Substrate stiffness affects the proliferation rate of fibroblast and epithelial cells in a cell type dependent manner.
By Elena Kassianidou

An Honors Thesis Submitted to the Department of Mechanical Engineering, Stanford University

ACKNOWLEDGMENTS
I would like to thank Professor Jan Skotheim, Amanda Amodeo and Professor Beth Pruitt for their valuable help and support throughout my ups and downs. Every moment you spent with me is truly appreciated. I would also like to thank UAR at Stanford University for a Major grant awarded to me in order to conduct this research during the summer of 2010.
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**Introduction:**

Adherent mammalian cells require a stiff substrate as well as the presence of growth factors in order to divide. They have the ability to sense the stiffness of their substrate due to the presence of integrin mediated focal adhesions and their downstream signaling cascades (the adhesion network). They can also respond to mitogens due to other cell surface receptors such as Receptor Tyrosine Kinases and their downstream signaling cascades (the mitogen network). While the mitogen network has been extensively studied and documented, the adhesion network and the process of mechanotransduction in the context of the cell’s decision to divide, have received much less attention. It is unclear how and when the adhesion and the growth factor pathways interact in order for the cell to determine whether to proceed to mitosis or not.

For my honors thesis, I have worked in Professor Jan Skotheim’s laboratory, part of the Biology Department, to develop an experimental set-up that will allow us to study both the adhesion and the cell cycle network in cells growing on polyacrylamide gels of specific substrate stiffness. Data suggests that the rate of proliferation is affected by substrate stiffness in a cell type dependent manner.

**Background:**

Cell adhesion, and more specifically substrate stiffness, is a regulator of growth factor signaling and therefore, of cell differentiation and cycle progression (Walker et al, 2005). The level of stiffness of a matrix can be a decisive factor in specifying lineage and committing to phenotypes. Stem cells growing on gels with an elastic modulus of ~1kPa differentiate into neurons. Cells growing on gels with an elastic modulus of ~10kPa differentiate into muscle whereas stem cells growing on gels of ~100kPA differentiate into collagenous bone cells (Engler et al. 2006). Cell cycle is also affected by substrate stiffness. Klein et al. have shown that cells that grow on a soft (low stiffness) substrate of an elastic modulus of ~2kPa do not...
proliferate irrespective of the amount of growth factors (Klein et al., 2009). In addition, tumors are known to be stiffer than wild-type tissue. Since tumor cells divide at much higher rates than wild-type cells, it can be argued that stiffness can be a proliferating cue for cells (Paszek et al, 2005).

It is well understood that G1 cells make an all or none decision to divide using an Rb-bound cell cycle switch with cyclin D, an upstream activator of cell cycle progression as input signal. Signals from both the adhesion and growth factor pathway activate cyclin D (Klein et al, 2009). It is suggested that signaling from both pathways results in sufficient cyclin D1 transcription whereas signaling from only one of the two pathways results in insufficient levels of cyclin D and thus cells arrest in G1 (Roovers and Assoian, 2000). This suggests that even though progression to cell cycle is a binary process of “off” and “on”, the decision to divide is made based on a collection of signaling events that considers the availability of growth factors, the adhesion of a cell with its environment and its intracellular tension. It is thus important to look at how mitogen signaling interacts with cellular adhesion and intracellular tension.

The mitogen network affects the transcription of cyclin D through a MAPK cascade. Growth factors bind to Receptor Tyrosine Kinases (RTKs) and activate Ras by allowing it to bind to GTP. Ras is a small GTPase that phosphorylates and activates a MAPK cascade, which in turn phosphorylates and activates the extracellular-signal-regulated kinase (ERK). Increased ERK activity leads to the phosphorylation of a group of transcription factors (Myc, Jun, etc), whose activation leads to increased transcription of cyclin D. The cyclin dependent kinases CDK4 and CDK6 then form a complex with cyclin D, to phosphorylate a set of substrates including the retinoblastoma protein (RB). Hypophosphorylated RB inhibits E2F, a family of transcription factors. Once RB is inactivated via CDK phosphorylation, E2F is able to activate downstream genes including cyclin E, a protein required for DNA replication (S-
phase). Figure 1 summarizes the mitogen network pathway (For a review of these pathways, see Bagosklonny and Pardee, 2002).

Studies have shown that the adhesion network involves two interconnected pathways, the MAPK and the Rho pathway (Assoian et al., 2008) that have the ability to activate ERK. The MAPK pathway is stimulated when integrin receptors on the cell surface bind to the extracellular matrix (ECM). Once integrins bind the ECM, they form pairs that contain α and β integrins. Eventually, the pairs gather together to form clusters.

