Figure 4. Bead twisting force diagram. Initially, a 1000 G magnetic pulse $\vec{P}$ magnetizes the bead. $\vec{H}(t)$, the magnetic field applied by the MTC, varies sinusoidally from 0-25 G or 0-50 G depending on the loading case. Increasing $\vec{H}(t)$ causes $\vec{M}(t)$, the magnetic moment of the bead, to rotate counterclockwise, increasing angle α . In a frictionless environment the beads would theoretically turn 90°, but when bound to a cell the resistance it provides keeps angle α very small.

2.5 Bleaching and Retinoic Acid Application

Retinoic acid was prepared at three different concentrations for application to undifferentiated mES cells. Stock RA dissolved in ethanol at a concentration of 10 mM was further diluted in ethanol to form a 100 μ M stock. Some of the 100 μ M stock was again diluted in ethanol to create a 10 μ M stock. Each of these stocks was added at a volume ratio of 1:1000 in ES medium free of LIF to create media with RA concentrations of 1 μ M, .1 μ M and .01 μ M. Initial brightfield (BF) and fluorescent images of pluripotent cells were taken 12 hours after plating the cells. After imaging, the target cells were bleached using 100W Hg lamp through the 475 nm filter at maximum intensity for ~8 minutes. Bleaching for much longer than 8 minutes caused significant blebbing and cell detachment, while shorter times did not sufficiently reduce GFP intensity. Fluorescent images were recorded every 30 seconds during the bleaching process. Conveniently, maximum aperture and fluorescent intensity worked well for both bleaching and imaging, so bleaching was not interrupted to capture images. After bleaching, the regular mES cell culture media was removed from the dish and exchanged with the desired concentration of RA supplemented LIF free medium. Follow up images were recorded at 1, 2, 4, 8, 12 and 24 hours after bleaching and RA application.

2.6 Image Acquisition and Analysis

Images were taken with a 40X .55NA air objective on a Leica inverted microscope with an MTC integrated into the stage. The cell border was focused in BF and a BF image was captured with a Hamamatsu CCD camera. The image was exposed for 10 ms and recorded at a resolution of 672 X 512 pixels. Keeping the focal plane and image position identical, the emission filter was set to 475 nm (to excite GFP) and cells were exposed to the 100W Hg lamp

at full intensity to obtain fluorescent images. Image brightness was allowed to stabilize for 1-2 seconds and then the image was captured at 200 ms exposure.

Images were imported into ImageJ (NIH) for analysis. Cell borders were carefully selected on the BF images with the polygonal lasso tool and then transferred to the fluorescent images for analysis. The measure function was used to record average fluorescent intensity as well as the area of each cell. These recorded intensities were normalized with respect to the initial intensity of each cell and plotted versus time to show the change in GFP expression. Cell areas were also normalized with respect to initial area before loading so that cell spreading could be plotted.

CHAPTER 3

RESULTS

3.1 Effect of Stress Application through FN on Oct3/4 Expression

Stress application through fibronectin coated beads downregulated oct3/4 expression, as demonstrated in Fig. 5. Chowdhury et al. have already shown that applying forces through RGD causes differentiation and spreading in undifferentiated mES cells [16]. However, RGD is a synthetic ligand and so the logical next step was to try repeating the experiment with a natural ligand. Fig. 5 shows that oct3/4 levels in mES cells decreased at nearly the same rate over 24 hours for the 17.5 Pa cases of RGD and fibronectin stress application. Surprisingly the 8.8 Pa fibronectin case resulted in statistically significant lower levels of oct3/4 than the 17.5 Pa case (~16% less at 12 hours and ~20% less at 24 hours.) The control case maintained a steady oct3/4 rate at the 12 hour mark and started to increase at the 24 hour mark, indicating the cells were still pluripotent and high expression could have been due to formation of colonies.

Another surprising finding was that stress application through fibronectin did not cause cells to spread. One would expect that a shared RGD binding site and similar temporal differentiation patterns would cause RGD and fibronectin to spread similarly. Fig. 6 shows that cell areas actually slightly decreased after cells were stressed at 0.3Hz with either 8.8 or 17.5 Pa for an hour with fibronectin coated beads. The only indication of spreading came long after stress application was finished. The second set of BF images in Fig. 7 show a cell finally spreading at the 12 hour mark, when the GFP expression is already quite dim. Cell spreading usually occurs quickly when cells are stressed. RGD stress application showed ~15% increase in cell area after only 20 minutes of loading.

