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Development of Photodegradable Hydrogel Platforms for Cardiomyocyte Culture

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Development of Photodegradable Hydrogel Platforms for Cardiomyocyte Culture

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A thesis submitted in partial fulfillment of the requirements for graduation with
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Abstract

It has been shown that the microenvironments to which cells are exposed influence their shape and gene expression. Traditionally, because of the difficulty of studying cells in the body, cells are removed from the organism and are cultured on glass or plastic surfaces such as tissue culture polystyrene (TCPS). One limitation to studying cells on TCPS is its non-physiologic ultra-high stiffness. In fact, TCPS is $10^6$ fold more stiff than a healthy heart. In the interest of studying cardiac disease in a more physiologic context, we used a photodegradable hydrogel cell culture platform that can be manipulated with light to more closely represent the environments that neonatal rat ventricular myocytes (NRVMs) are exposed to in vivo. In many cases, as cardiac disease progresses, the cardiac muscle becomes stiffer and the cells become less organized. Our research used UV light to create micron-scale patterns in these hydrogels in order to better mimic diseased and healthy states of a heart and thus encourage cells to maintain their native phenotypes. The hydrogels that were created influenced both the degree of cell alignment and expression levels of genes known to be sensors of health and disease (MHY6, MYH7, ANF, COL1A1, ACTA1, CCN2, ATP2A2, CACNA1C, and MMP2). Over all, alignment indices and expression levels of many of the genes offer strong evidence in support of the claim that a PEG hydrogel system is a healthier environment for NRVMs than TCPS is. In the future, these gels may be used as a more physiological alternative for culturing NRVMs for cardiac disease research.
1. Introduction

Worldwide, the leading cause of death is ischemic heart disease, a general title for the complication that causes limited blood flow to and from the heart. [1] In an effort to reduce the nearly one million heart attacks that occur each year in the U.S. alone, the National Institute of Health has made research on heart disease one of its top priorities. Over the last five years, each year the NIH has spent roughly two billion dollars on funding for cardiac disease research, making this topic one of the most important in the industry. [2]

For decades, researchers have been growing cells on TCPS dishes as an alternative to in vivo studies, which can be quite difficult and costly. However, it is known that a cell’s microenvironment has effects on its gene expression, growth rate, growth pattern, and overall function. [3] In healthy cardiac tissues as compared to diseased, cells are more aligned and the overall tissue is softer. When a cell is removed from the body, it has the potential to behave differently due to environmental factors. In more recent years, labs across the country have been making an effort to study cells in environments that better resemble that of the cells’ natural environment. [4] Particularly for heart disease, this is important. TCPS, being a rigid plastic, is a million times stiffer than healthy cardiac tissue. To be more specific, the Young’s Modulus of tissue culture polystyrene and glass ranges from 1-70 GPa, whereas cardiac tissue ranges from ~10-100 kPa, many orders of magnitude less than standard tissue culture surfaces. In order to more accurately study the progression of heart disease, we think that it is important to study cardiomyocytes on softer platforms. Thus, we are using photodegradable hydrogel cell platforms to which we can adjust the modulus and pattern. From analysis of the
cells’ gene expression and alignment, we can deduce whether our platforms better mimic healthy or diseased states of a heart as compared to TCPS. By using these adjustable platforms, we are creating a foundation upon which can be built many years of directed, accurate, and more advanced research on cardiac diseases.

1.1. Cellular Model of Heart Disease:

Our study concentrated on the gene expression analysis and alignment of NRVMs. Our NRVMs were isolated from one to three day-old Sprague-Dawley rat pup hearts. Myocytes are the contracting cells of cardiac muscle. NRVMs are an established in vitro system for studying heart disease and our labs have several decades of expertise and prior data on NRVM biology. In addition, previous results from NRVMs have been closely correlated with observations in human disease.

1.2 Effects of Different Platforms on Gene Expression

As a mechanism of adapting to their environment, cells control their levels of expression of various proteins by modifying gene transcription and translation. During cardiac disease or post-cardiac infarction, expression levels for genes expressed by cardiomyocytes may be increased or decreased. In our study, we analyzed the gene expression levels of nine genes in NRVMs.