Formation of clusters results in the recruitment of mechanosensor proteins such as vinculin and focal adhesion kinase (FAK) in a force-dependent manner. Interaction of the actin cytoskeleton and the intracellular domain of integrins results in tension that enhances
protein recruitment. When drugs that inhibit actin-myosin interactions are introduced, recruitment of proteins to the focal adhesion sites is inhibited indicating the force-dependent nature of the recruitment (Chicurel et al., 1998).

Active FAK and vinculin are required for the formation of active focal adhesion sites. Recruitment and activation of FAK leads to ERK and Rac phosphorylation. These proteins are able to initiate transcription of cyclin D (Balmanno and Cook, 1999). FAK by itself is able to initiate an ERK pulse activation but cannot sustain it for long periods. Cyclin D transcription will stop once the ERK pulse falls below a certain value. Figure 1 summarizes how integrin binding can lead to production of cyclin D.

The length of the ERK pulse is thus important for cyclin D transcription (Roovers and Assoian, 2000). Research has shown that in order to sustain ERK activity, the Rho pathway must also be activated via intracellular tension. The Rho kinase pathway increases actin polymerization, which results in the formation of stress fibers and a built-up in intracellular tension (Assoian et al., 2008). The presence of stress fibers encourages the clustering of integrins and leads to a sustained high level of MAPK pathway activity (described above). In addition, Rho kinase activity increases with intracellular tension and results in sustained ERK activation and thus increased transcription levels cyclin D activity. This suggests that both MAPK and Rho signaling are required for sufficient cyclin D levels (Figure 2).

Some papers suggest that without Rho activation, there is no sufficient ERK activation to result in cyclin D1 synthesis. This is shown in cells growing in suspension where only a short pulse of ERK is generated after exposure to growth factors (Roovers and Assoian, 2000). As a consequence, the cells are unable to progress in mitosis. Welsh et al. (2001) however, have shown that even though inhibition of Rho prevents stress fiber formation and sustained ERK activity, Rac, a protein normally inhibited by Rho, is now allowed to activate transcription of cyclin D1 (Figure 2). Cells, thus, still divide even at a low
tensional environment. This suggests that MAPK activation, as a direct result of cellular adhesion, is sufficient for cell cycle progression under normal growth factor stimulation. Studies of the effect of MAPK and Rho pathways on cell cycle regulation have been contradictory and the relative contribution of the two pathways remains unclear.

![Figure 2: Schematic of the interaction of the MAPK and Rho pathway (Assoian and Klein 2008).](image)

Previous studies have hypothesized that ERK is the interaction point between the mitogen and the adhesion pathways since it is present in both and the magnitude and duration of ERK have been shown to induce proliferation. Klein et al. (2009) however, provided evidence contradicting this idea. The authors have shown that cells growing on soft gels can produce a sustained oscillation of ERK activity of similar amplitude as in cells growing on stiff gels. Even though there is sustained ERK activity, cells growing on soft gels have reduced cyclin D mRNA levels that prevent them from dividing. This indicates that ERK is resistant to stiffness changes and additional regulation links adhesion to cell cycle.

Klein et al. (2009) conducted experiments using crude methods for disrupting cell attachment such as trypsinising and analyzing cell populations. Trypsin is an enzyme that breaks peptide chains at lysine or arginine residues and it is able to break down the integrin-ECM bonds of cells. The authors analyzed protein content using Western Blotting, which may mask potentially behavior of individual cells. As demonstrated by Skotheim et al.
(2008), cell-to-cell variability may be considerable so that population-level studies can give incorrect results. Thus, single cell analysis is likely required to determine how cell adhesion and intracellular tension interact with the mitogen network and how they affect cell cycle progression.

My thesis project aims to address the effect of substrate stiffness on the rate of proliferation of different cell types. I examined individual human cells grown on polyacrylamide hydrogels of varying stiffness using time-lapse microscopy. By creating hydrogels of varying stiffness levels while providing enough growth factors, I studied the effect of substrate stiffness on cell division kinetics. I showed that cell division timing changes based on substrate stiffness. Human foreskin fibroblasts (HFFs) growing on polyacrylamide gels of elastic modulus of ~27kPa divided significantly faster than cells growing on gels of elastic modulus ~3kPa. Human epithelial cells (RPE1s) showed a lower stiffness requirement for proliferation since the difference in cell cycle kinetics of cells growing on stiff (~27kPa) and soft (~3kPa) was not significant. My research shows that substrate stiffness affects cell cycle kinetics but different types of cells have different stiffness requirements. Thus, the effect of stiffness on cell cycle and proliferation changes depending on the cell type. The experimental set-up may be used in the future to answer questions of regulation of the adhesion network, its interaction with growth factor induced signaling and its effect on cell cycle.