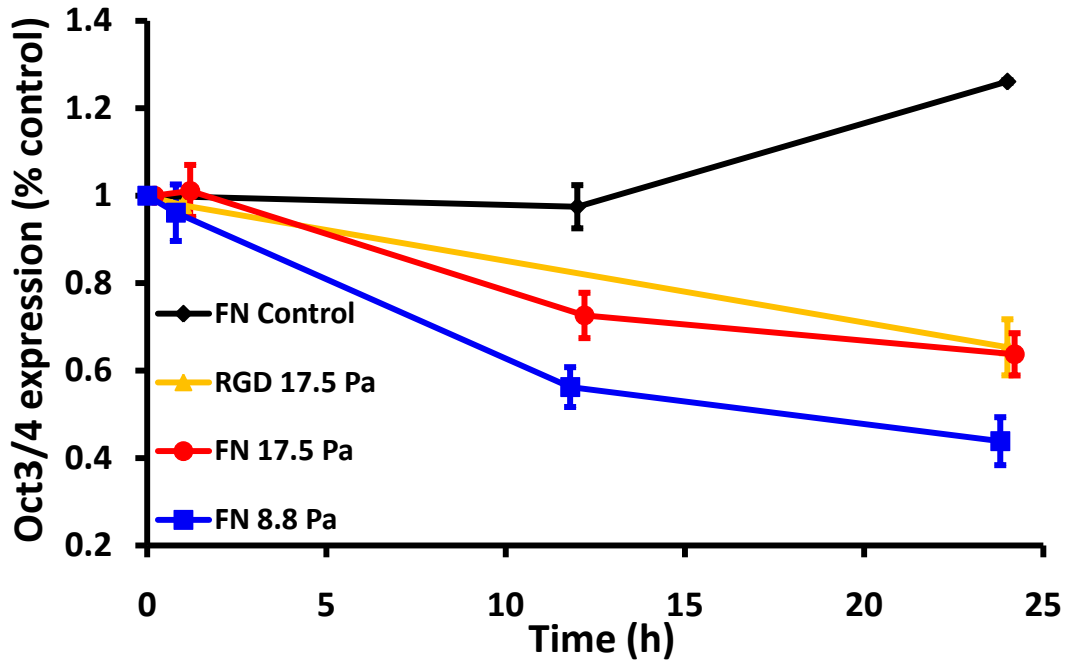


Figure 5. Oscillating stress applied through fibronectin reduced oct3/4 expression in mES cells. Undifferentiated mES cells were plated on collagen-I coated 0.6 kPa polyacrylamide gels, bound with fibronectin coated beads, and stressed at 0.3 Hz for 1 hour at 8.8 and 17.5 Pa (FN 8.8 Pa; $n = 6$), (FN 17.5 Pa, $n = 6$). Oct3/4 expression as a function of GFP intensity was normalized with respect to initial intensity for each cell. Both stressed fibronectin cases showed a large decrease in oct3/4 expression, a strong indicator that cells differentiated. The FN 17.5 Pa stress case decreased in oct3/4 expression at a very similar rate to the RGD case. Unexpectedly, the FN 8.8 Pa stress case showed the greatest decrease in intensity. A two-tailed Student's *t*-test revealed that the difference between the 17.7 and 8.8 Pa FN stress cases was statistically significant at both 12 and 24 hours ($p < .04$ and $p < .008$ respectively). For the control case (FN Control; $n = 3$), cells were bound with beads but not stressed, and oct3/4 expression increased. RGD data (RGD 17.5 Pa, $n = 5$) was obtained from Chowdhury et al. [16].