**NPPA (ANF): Natriuretic peptide precursor A**

The atrial cells of the heart secrete the natriuretic peptide product of this gene. The atrium of the heart is where blood enters. When secreted, the peptide acts to reduce
water and sodium loads on the circulation system, effectively decreasing blood pressure. ANF has been shown to increase in many forms of heart disease. [5]

MYH6 (αMHC): a myosin heavy chain subunit of cardiac myosin

Cardiac myocytes are vital to the composition of the cardiac muscle tissue, and are thus rich in sarcomeres. Sarcomeres, in essence, are the basic contractile unit of a muscle and are composed of interacting thick and thin filaments. These filaments are composed of myosin and actin, respectively. While there are multiple types of myosin, their basic structure includes a long fibrous tail and a globular protein head, which “walks” along the actin filaments during muscle movement. ATP hydrolysis is the source of energy for this process. [6] In the heart, myosin fibers in the muscles are composed of six subunits: two each of heavy chains, essential light chains, and regulatory light chains. Figure 1 shows the general structure of a myosin molecule. The MYH6 gene encodes the α myosin heavy chain subunit and is located on chromosome 14, just 4kb downstream of MYH7, the β myosin heavy chain subunit. As α myosin heavy chain is the major isoform found in healthy adult rodents, it has been of particular interests to scientists using rats and mice as models for heart disease studies. Decreased expression levels of this gene have been observed in human heart disease and have been speculated to be a cause of heart disease directly. [7]
Figure 1) Structure of a myosin molecule. MYH6 and MYH7 encode heavy chain (MHC) proteins that coil together to form the tail. α and β heavy chain subunits are nearly identical and differ mostly in their motor domains (heads), which interact with actin. [8]

**MYH7 (βMHC): β myosin heavy chain subunit of cardiac myosin**

This gene encodes the β subunit of the heavy chains for cardiac myosin filaments. This subunit is an alternative isoform to the α subunit and plays a role in the contraction of slow-twitch skeletal muscles. In heart disease in rodents, induction of the β myosin heavy chain isoform has been described. [9] In adult humans, β is the major isoform.

**CACNA1C (CaV1.2): α 1C subunit of the voltage-dependent calcium channel**

The CACNA1C gene encodes the α 1C subunit of an L-type, voltage-dependent calcium channel (CaV) found in cardiac muscle. The α subunit of the channel is essential to the protein’s structure as it determines most of the channel’s properties. Though an important channel, CaV1.2 is not the only calcium transporter on the cell membrane. Upon membrane depolarization, this channel mediates the influx of Ca$^{2+}$ ions into the cell to couple the process of excitation and contraction of the muscle. This particular channel is specific for long-lasting activation. Defects in this gene have been known to cause low
blood pressure, arrhythmia, and structural defects. Overall, the CaV1.2 channel plays a major role in maintaining a regular heartbeat.

**COL1A1: a subunit of type 1 collagen**

The COL1A1 gene is located on chromosome 17 and encodes the pro-α chains of collagen type 1. While there are various kinds of collagen in the body, collagen type 1 is the most abundant and is found in scar tissue and connective tissues. A particular type of connective tissue called the endomysium ensheaths individual muscle fibers in the heart and is composed of collagen type 1. As a result of tissue damage, scar tissue may be present and thus an increase in the presence of collagen 1 and the expression of COL1A1 is expected. [11]

**MMP2: Matrix metalloproteinase 2**

As a group, matrix metalloproteinases (MMPs) function to degrade structural components of the extracellular matrix. Matrix remodeling can occur normally or as a result of processes including, but not limited to: angiogenesis, ventricular remodeling post-infarction, metastasis, and reproduction. During the development of many cardiac diseases, increased amounts of tissue remodeling occur, eliciting increased expression levels of the MMP2 protein in order to produce the peptidase. The MMP2 peptidase is a collagenase and thus degrades type IV collagen, which is a major structural component of basement membranes. This gene is found on chromosome 16. [12]
CCN2 (CTGF): Connective tissue growth factor

Located on chromosome 6, the CCN2 gene encodes a mitogen, which works to promote cell division by triggering mitosis. Thus, this gene product is considered a cellular growth factor. In cases of cardiac disease, CTGF is secreted by vascular endothelial cells as a way of encouraging tissue repair after damage. [13] As a part of the repair, CTGF works with TGF-β to induce sustained fibrosis (scarring). It follows then, that in cases of heart diseases where there has been damage, the gene is upregulated. [14]

ATP2A2 (SERCA2): Sarcoplasmic reticulum Ca$^{2+}$-ATPase

The SERCA2 gene, found on chromosome 12, is part of the SERCA family, whose products are Ca$^{2+}$-ATPases that reside in the sarcoplasmic reticula of cardiac muscle cells. The gene we studied encodes a Ca$^{2+}$ pump, which uses ATP hydrolysis as its driving force to move calcium ions across the membrane, out of the cytosol, and into the lumen of the sarcoplasmic reticulum (SR). The SR is a type of smooth endoplasmic reticulum (ER) and is found in muscle fibers. In the process of muscle contraction and relaxation, the SR releases and absorbs calcium ions, respectively. To elaborate, the system of muscle contraction is based on action potentials and voltage-gated ions channels. Once the muscle cell has been activated by the neurotransmitters from a neighboring nerve cell, it propagates its own action potential via Na$^{2+}$ channels. Once the action potential has reached the T-tubule (a membrane invagination), the SR is induced to release Ca$^{2+}$ ions into the cytosol, where the ions can interact with and allow myosin to walk along actin filaments and cause muscle contraction. [15] Without a functional
SERCA2 calcium channel, as evidenced by a decrease in gene expression during disease, cardiac muscle contractility may be abnormal.