Methods:
Reactives coverslips:
In order to look at how substrate stiffness affects cell cycle, it is important to grow cells under time lapse microscopy for at least 5 days to obtain data for multiple divisions. In order to achieve this, the surface area of a 20mm glass bottom in a 35mm cell culture dish needs to be functionalized to allow for the polyacrylamide gel to remain attached for long periods of times. Pelham and Wang (1997) provide a detailed protocol of the process. Briefly,
the glass bottom is coated with a thin layer of 0.1M sodium hydroxide (NaOH) for three minutes. A layer of 0.5% 3-aminopropyltrimethoxysilane (APTMS) is added for 10 minutes and is subsequently washed away (3 washes with distilled water, 10 min. each). Afterwards, the glass bottom is incubated in 0.5% glutaraldehyde in phosphate-buffered saline (PBS) at room temperature for 30 minutes. The dishes are then rinsed thoroughly with distilled water.

Polyacrylamide Gel:
I aimed to make substrates of stiffness that samples the dynamic range over which cell cycle kinetics change. From the literature, polyacrylamide gels can have a range of elastic moduli between ~2kPa and ~24kPa (Klein et al. 2009). Polyacrylamide gels are made as described in Pelham and Wang (1997). The 40% acrylamide monomer concentration is held constant at 10% whereas the 2% bis-acrylamide cross-linker concentration varies from 0.03% to 0.6%. The stiffness of the gel depends on the concentration of bis-acrylamide, thus the 0.6% gel has the highest elastic modulus. Briefly, a polyacrylamide batch is created based on the values show in Table 1. 8µl of 10% ammonium persulphate (APS) and 1 µl of NNN’N’-tetramethylethlenediaine (TEMED) are added. 30µl of the solution is transferred on the glass bottom of the dish and carefully spread out by placing an 18mm-diameter glass coverslip on top. Gels are allowed to polymerize for 30 minutes and are then washed to remove any non-crosslinked acrylamide.

Rheology Device Readings:
The stiffness of the polyacrylamide gels was determined using a Physica MCR 300 rheometer device while carrying out an Amplitude Sweep test. The moving part of the device oscillates at very low amplitude to induce a defined shear strain within the polyacrylamide gel. The resistance to these shear deformations is measured and used to determine the gel’s viscoelastic properties. In an Amplitude Sweep test, the amplitude of deformation varies while the frequency remains constant allowing us to obtain information about the structural strength of the sample via the shear storage modulus value G’.

In our measurements, the
viscous resistance determined by the shear loss modulus $G''$ is significantly lower than the elastic resistance measured by the storage modulus $G'$. This allows us to approximate shear modulus $G$ with shear storage modulus $G'$. Given $G'$ and the Poisson’s ratio $v$, we can determine the elastic modulus $E$ of a gel using

\[ E = G' \ast (2 + 2v) \]

The Poisson ratio of polyacrylamide gels is approximately 0.5 since the gels are nearly incompressible (Boudou et al., 2006). This simplifies our formula to

\[ E = 3G' \]

<table>
<thead>
<tr>
<th></th>
<th>0.03% (soft)</th>
<th>0.06%</th>
<th>0.3% (inter)</th>
<th>0.6% (stiff)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>To 8ml</td>
<td>5.79ml</td>
<td>5.67ml</td>
<td>4.71ml</td>
</tr>
<tr>
<td>40% Acrylamide</td>
<td>10%</td>
<td>2.00ml</td>
<td>2.00ml</td>
<td>2.00ml</td>
</tr>
<tr>
<td>2% Bis-acrylamide</td>
<td>Variable</td>
<td>120μl</td>
<td>240μl</td>
<td>1.20ml</td>
</tr>
</tbody>
</table>

*Table 1*: Table showing the amount of acrylamide and bis-acrylamide used to create each type of polyacrylamide gel described in this thesis.

In order to get accurate readings, the area of the gel has to be completely covered by the fitting device of the rheometer. Since the device has a 25mm diameter fitting, gels studied on the rheometer have a 25mm diameter instead of 18mm and are made up of 200μl instead of 30μl. Assuming that the stiffness is homogeneous throughout the gel, there should be no difference in stiffness due to the change in thickness.