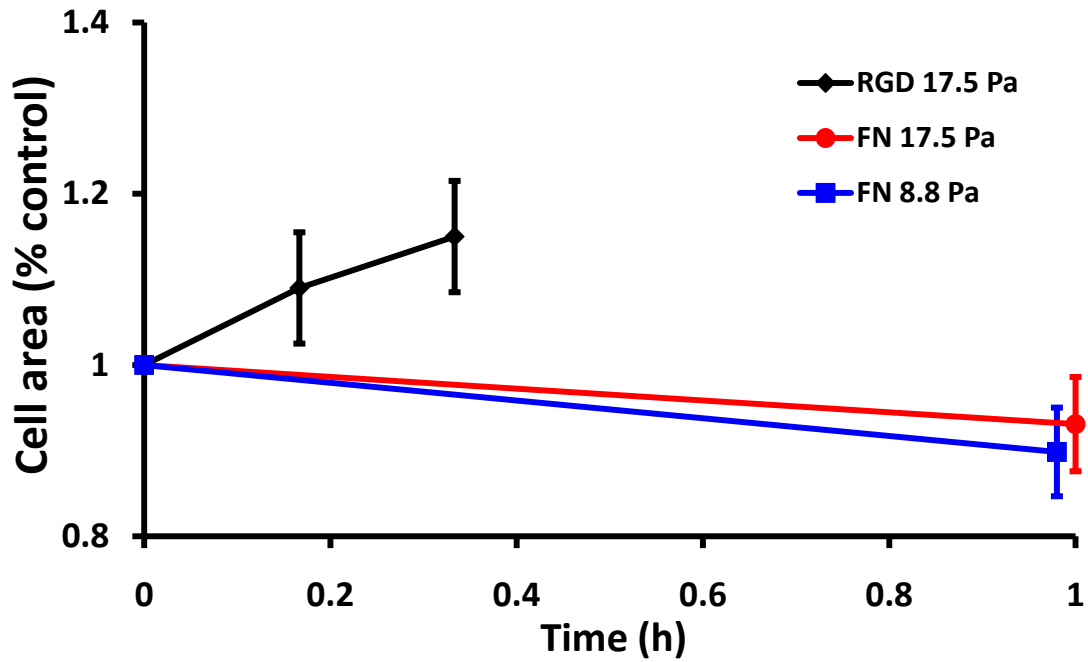


Figure 6. Oscillating stress applied through fibronectin did not result in cell spreading. Undifferentiated mES cells were plated on collagen-I coated 0.6 kPa polyacrylamide gels, bound with fibronectin coated beads, and stressed at 0.3 Hz for 1 hour at 8.8 and 17.5 Pa (FN 8.8 Pa; $n = 6$), (FN 17.5 Pa, $n = 6$). Cell areas were measured based on BF images and normalized with respect to initial area. Both fibronectin cases showed a slight decrease in cell area. Cells stressed through RGD (RGD 17.5 Pa, $n = 5$) showed an average increase of ~15% after loading. RGD data was obtained from Chowdhury et al. [16].