ACTA1: α I skeletal muscle actin

The ACTA1 gene, located on chromosome 1, encodes the α isoform of skeletal muscle actin. This particular actin isoform is important for the contractility functions of a cell. These contractions in the heart help produce the heart beat. Actin, as the other major molecule in muscle fiber structure, composes the thin filaments of sarcomeres. It is worth noting that there are two types of α actin found in the heart: cardiac α actin and skeletal muscle α actin. To date, these two isoforms are accepted as being nearly identical, with no known functional differences. [16] However, our studies included only the gene that encodes the skeletal muscle actin. Based on previous research, ACTA1 expression increases as a result of cardiac diseases that are characterized by dedifferentiation or increased growth of the muscle cells. These effects are largely the result of increased blood pressure or volume. [17]

1.3 Effects of Different Platforms on Alignment Index

In healthy cardiac tissue, the cardiac muscle cells of the heart are striated with the sarcomeres showing alignment. As disease progression occurs, the myocytes become increasingly less organized and assume asymmetric shapes. When plated on a smooth TCPS surface, cells have no directional cues to guide their growth and thus exhibit more disorganization. In an attempt to help the cells achieve the alignment they have in a healthy heart, we have cultured them on gels with rectangular and channeled patterns that
have ranging aspect ratios. The cells’ alignment can then be quantified and compared to non-patterned gels and TCPS cultures. Typically, we assume that cells that are more aligned are exhibiting healthier characteristics and how aligned they are on certain gels can help inform us as to how “healthy” that platform is.

1.4 Poly(ethylene glycol) Hydrogels

Our platforms for modeling the diseased and healthy states of the heart are poly(ethylene glycol) (PEG) hydrogels that are functionalized with photolabile acrylate groups that allow them to be cross-linked into polymer networks. [18] These acrylate groups can also be cleaved with UV light. [18] Additionally, PEG hydrogels have high water content so they are ideal tissue mimics, and they are bioinert so they have a low tendency to adsorb non-specific proteins.

2. Materials and Methods

Neonatal rat ventricular myocytes were isolated from the hearts of 1-3 day-old Sprague-Dawley rat pups and used for all experimental procedures. Neonatal cells were used because they have robust responses to stimuli in culture and they are able to be in culture for 7-10 days instead of 2-3 in the case of adult cells. Total, there were nine gel samples across three cell preparations (n=9).

2.1 Hydrogel Preparation

Our PEG hydrogels were 18mm in diameter and ~150 microns thick after polymerization (measurements of thickness is an area for potential further study). Before
the gels were synthesized, the coverslips that the gels were attached to were acrylated. Acrylation is the process of adding an acryl group. In our case, the acryl group comes from acryloxypropyltrimethoxysaline (APTS), which is functionalized to the surface of the glass. Each 18mm coverslip was passed through a natural gas flame 4-5 times before acrylation. The purpose of this is two-fold: the flame cleans the glass and also adds hydroxyl groups to its surface to better improve the efficiency of the silanization reaction. Ten at a time, the coverslips were submerged in 95% ethanol, adjusted to a pH of 4.5-5.5 with acetic acid, and combined with 500 µL APTS. After reacting for 2-3 minutes, the coverslips were rinsed with 95% ethanol and baked at 60-80 °C for 15 minutes to dry. The purpose of adding the functional group is to allow the gel to form a covalent bond with the glass.

Master mixes for the gels were composed of PEGdiPDA, PEG400A, TEMED, and gelatin (1mg/L), and were dissolved in PBS. Each gel consisted of 38µL of polymerized solution, and enough master mix for three gels was added to each eppendorf tube. Ammonium persulfate (AP) was added to each eppendorf tube to initiate the polymerization reaction. A 10% AP 2M solution was used with a 90% PEGdiPDA mastermix (actual PEGdiPDA weight percent was 6-8%). Upon addition, TEMED (tetramethylethylenediamine) catalyzed the reduction of AP (formation of a radical), which initiated the polymerization of the PEG groups to create cross-links. [18] The PEG macromer and its subunits are represented in Figure 2a-b. While the gel was polymerizing, three 38µL aliquots were quickly pipetted onto a clean glass slide and 18mm round acrylated glass coverslips were placed on top of the gel. After waiting 3-4 minutes for the gels to polymerize fully, the gels were submerged in PBS so as to make
their separation from the glass slide easier. A razor blade was used to gently remove the gels from the glass. Gels were then stored in a 6-well plate, in the dark, at 4°C, and in PBS with fungizone and penicillin and streptomycin antibiotics.