The gels are placed in PBS until the rheometer test is performed. In order to reduce slipping effects, the gel surface is gently dried using kim-wipes. In addition, sand paper is attached on the surface of the rheometer and the fitting to increase surface friction.

**Incorporation of ECM proteins on the surface of the gel**

ECM proteins must be attached to the gel surface in order for cells to adhere and proliferate. I chose to coat polyacrylamide gels with fibronectin because human foreskin fibroblasts (HFFs) adhere to fibronectin. Experiments were also carried out using RPE1s
(human epithelial cells) in order to see whether different cell types exhibit similar cell cycle kinetics when grown on substrates of different stiffness.

In order for fibronectin to attach to the gel, the gel surface needs to be covered with a cross-linker that will bind to both the polyacrylamide surface and fibronectin. I used four different cross-linkers on the polyacrylamide gel surface. I was not successful in replicating the first two methods. The first method was obtained from Damljanovic et al. (2005) and involved the immersion of gels in pure hydrazine hydrate for at least 2 hours and then for 1 hour in 5% glacial acetic acid and 1 hour in water. 50μl of fibronectin was added on the gel surface immediately after the water immersion ended. The second method was adopted from Pelham and Wang (1997) and it involves the photo-activation of sulfo-SANPAH using ultraviolet light.

- **NHS dissolved in Ethyl Acetate:**
  Data was collected using the third method, taken from Chelsey Simmons, a graduate student in Professor Beth Pruitt’s laboratory in the Mechanical Engineering Department (work is unpublished). Chelsey suggested functionalizing an 18mm glass coverslide with 20μl of NHS-ester in a 1% suspension of ethyl acetate (NHS does not dissolve completely in ethyl acetate). I decided to use 100μl of 0.2% because it was easier to spread this quantity evenly on the coverslip at this quantity. Even with this amount, I was unable to achieve consistent results so I spread 100μl of 0.2% NHS-ester twice on each coverslip.

Several methods of drying out the NHS-ester on the glass slide were tried to see the effect of each method on having an equally spread out layer of NHS-ester-ethyl acetate on the surface of the glass slide. Coverslips were left to dry at room temperature, nutated at room temperature or at 4°C, or left to stand at 70°C. Evaporation rate was too high at 70°C and NHS did not spread evenly throughout the surface of the coverslip. When the coverslips were left to dry at room temperature, the center of the coverslip had more NHS than the outer parts
did. Swirling sometimes resulted to even distribution of NHS and sometimes to circular rings being formed on the surface. Coverslips that were left to dry on a rotating machine at 4°C had the most even distribution. Gels were made using coverslips nutated at room temperature and 4°C. This allows incorporation of NHS-ester groups onto the gel surface that can interact with fibronectin and allow ECM attachment to occur successfully. 50μL of 1mg/ml fibronectin was added to the surface of each gel and left over night.

- **Fibronectin incorporated within the gel**
  The last method of incorporation of ECM was tested within the last month. This method was obtained from Reinhart-King et al. (2005) and it allows for the incorporation of ECM protein into the polyacrylamide mixture before the polyacrylamide gel polymerizes. I created gels that contained 10% and 20% fibronectin (Table 2). Our fibronectin stock contains Tris, an amine group that reacts with Acrolyl-X and should be removed before the protein is added in the gel. Removal of Tris occurs through dialysis of fibronectin overnight against PBS using a Thermo Slide-A-Lyzer cassette. Once the dialyzed protein is collected, it is incubated with 100μl of 1M sodium bicarbonate (NaHCO₃) buffer per ml recovered and 75μl of 10mg/ml Acrolyl-X succinimidyl ester dissolved in dimethyl sulfoxide (DMSO) per ml recovered for 1 hour at room temperature on a tube rotator. A nanodrop device is used to determine the concentration of protein recovered. The protein is added to the polyacrylamide gel solution. The stiffness of the gel is expected to remain unchanged since the ratio of acrylamide to bis-acrylamide present in the gel is kept constant.