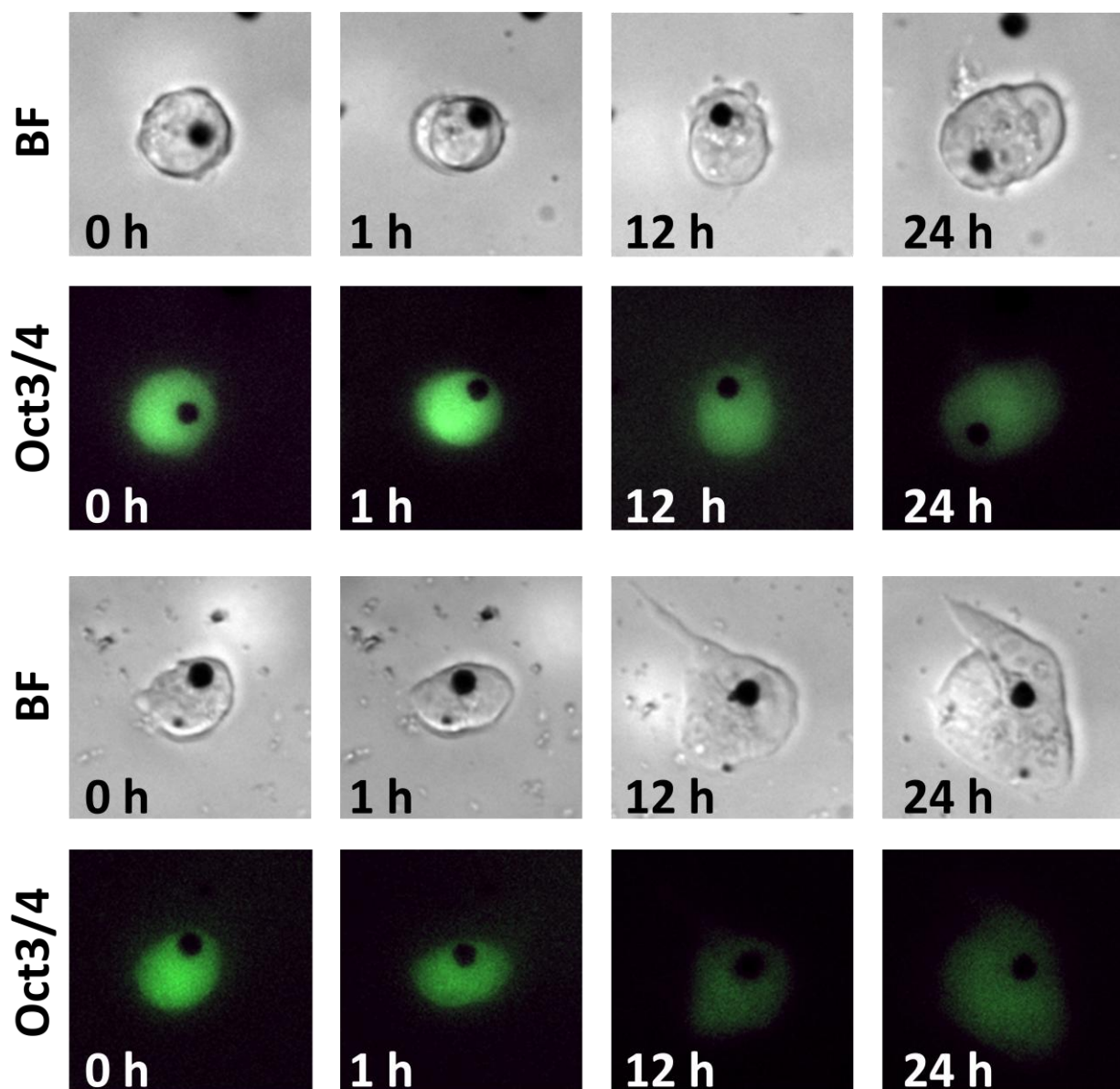


Figure 7. Brightfield and GFP images of stressed cells. BF images show that cells were initially rounded and continued to divide after stress application. GFP images show both cells were initially expressing high levels of oct3/4 indicating pluripotency. A visible decrease in GFP intensity occurs over the course of 24 hours.

3.2 Bleaching and Recovery of Oct3/4 in Retinoic Acid Treated mES Cells

Treatment of photobleached, undifferentiated mES cells with various concentrations of retinoic acid allowed some minor recovery of oct3/4 within the first four hours after exposure, as can be seen in Fig. 8. However, every concentration tested ultimately caused the mES cells to differentiate. As expected, the 1 μ M case was more effective at reducing oct3/4 expression than the other two concentrations. RA concentrations as high as 1 mM were tested, but they caused cells to bleb and detach from the substrate in large numbers. The bleaching alone was not toxic to cells if kept to 8 minutes or under. The control cells recovered normally after being bleached and were unable to be measured after 8 hours because they became confluent. Bleaching for periods longer than 8 minutes was not healthy for the cells, though, as they generally started to bleb and their borders became poorly defined.

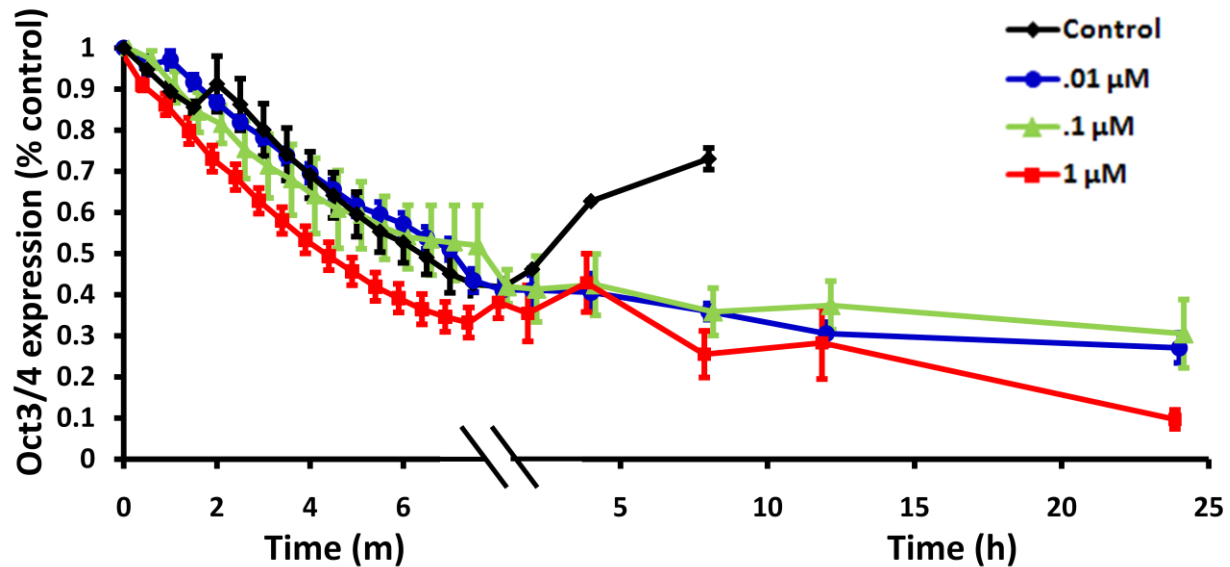


Figure 8. Bleaching and retinoic acid application reduced oct3/4 expression in undifferentiated mES cells. Pluripotent mES cells were plated on collagen-I coated 0.6 kPa polyacrylamide gels. Cells were then bleached at full intensity and aperture for 8 minutes with a 100W Hg lamp