Figure 2  

Kloxin et al., 2009

2.2 Gel Patterning via Photolithography

To create patterns in the gels before the cells were plated, an OmniCure light source and a photomask were used. The wavelength of light generated by the OmniCure was 365 nm, which is in the UVA range. A collimator was used to focus the light so that the edges of the beam were sharp and the light was evenly distributed over four wells at a time. Gels were placed gel-side down on the photomask, so that the light would pass
through the mask before reaching the gel and only irradiate parts of the gel that were not blocked by the pattern on the mask (like a stencil). Irradiation of the gels occurred from the bottom up for 7.5 minutes each. The chemical effects of the irradiation process are depicted in Figure 2c. The depth of the channels has not been measured, but remains an area of further study. The gel patterns included channels, rectangles, and squares. The channels stretched across the entire cell and were 2, 5, and 10 µm in width (both the peaks and valleys were the same width). These channels can also be considered rectangles of 2, 5, and 10 µm width by infinite length. The other rectangles patterned were 10x5x5, 20x5x5, and 40x5x5 µm. The square 5x5x5 µm pattern was also used. All rectangular (not channels) and square patterns had valleys of 5 µm width between each raised rectangle. The overall patterning process can be seen as a simplified version in Figure 3.

Modified from Scheler and Strohriegl, 2009 Figure 3)
Visualization of a photolithography process. The actual process takes place with the light shining up from beneath rather than from above.
2.3 NRVM Isolation and Culture

1-3 day old Sprague Dawley rat pups were sacrificed for the use of their cardiac myocytes. Their hearts were removed, washed in a solution of Heparin and CBFHH (Calcium and Bicarbonate Free Hanks and Hepes) buffer, and dissolved in Trypsin. Once the fibroblasts and myocytes were isolated in solution, the mixture was pre-plated. Pre-plating allowed the fibroblasts to adhere to the plate while the myocytes remained in solution. The solution containing the myocytes was then removed and ready to be used. Cells were plated in growth media consisting of 500mL Minimum Essential Media (Life Technologies, 11575), 1.5mL 10mM BrdU (Sigma, B5002-1), 1.0mL PB12 (1:1 mixture of Penicillin G (Sigma, P7794-10MU) and Vitamin B-12 (Sigma, V6629-250mG)), and 26.5mL calf serum. On the second day of culture, cells were washed and the media was replaced with media containing 500mL Minimum Essential Media, 10mL 1M HEPES stock (Fisher, 1688449), 1mL PB12, 10ug/mL Insulin, 10ug/mL Transferrin, 0.1% by weight BSA, 30mM BrdU, and 10% fetal bovine serum.

2.4 RNA Isolation and Nanodrop Verification

After culturing cells for four days on our gels, their RNA was isolated using the procedure for the QuickRNA Microprep kit from Zymo Research (ZRC178208). Standard protocol was followed as directed by the kit for adherent cells. To lyse the cells, 300µL of lysis buffer was added to empty untreated TCPS wells. Gels were rubbed against the bottom of the dish to loosen the cells. For TCPS samples, 300µL of lysis buffer was added to every other well (600µL per 4 TCPS samples) and a cell scraper was used. The RNA in lysis buffer was added to QiaShredder columns and centrifuged for
120 seconds to remove gel debris. All centrifugation was done at 12,000xg in an EIC Micromax RF Refrigerated Microcentrifuge (rotor 851). The liquid was then transferred to RNase-free tubes and one volume of ethanol was added and the solution was vortexed. The mixture was transferred to a spin column with a collection tube and centrifuged for 30 seconds. An in-column DNase treatment was performed to remove trace DNA. After being washed with buffer, RNase/DNase-free water was added to the column to elute the RNA.

A NanoDrop 1000 spectrophotometer was then used to analyze the RNA concentration of the liquid product obtained from RNA isolation. 1.3-1.5μL of sample was pipetted onto the instrument. The instrument displayed protein concentrations and contamination ratios. Contamination ratios were calculated as the ratio of absorbance at 260nm and 280nm; a ratio of ~2.0 was accepted as pure for RNA. If the ratio was lower, contaminants such as other proteins or phenol may have been present. (ThermoScientific NanoDrop 1000 Technical Bulletin) Overall, the concentration of our RNA was necessary for knowing how to properly dilute the sample for qPCR.