<table>
<thead>
<tr>
<th></th>
<th>10%</th>
<th>20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>67μl</td>
<td>47μl</td>
</tr>
<tr>
<td>40% Acrylamide</td>
<td>50.6μl</td>
<td>50.6μl</td>
</tr>
<tr>
<td>2% Bis-acrylamide</td>
<td>60μl</td>
<td>60μl</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>20μl</td>
<td>40μl</td>
</tr>
<tr>
<td>10% APS</td>
<td>4μl</td>
<td>4μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.5μl</td>
<td>0.5μl</td>
</tr>
</tbody>
</table>

*Table 2: Table showing the amount of acrylamide, bis-acrylamide, fibronectin and water necessary to make the gels incorporating fibronectin into the whole gel rather than just the surface.*
Once the ECM was incorporated onto/into the gel, the gels were left to polymerize and were washed with PBS three times for 10 minutes to remove unattached fibronectin. Cells were then seeded at a rate of 50,000 cells per gel of 18mm diameter. The approximate total number of cells in the culture was determined using a hematocytometer.

Microscope set up:
All time-lapse microscopy experiments are carried out using a Zeiss Axiovert 200M fitted with a custom heat and CO₂ incubation chamber. The temperature was maintained at 37°C with 5% CO₂ Balance Air. In all cases, cells were monitored for 72 hours (3 days) or until cell death while growing in 2ml of DMEM 10% Fetal Bovine Serum (FBS), 1% PS media. The microscope tracks 12 locations in each gel at a frame rate of 10 minutes. All pictures and movies used in this paper are phase images. For phase imaging, exposure time used is 10ms. The microscope is connected to a Hamamatsu CCD camera that takes images and sends them to a computer. In all experiments carried out, the culture medium was left unchanged.

Statistical Methods:
To determine differences between the stiffness distributions of the three types of gels discussed in this paper, I used a Two-sample Kolmogorov-Smirnov goodness-of-fit hypothesis test (alpha = 0.05). To determine the significance in the change of division rate on specific cell type growing on 0.6% and 0.03% bis-acrylamide gels, a Student t-test was carried out with 4 degrees of freedom. T-values were obtained at a level of confidence of p = 0.05. The same test was used to determine whether the change in division rate in the two cell types was significant.

Results:

Gel Stiffness:
I measured the elastic modulus of 16 stiff (0.6% bis-acrylamide), 15 intermediate (0.3% bis-acrylamide gels) and 24 soft (0.03% bis-acrylamide) gels as described in the Methods section. Figure 3 illustrates an example of a 0.6% and a 0.03% bis-acrylamide gel
analyzed using the Amplitude Sweep program. Both graphs (a) and (b) follow the same pattern. At small strains, the shear storage modulus is relatively constant and as the strain increases, the storage modulus decreases. The 0.6% bis-acrylamide gel has a higher storage modulus (~9 times more) than the 0.03% bis-acrylamide gel indicating that the 0.6% gel is expected to have a stiffness that is about 9 times greater than that of the 0.03% bis-acrylamide gel.

**Figure 3:** Data obtained from the Amplitude Sweep for (a) 0.6% bis-acrylamide and (b) gel. The average storage modulus $G'$ value was extracted from the linear region of the graphs under small strain conditions.

(a) The $G'$ value is 91,600Pa. Using (2), the elastic modulus $E$ of this gel is 27.5kPa
(b) The $G'$ value is 1050Pa. Using (2), the elastic modulus $E$ of this gel is 3.15kPa
Figure 4 shows the distribution of stiffness values of 0.6%, 0.3% and 0.03% bis-acrylamide gels. Statistical analysis shows clear separation between 0.6% and 0.03% bis-acrylamide gels ($p = 1.8 \times 10^{-9}$) and between 0.3% and 0.03% bis-acrylamide gels ($p = 3.7 \times 10^{-9}$). Even though there is some overlap between the 0.6% and 0.3% bis-acrylamide gel distribution, the difference is significant ($p = 3.5 \times 10^{-6}$). This indicates that the stiffness felt by cells growing on soft gels is significantly different to that felt by cells growing on stiff gels. As a result, these gels are a good experimental set-up to study how cell cycle kinetics change based on stiffness.

**Figure 4:** Box plots showing the first quartile, the median and the third quartile for 0.6%, 0.3% and 0.03% bis-acrylamide gels. The respective values for each gel type are: for 0.6%, $q_1=22.189$, median $= 26.329$, $q_3=2.294$. For 0.3%, $q_1 = 14.06$, median $= 15.79$, $q_3 = 18.204$. For 0.03%, $q_1 = 3.049$, median $= 3.7$ and $q_3=4.484$. Error bars show 95% degree of confidence.