through a 475 nm filter. The x-axis of the figure was divided into timescales on the order of minutes (during bleaching) and hours (after bleaching) so that the bleaching curve would be easier to see. After bleaching, normal mES cell medium was replaced with retinoic acid supplemented – LIF medium at three concentrations (1 μ M RA; n = 6), (.1 μ M n = 3), and (.01 μ M n = 8). The control case (n = 2) also received new medium, but with no retinoic acid added. Oct3/4 expression as a function of GFP intensity was normalized with respect to initial intensity for each cell. Addition of retinoic acid caused cells to maintain or decrease oct3/4 expression, while the control case increased. The 1 μ M RA case decreased in oct3/4 expression the most, with cells expressing an average of 9.7% of their initial intensity after 24 hours.

CHAPTER 4

DISCUSSION

Plating mES cells on fibronectin or laminin is known to activate integrin signaling, so it was expected that applying stress through a bead coated with fibronectin causes mES cells to differentiate [5]. However, plating mES cells on fibronectin is also known to cause cell spreading. Thus it was surprising to see that the cells actually decreased in area when stressed with fibronectin coated beads. Spreading was only seen in a few cells ~12 hours after loading, so it may have been a response to differentiation rather than an early response to force application. Fibronectin uses the same RGD binding site as synthetic RGD, so why did cells shrink instead of spreading? One property of fibronectin that could play a role is that fibronectin changes configuration when placed in tension. This configurational change reveals extra binding sites that could affect cell spreading.

Cells can generate tension by pulling against a substrate with their focal adhesions. Shaub demonstrated that the magnitude of this force is sufficient to unfold a fibronectin molecule [34]. Olberhauser et al. found that 80 pN and 200 pN were required to unfold the weak and stable FNIII domains, respectively [17]. These domains unfold in a “sawtooth” fashion, with each peak requiring 16.2 more pN to unfold than the last [17]. It would be interesting to find out whether the force applied to individual fibronectin molecules during stress application with the MTC is sufficient to unfold them. Fibronectin can extend from a length of 160 nm folded out to 950 nm when unfolded, which according to Olberhauser is important for “tissue preservation under deforming loads.” If this is the case, it could have unknown effects on the propagation of stress

from the bead to the cell. If the force applied were too high it could even break fibronectin, which could reduce the effects of stress on the cell.

The original goal of the bleaching and recovery experiment was to complete the curve for both retinoic acid and stress application through RGD coated beads. This would have allowed the chemical and mechanical differentiation processes to be directly compared. By bleaching down the oct3/4 levels to a baseline, the effect of chemical and mechanical cues on production of oct3/4 could have been studied. Furthermore, the chemical concentration of retinoic acid could have been easily adjusted in an effort to match the level of chemical and mechanical input required to get a similar cellular response. Unfortunately, the combination of bleaching and stress application killed cells. After bleaching cells looked normal, but stress application produced a large ring of dead cells the same size as the bleaching area. Cells outside the bleaching area appeared normal after force application.

The results obtained from bleaching and recovery were in line with other mES cell RA differentiation data. Micallef et al. reported an ideal differentiation concentration of 10^{-5} M and Jones Villeneuve et al. reported a concentration of 10^{-7} M as being the best for differentiation [24, 27]. Our quickest downregulation of oct3/4 came with 10^{-6} M, which falls right in between the two.

CHAPTER 5

CONCLUSIONS

Countless experiments have demonstrated that mechanical factors exert great influence over the fate of cells. As Na et al. demonstrated, even small locally applied forces can propagate the length of the cell to activate molecules [14]. These mechanotransduction processes are particularly important in embryonic stem cells, because soft cells are more sensitive to force application and because mechanical cues influence their differentiation [16]. If there is to be progress in deriving embryonic stem cell based cures, the mechanotransduction process must be further explored. The MTC is an ideal tool to accomplish this, as it can probe specific integrins in a cell that act as the cell's connection to its mechanical environment. We have demonstrated that oscillating forces applied through fibronectin can differentiate mES cells, but more questions remain to be answered. More experiments need to be conducted to find out why fibronectin does not cause ES cells to spread, and whether a smaller force applied through fibronectin can repeatedly downregulate oct3/4 more than a higher force or if it is just a statistical anomaly. Once these questions are answered, the next step is to explore more natural ligands. Laminin and E-cadherin are two possibilities that may help us gain a better understanding of mechanotransduction.

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