2.5 cDNA Synthesis (Super Script III First-Strand Synthesis protocol)

Standard protocol was followed according to the SuperScript III First-Strand Synthesis for RT-PCR kit (Invitrogen, 18080-051). The general process included combining the RNA we isolated with random hexamers, buffer, MgCl₂, dNTPs, RNase OUT, and the SuperScript III reverse transcriptase. The random hexamers acted as non-specific primers for the reverse transcriptase to begin elongation of the cDNA complementary to the RNA. The samples were then placed in a thermal cycler that
regulated the synthesis process. Because we used random hexamers instead of specific gene primers, the specificity for the target genes arose through the use of specific gene primers during the qPCR process. The overall purpose of cDNA synthesis was to create DNA that was complementary to the isolated RNA to be used in qPCR. DNA is more stable than RNA and is thus preferentially used for quantitative analysis.

2.6 Quantitative Polymerase Chain Reaction (qPCR)

A master mix containing SYBR Green, mH2O, and forward and reverse primers was added to the cDNA to initiate the polymerase reaction. qPCR was done according to the instructions from the SYBR Green mastermix. Nine genes were analyzed: NPPA (ANF), MYH6 (αMHC), MYH7 (βMHC), CACNA1C (CAV 1.2), COL1A1, MMP2, CCN2 (CTGF), ATP2A2, and ACTA1. 18S, GAPDH, and RPL30 were used as reference genes, though, ultimately; RPL30 was more appropriate for our data analysis. All primers can be found in the supplementary materials section.

2.7 Staining NRVMs

We took both brightfield and fluorescence images of NRVMs. ActinGreen (Life Technologies, R37110) was used to stain for filamentous actin in the sarcomeres, DAPI (Life Technologies, D1306) was used to stain the cells’ nuclei, and CellTracker Red (CTR) (Life Technologies, C34552) was used to stain the cytoplasm. After washing cells in PBS, they were stained with CTR that was reconstituted with DMSO (7.3μL DMSO to 50μg CTR) and diluted to a 1:5000 concentration with media (media was serum free media with 10% FBS added). The cells were then washed with PBS and fixed with a 4%
paraformaldehyde solution. Fixing serves to preserve the cells and prevent them from undergoing decay or proceeding with biological processes that may affect the results. Cell membranes were then permeabilized with a 0.1% Triton X-100 solution. Incubation with ActinGreen and DAPI according to the manufacturers’ instructions followed, with PBS rinses in between. Cells were then stored in PBS at 4°C.

2.8 Imaging

Brightfield and fluorescence images of the CTR, ActinGreen, and DAPI channels were taken on a Zeiss 710 or Nikon A1R confocal microscope. These images were used as a means of visualizing the sarcomeres, nucleus, and overall cell directionality in relation to the gel pattern orientation.

2.9 Image Analysis

Matlab code was written for the analysis of f-actin alignment and orientation. Once pictures were taken with the microscope, square sections of the images were cropped from the original pictures using FIJI (http://imagej.nih.gov/ij/) software. The square images were intended to have well-defined sarcomeres so that Matlab could easily identify them. Once in Matlab, the software highlighted the brightest lines in the picture, which should have been sarcomeres. Based on a formula, it then determined at what angle each filament/line was extending. Then, it calculated the average angle of the sarcomeres in each square picture. Using another algorithm, the software output the value for the alignment index (AI) and the dispersion for each picture. The alignment index and dispersion are two ways of measuring how aligned the cells are. The higher
the value of the AI, the more aligned the cells should be. [19] Dispersion, on the other hand, is a different tool that we used to measure how randomized the cell directionality was. This value ranges from 0 to 1, 1 being that all of the cells/filaments are pointing in different directions. With this information we can determine how effective our gels and patterns are at directing the orientation of cell growth. Data was then compiled and graphed using both GraphPad and Excel software.

2.10 Statistical Analysis

Data are presented as mean ± standard error of the mean with nine experimental replicates across three biological isolations. Data were compared using a 2-way ANOVA followed by post-hoc pairwise comparisons as well as multiple linear regressions to assess the influence of both the pattern and modulus. Significance was established for $p \leq 0.05$.

3. Results

3.1 Cell Alignment

The purpose of this research was, in part, to create a photodegradable hydrogel system that would allow cells cultured on it to exhibit growth behaviors similar to those seen in vivo. One of the major aspects of this is the orientation of the cells. In the heart, cells are highly aligned. On TCPS though, cells have no directional cues and are free to spread out in all directions. By patterning the gels, we were able to add directional cues to the gel in hopes of encouraging them to align with a more consistent and in vivo-like orientation.
Figure 4 shows the results of our efforts. We used Matlab to analyze the pictures taken of cells stained with ActinGreen and the alignment index was calculated for each image. Samples of the pictures can be seen in Figure 5. For three cell isolations, each gel type’s index was averaged. A higher index value correlates to more f-actin alignment within the cells. A direct correlation was assumed between the degree of cell alignment and the alignment of f-actin. The alignment index for TCPS was used as a reference point. Both smooth gel moduli seemed to yield about the same amount of alignment as the TCPS. As the aspect ratio of the pattern increased though, so did the alignment index. The stiffer gels had higher indices than their respective 10kPa counterparts. Ultimately, the 35kPa gel with the 5µm channels had the highest alignment index. For the results of the 2, 5, and 10µm channels, please see the supplementary material, as these data were less conclusive.