**Reactive Coverslips:**

I was successful in replicating the protocol of Pelhalm and Wang (1997) and gels stuck on the glass slide. Even though, literature suggested that the coverslips could be stored for up to 2 weeks, my coverslips were sometimes non-functional after day 3. In order to figure out why this was happening, I conducted experiments where one of the reagents
(NaOH, glutaraldehyde or APTMS) was changed while the other two were kept constant. My experiments showed that when glutaraldehyde is stored for more than a month, it does not work as effectively and the coverslips do not last for as long as expected, preventing us from being able to image the gels.

**Results using NHS-ester dissolved in ethyl acetate gel:**

This method of coating the gel surface with fibronectin had variable success. I was faced with two problems. At too high concentrations of NHS-ethyl acetate, NHS did not spread evenly on the coverslip. As a result, there were areas on the gel that had too much NHS leading to crystallization that made imaging difficult. At too low concentrations of NHS-ethyl acetate, no crystals formed but the cells peeled of the surface. The cells divided during day 1 and then started migrating towards specific parts of the gel. Eventually, the cells formed clusters and peeled completely. I hypothesized that the NHS-polyacrylamide bond may not be strong enough to support the tension applied by the increasing number of cells on the gel. As a result, the cells are able to “pull” the NHS off the gel, cluster and eventually peel off. Figure 5a and Movie 1 are an example of cells peeling off the surface of a 0.6% bis-acrylamide gel. Sometimes, the gels did work as demonstrated in Figure 5b. The same issues were true for the 0.03% gels as well. An example of a successful 0.03% bis-acrylamide gel is shown in Figure 5c.

In order to balance the two problems, I decided to vary the amount of NHS-ethyl acetate added and the way the coverslip dried to see how crystallization, cell attaching and survivability changes. Table 4 shows the results of these experiments. Coverslips made at room temperature were sometimes successful in attachment and cell proliferation. It rarely resulted to levels of crystallization that would make imaging difficult. Coverslips made at 4°C has the most even distribution of NHS-ethyl acetate. The cells attached and proliferated on the gel surface but there was extreme crystallization. It is possible that during room temperature evaporation, some of the NHS escapes to the atmosphere, whereas in 4°C, more
NHS remains on the surface leading to more crystallization on the gel. Given these results, all data was collected from gels made of coverslips that were nutated at room temperature.

Figure 5: Frames from movies of cells growing on a) and b) 0.6% bis-acrylamide gel and c) 0.03% bis-acrylamide gel. For all gels, fibronectin was incorporated onto the surface using 2 times 100μl of 0.2% NHS-ester dissolved in ethyl-acetate. 50μl of 1mg/ml fibronectin were added on the surface of the gel after polymerization.

a) Frame 2 and 3 show cell proliferation. Frame 4 and onwards, cells started curling up, forming balls and eventually detaching completely from the gel.

b) Cells remain attached and spread out throughout the experiment.

c) Cells take longer to adhere and spread (frames 1–4). Cells remain attached and proliferate throughout the experiment.
Despite the drawbacks of the NHS method, I was able to produce enough functional gels to analyze the effect of stiffness on proliferation rates by comparing cell behavior at 0.03% and 0.6% bis-acrylamide gels using time-lapse microscopy. To measure the proliferation rate, I counted the number of divisions occurring in each frame. Cells growing on stiffer (0.6%) gels had a larger spreading area; they moved faster and divided more frequently (See Movie 2 and Movie 3). This experiment was conducted for both HFFs and RPE1s to see whether different cell types would show similar dependences on substrate stiffness (See Movie 4 and 5 for RPE1 cells).

Figure 6a shows how stiffness affects cell division of HFFs. Cells growing on stiff gels divide significantly more than cells growing on soft gels. Using a t-test, I determined that the change in rate of division in cells growing on firm and soft gels is significant in all cases (for 0-12hrs \( p = 0.0007 \), for 12-24hrs, \( p = 0.0021 \), for 24-36hrs \( p = 0.0036 \) and for 36-48hrs \( p=0.0011 \)). This shows that HFF cell cycle kinetics are affected by the stiffness of the substrate.

<table>
<thead>
<tr>
<th>Amount of NHS added</th>
<th>Day 3</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 gels using 100μl, twice, coverslip dried at room temperature</td>
<td>1 gel did not have cells attached at center. 2 gels had cells spread throughout the surface. Gel surfaces were imageable.</td>
<td>Only one of the gels did not experience peeling but did not reach confluence. Gel surfaces were imageable.</td>
</tr>
<tr>
<td>2 gels using 90μl, twice, coverslip dried at room temperature</td>
<td>1 of the gels showed signs of peeling. 1 of the gels did not have cells attached near the center. Gel surfaces were imageable.</td>
<td>All gels show signs of peeling. Gel surfaces were imageable.</td>
</tr>
<tr>
<td>3 gels using 80μl, twice, coverslip dried at room temperature</td>
<td>All gels had cells attached and spread out. Gel surface was imageable.</td>
<td>All gels show signs of peeling. Gel surfaces were imageable.</td>
</tr>
<tr>
<td>3 gels using 75μl, twice, coverslip dried at room temperature</td>
<td>All gels had cells attached and spread out. Gel surface was imageable.</td>
<td>All gels show signs of peeling. Gel surfaces were imageable.</td>
</tr>
<tr>
<td>3 gels using 100μl, twice, coverslips dried at 4°C</td>
<td>All gels had cells attached and spread out. Gel surface was not imageable.</td>
<td>Gels did not experience peeling. Gel surfaces were not imageable.</td>
</tr>
<tr>
<td>3 gels using 85μl, twice, coverslips dried at 4°C</td>
<td>All gel had cells attached and spread out. Gel surface was somewhat imageable.</td>
<td>All gels show signs of peeling. Gel surfaces were not imageable.</td>
</tr>
</tbody>
</table>

*Table 4:* Different amounts of NHS and methods of drying the coverslip were used per gel to study the effect of NHS and cell attachment and survivability. Results are inconclusive.
Figure 6b shows how stiffness affects cell division of RPE1s. RPE1s divided faster on the firm gels than the soft gels. Using a t-test, the change in rate of division in RPE1s growing on stiff and soft gels per 12-hour periods is not significant (for 12-24hrs $p = 0.29$, 24-36hrs $p = 0.13$ and for 36-48hrs $p = 0.94$).

Figure 6: Division per (a) HFFs and (b) RPE1s within a 12-hour time frame. Number of cells and divisions counted in 3 time-lapse microscopy positions within a gel. This shows that stiffness affects HFF cell division. Graph (a) shows that stiffness affects HFF cell division and all comparisons are significant. Graph (b) shows that RPE1 cells are affected by substrate stiffness but not to the same extent as the HFF cells. All comparisons between 0.6% and 0.03% bis-acylamide gels for HFF and RPE1 cells are statistically significant ($p<0.05$). All comparisons between HFF and RPE1 division rates on 0.6% bis-acylamide gels are statistically insignificant whereas comparisons on 0.03% bis-acylamide gels are statistically significant ($p<0.01$).
Comparing the two cell types, results show a significant difference between the rate of division rate of HFFs and RPE1s on 0.03% bis-acrylamide gels (all p values are ~0.01). There is a significant difference between the division rates of HFFs and RPE1s growing on 0.6% bis-acrylamide gels during 12-24hrs (p=0.0470) but no statistical difference at 24-36 (p=0.1794) and 36-48hrs (p=0.9132). This shows that HFFs and RPE1s growing on stiff substrates divide in similar rates but when growing on soft substrates, they divide at different rates, specifically the RPE1s divide at significantly higher rates than HFFs. This indicates that the effect of substrate stiffness on HFF cell division is stronger than on RPE1 cell division. Different cell types are thus, affected differently by substrate stiffness.

**New method of incorporating ECM into the gel:**

I started exploring this option a couple of weeks ago and was only able to make a couple of gels so far. I made 3 gels with 10% of fibronectin and 3 gels with 20% fibronectin. All of the gels had cells attached on them and relatively spread out but the majority of the cells did not adhere. None of the gels reached confluence even after a week of cells growing on them (data not shown). This indicates that the cells were not dividing properly. This may be due to the stiffness of the gel; I have not checked to see whether addition of fibronectin within the gel affects the overall stiffness. More work is necessary to see whether this method can be more reliable than NHS-ethyl acetate.

**Discussion:**

For my thesis project, I studied the effect of substrate stiffness on cell cycle kinetics in different primary cell lines. To perform this research, I needed to create hydrogel substrates of constant stiffness. I found that polyacrylamide gels are a suitable substrate to study the effect of stiffness on cell proliferation rates. By varying the bis-acrylamide concentration, gels can be made within an elastic modulus range corresponding to the physiological ranges of human tissue. The gels made for this experiment had an elastic modulus of ~27kPa, ~16kPa and ~4kPa, similar to fatty tissue surrounding the prostate gland.
(~30kPa), thyroid gland tissue (~12.3kPa+/-4.8) and breast tumor tissue (~4kPa) (Alterovitz et al., 2009, Levental et al., 2006, Lyshchik et al., 2005).