![Mean Alignment Index vs. Channel Width and Modulus](image)

Mean alignment index (Al) for rectangular gel patterns in order of increasing aspect ratio. All indices are normalizes to TCPS. N=9 Arrows indicate that all patterns are notably different compared to the infx5µm pattern. The asterisks indicate patterns where the moduli have notably different alignment indices.
<table>
<thead>
<tr>
<th>Pressure</th>
<th>Magnification</th>
<th>ActinGreen and DAPI</th>
<th>Bright-field</th>
</tr>
</thead>
<tbody>
<tr>
<td>10kpa</td>
<td>5x5x5 µm</td>
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<tr>
<td></td>
<td>10x5x5 µm</td>
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<td>20x5x5 µm</td>
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</tbody>
</table>
Figure 5) A summary of the ActinGreen, DAPI, and Bright-field channels for the 10kpa and 35kpa gels. F-actin alignment can be seen along the direction of many of the gel patterns. TCPS is shown last as a reference exhibiting disorganized sarcomere orientation. All scale bars are 10µm.
3.2 Gene Expression

Figure 6a-j show the gene expression levels for the nine genes investigated on patterned gels with increasing aspect ratios. The aspect ratios are calculated by dividing the pattern’s length by its width (i.e. a 40µm x 5µm rectangular pattern would have the aspect ratio of 8). The expression levels of these genes were normalize to the expression of the RPL30 reference gene and then normalized to expression levels of cells cultured on TCPS. The statistical significance of the patterns seen was analyzed using an R script developed by William Wan. Not all genes show significant differences with respect to TCPS.

**Genes With Significant Trends**

Gel pattern, modulus, or a combination of both, significantly (p≤0.05) influenced the expression levels of some genes. MYH6 (αMHC) expression had consistently lower expression levels in cells plated on 35kPa gels compared to the 10kPa gels with the same pattern. NPPA (ANF) expression showed a significant correlation with the aspect ratio of the pattern. As the aspect ratio increased, so did expression. As expected, the highest expression was seen on the gels without any patterning, even though the 5x5µm (aspect ratio = 1) pattern is thought to act like a smooth surface because the cells are big enough to sit on top of it. CCN2 (CTFG) showed a similar trend, but with an even stronger correlation to aspect ratio and modulus. ACTA1 expression was the last gene to show a significant dependence on aspect ratio, as its expression went up with increased pattern length. It is also worth noting that the modulus may play a significant role as well, though its p value was slightly higher than our threshold.
**Genes Without Significant Trends**

Though many genes had p values of more than 0.05 for their dependence on pattern size or modulus, some still exhibited general trends. For example, ACTA1, COL1A1 and MYH7 expression levels were higher on the 35kPa gels with patterns. The smooth gels differed less in their expression between the two moduli, which could be a contributing factor to the higher p values for those genes. None of the genes analyzed seemed to have a trend that was dependent on the combined effects of the pattern size as well as the modulus, as evidenced by the high “Interaction” p values.
## MYH6 Expression

![MYH6 Expression Graph](image1.png)

- **a)** MYH6 (αMHC) gene expression
  - Significance: Aspect Ratio ($p=0.176$), Modulus ($p=0.019$)*, Interaction ($p=0.863$)

## MYH7 Expression

![MYH7 Expression Graph](image2.png)

- **b)** MYH7 (βMHC) gene expression
  - Significance: Aspect Ratio ($p=0.080$), Modulus ($p=0.152$), Interaction ($p=0.875$)

## MYH6 : MYH7 Expression Ratio

![MYH6 : MYH7 Expression Ratio Graph](image3.png)

- **c)** MYH6/MYH7 gene expression ratio
  - Significance: Aspect Ratio ($p=0.233$), Modulus ($p=0.752$), Interaction ($p=0.974$)
  - A horizontal line showing the MYH6/MYH7 ratio on TCPS is shown ($y=1$).