I examined two different cell lines, human foreskin fibroblasts (HFFs) and human epithelial cells (RPE1s) and found that cell proliferation depends on substrate stiffness and cell type. While HFFs divide faster on 27kPa gels than on 4kPa, RPE1 proliferation was relatively unaffected. There is no significant difference when comparing the HFF and RPE1 cell division rates on 0.6% bis-acrylamide gels indicating that on substrates of ~27kPa, both cell lines divide at a similar rate. There is a significant difference, however, when comparing the HFF and RPE1 division rates when growing on 0.03% bis-acrylamide gels. HFF cells proliferate at a much smaller rate. This shows that the effect of substrate stiffness on the proliferation rates of HFF and RPE1 is different; specifically HFF cells are much more affected than RPE1s. This suggests that the critical stiffness requirement for proliferation is cell type specific.

In order to determine the exact nature of the rate of proliferation for HFFs, more data has to be collected at intermediate stiffness gels. Data can be used to determine how sharp the effect of substrate stiffness is on HFFs. As determined by Roovers et al. (2001, 2003), the G1 phase of cell cycle increases as stress fibers within the cell and cell adhesion were disrupted. This indicates that the rate of proliferation can be tuned as a function of stiffness. Figure 7 suggests two possible ways of tuning, graded or two-valued. A two-valued tuning indicates the presence of a stiffness threshold value that cells need to sense in order to divide normally. Above that value, cell proliferation rate is at a constant high level (assuming that enough growth factor is provided) and below that value, it is at a constant low. If it is a two-valued process, looking at cell division rates using various stiffness values will allow us to determine the threshold stiffness value and compare among different cell lines. If the process is graded, there should be a minimum substrate stiffness value and above that, proliferation rates will
increase until it plateaus to a maximum value. Our preliminary data suggests that the relationship between proliferation rate and stiffness is likely not a two-valued process, but rather a graded process where the rate of proliferation is tuned as a function of substrate stiffness.

Throughout my research I was faced with the problem of successfully incorporating the ECM on the gel surface. Even though I was able to collect data, the NHS-ethyl acetate method is unreliable and not reproducible. I was faced with two main problems; crystallization on the gel surface and cell peeling. Further work needs to be done to find a way to balance the two issues by using enough NHS to allow for cell attachment and proliferation but not too much to form crystals. It is possible that peeling may result due to tension applied from cells on fibronectin. This force may be strong enough to pull fibronectin off the gel. It is necessary to come up with a method that will result to polyacrylamide-fibronectin bonds that are strong enough to withstand any forces imposed on fibronectin from the attached cells.

Future work will also concentrate on gels that have fibronectin incorporated within rather than on the surface. The main advantage of this method over the NHS method is that there is

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**Figure 7**: a) Schematic of the two-valued tuning process. There are two constant values cell cycle time, one high and one low. Once stiffness reaches the critical threshold value, the cell cycle time switches from one time point to the other rapidly.
b) Schematic of the graded tuning process. As stiffness decreases, cell cycle time increases in a graded manner. Once stiffness reaches the minimum value (dotted line), there will be no proliferation and cell cycle time will be infinitely large.
no crystallization formed ensuring high quality imaging. The main disadvantage is that when making such a gel, it is difficult to determine how much fibronectin is available at the surface (how many binding sites are available to the cell membrane).

Preliminary data obtained from these experiments suggest that stiffness does affect cell cycle proliferation in certain cell types. This indicates an interaction between the two network signal pathways (mitogen and adhesion) that ultimately affects cell cycle kinetics. This observation brings up multiple questions. How does the cell sense the stiffness of its substrate? What is the point of interaction between the cell cycle and the adhesion network and how is it regulated? Why are certain cell lines more sensitive to stiffness than others? Using the experimental set-up described in this paper, some of these questions can be answered. Through integration of fluorescent reporters for specific proteins of each pathway such as cyclin D and ERK and targeted RNAi of known signaling components, we can analyze how cell proliferation rates are affected and determine possible points of interaction. We can determine how the amount of cyclin D varies based on the amount of FAK and ERK within the cell. This will allow us to determine the adhesion requirements the cell needs to meet in order to commit to division and which pathway contributes the most to this decision (Rho – intracellular tension Vs MAPK – adhesion related).
Works Cited


8. Roovers K and Assoian RK, 2000, Integrating the MAP kinase signal into the G1 phase cell cycle machinery, *Bioessays, 22:818-26*