## NPPA Expression

![NPPA Expression Graph](image4.png)

- **d)** NPPA (ANF) gene expression
  - Significance: Aspect Ratio ($p=0.009$)*, Modulus ($p=0.284$), Interaction ($p=0.905$)

## COL1A1 Expression

![COL1A1 Expression Graph](image5.png)

- **e)** COL1A1 gene expression
  - Significance: Aspect Ratio ($p=0.168$), Modulus ($p=0.111$), Interaction ($p=0.531$)

## CCN2 Expression

![CCN2 Expression Graph](image6.png)

- **f)** CCN2 (CTGF) gene expression
  - Significance: Aspect Ratio ($p=0.002$)*, Modulus ($p=0.026$)*, Interaction ($p=0.919$)
g) ACTA1 gene expression
Significance: Aspect Ratio (p=0.0002)*, Modulus (p=0.056)*, Interaction (p=0.973)

h) ATP2A2 gene expression
Significance: Aspect Ratio (p=0.973), Modulus (p=0.752), Interaction (p=0.974)

i) CACNA1C (CaV 1.2) gene expression
Significance: Aspect Ratio (p=0.754), Modulus (p=0.937), Interaction (p=0.981)

j) MMP2 gene expression
Significance: Aspect Ratio (p=0.298), Modulus = (0.331), Interaction (p=0.601)

Figure 6)
Gene expression analysis for all genes analyzed. For all graphs, the X-axis is the aspect ratio of the gel pattern (ranging from a 5µm x 5µm square being 1 to a gel-length 5µm channel being infinitely long). The Y-axis is the fold change for the gene expression relative to its expression level in cells cultured on TCPS. As all expression levels have been normalized to TCPS, TCPS=1 on the Y-axis. Lines at the top of the graphs indicate that there was a significant difference between the samples at each end of the line. Significance was defined as p ≤ 0.05 and significant values are marked with an asterisk in the captions. All p values were calculated based on a regression analysis.
4. Discussion

4.1 Cell Alignment

All data were collected and averaged from several replicates from three independent cell preparations. Figure 4 is a graphical representation of the cell alignment data. For the smooth surfaces, modulus does not seem to have much of an affect on the alignment, as both 35kPa and 10kPa have the same alignment index as TCPS (≥1). As the channel size (aspect ratio) increases, the alignment increases as well. Additionally, the 35kPa gels allow for more alignment than the 10kPa gels.

With these data, we can make a few observations. The first observation that can be made is that, the longer the channel size, the more directional cues the cells get, which produces more alignment. The second claim we may make is that the 35kPa gels encourage more cell alignment than the 10kPa gels, perhaps due to their rigidity and subsequent decreased ability to be deformed by the cells. Keeping in mind that cells in healthy cardiac tissue in vivo are aligned and those found in diseased tissue are less so, we can claim that, of our gel types, the 35kPa gel with the 5µm channels promotes the alignment of cells that are the most likely representatives of cells in a healthy heart (with respect to alignment only). The other patterned gels seem to encourage healthier growth than TCPS as well. The 5x5µm square patterned gel likely did not produce more aligned cells because its squares were small enough for cells to sit on top of them and “see” a surface with virtually no directional cues. The decrease in alignment compared to TCPS is an area for further investigation. One possible explanation is that, after patterning, the modulus and stiffness of the gel changed in a way that allowed the cells to manipulate and deform the gel more.
4.2 Gene Expression

In our research, nine genes were chosen that were predicted to have either increased or decreased expression as a result of disease. Not all the genes we studied showed such differences. This may be because the expression of these genes does not depend on a change in substrate stiffness (modulus) or cell alignment or that some genes are less sensitive to changes in substrate under these conditions. Another possibility is that the actual protein expression can change in a way that is not directly correlated with mRNA expression.

*MYH6*

From previous literature, the expression of this gene is expected to decrease in heart disease. [20] Though there is no increasing or decreasing trend in expression relative to the increase in pattern aspect ratio, nearly all conditions produced increased expression relative to TCPS. Additionally, expression on 35kpa gels was less than that on the 10kpa gels with the same pattern.

*MYH7*

MYH7 expression was observed to increase in cases of cardiac stress [21], and this finding is consistent with the heightened expression seen in smooth gels and stiffer gels. The cause for the increase in expression for gels with longer aspect ratios is unclear though.
**MYH6/MYH7 Ratio**

The purpose of calculating this ratio is to allow us to more easily measure the overall cell characteristics if and when each gene is changing in a way that is counterintuitive. In rodents, MYH6 decreases in disease while MYH7 increases; thus, a decreased 6:7 ratio should reflect cells cultured on substrates that represent more diseased environments. TCPS has a lower ratio than the majority of our engineered hydrogel environments; thus, our hydrogel substrates seem to represent a healthier microenvironment.

**NPPA (ANF)**

As a gene that has increased expression during increased blood pressure or cardiac stress, ANF expression has been shown to increase in disease [5]. All of our gels, regardless of modulus or pattern, produced cells that had decreased expression levels of NPPA compared to those on TCPS. Additionally, with the exception of the smooth ones, the 35kPa gels have a higher expression compared to the 10kPa gels, but still less than TCPS. This makes sense because diseased tissue is most often stiffer than healthy tissue, and would thus indicate that the 35kPa gels may mimic diseased characteristics in NRVMs compared to 10kpa gels (with respect to gene expression). However, it must be noted that, as the aspect ratio of the pattern increased (and thus, the alignment of the cells), the expression increased as well.
**COL1A1**

As a result of increased scar tissue in cardiomyopathy, this gene is expected to increase in diseased environments. This idea is supported by evidence of lower expression for cells cultured on patterned 10kPa gels. However, the data for lower expression are not significant, as can be seen by the larger error bars, and warrant further investigation before substantial conclusions can be drawn.

**CCN2 (CTGF)**

According to previous evidence, the expression of the CCN2 gene is upregulated in various forms of heart disease. [14] The fact that all gel conditions produced cells with expression levels lower than those on TCPS indicates that the gel system may provide a healthier environment for the cells. Additionally, we see that the stiffer gels have increased expression relative to the softer ones and that the gels without patterns also have increased expression. All of these factors support the claim that gels, because of their substantially softer modulus and growth-directing patterning, they may be better than TCPS at cultivating healthy NRVMs.

**ACTA1**

Being a gene that is a marker of heart disease and cardiac growth, ACTA1 expression levels are expected to increase in less healthy cell culture environments. As an indication that our gels provide a healthier environment for NRVMs, we see lower expression from cells on the gel substrates compared to those on TCPS. With the exception of the smooth gel, the 35kPa gels yield higher gene expression than the 10kPa
and consistently increase as the aspect ratio of the gel increases as well. According to our statistical analysis, the pattern plays a significant role in changing the gene expression, and so too does the modulus, but to a lesser extent.

\textit{ATP2A2}

Although the specific effects of the pattern and modulus of the individual gels are indeterminable at present, all gels showed an increase in gene expression relative to TCPS. This, again, supports the claim that our gel systems are healthier overall than TCPS because expression of ATP2A2 decreases in instances of disease.

\textit{CACNA1C (CaV 1.2)}

Multiple investigations in the past have come to different conclusions concerning the expression levels of Cav1.2 as a result of cardiac disease. Cav1.2 has been seen to increase in exercised-induced cardiac remodeling [22], but has also been shown to not change in end-stage heart failure [23]. Thus, conclusions are still hard to draw from our data. Two things are clear though: neither the gel’s modulus nor its pattern plays a significant role in the alteration of expression, but also that our gels have an effect on the expression because all gels exhibit increased expression relative to TCPS.

\textit{MMP2}

Expression level changes for this gene in cardiac disease are inconclusive, as there are a variety of different diseases of the heart and each may influence the gene differently. There is literature that supports both its increase and decrease. MMP2 may
increase as a result of matrix remodeling post-infarction, but may decrease as a result of cardiac fibrosis [24]. With conflicting information and extensive uncertainty, conclusions cannot be drawn about our gel system and how it affects MMP2 at this time. However, it is worth noting that the gene’s expression is increased relative to TCPS on all gels.

5. Conclusions

The photodegradable PEG hydrogel system that we have developed creates a different environment for the culture of NRVMs that affects both their alignment and their gene expression levels. Patterned gels produce cells with increased alignment and cells become more aligned as the aspect ratio of the patterns increase. Additionally, the stiffer gels are more effective at aligning cells than the softer ones. In terms of alignment behavior, the gel system is successful in promoting healthy morphological characteristics in cardiomyocytes.

Due to the high amount of variation in some of the genes studied, the effect of the gels on gene expression in NRVMs is less conclusive. However, many of the genes showed the predicted expression changes, indicating that gels, as a whole, are a healthier substrate for cells than TCPS. The effect of the patterns themselves is less clear and warrants further investigation. Ultimately, to further confirm that these gels are more characteristic of healthy or diseased cardiac tissue than TCPS, other genes with known behavioral changes should be studied. Further experiments should also be done comparing the 10kPa gels to the 35kPa gels to more concretely determine whether a softer gel is necessarily more indicative of a healthy environment. Moreover, attention
should be given to how stiffer substrates, when patterns are cut into them, act relative to
the softer substrates. Modulus and stiffness are two different aspects of these gels that
should be considered separately, as they are two different things. While modulus
(measured by the Young’s Modulus) is a measure of density of the PEG crosslinks and is
dependent on the type of material we are using, stiffness is dependent on the extent to
which the cell can deform the gel. When 35kPa gels are irradiated with UV light,
crosslinks within the gel network are broken, and the decrease in crosslink density results
in a decrease in modulus. When an area is exposed to UV light for very long periods of
time or very high intensities, all of the crosslinks in the area can be broken, resulting in
physical erosion of the gel surface. Eroding the gel surface through a photomask lets us
create different topographical patterns. For this reason, 35kPa gels, when irradiated, may
be of stiffness similar to that of the 10kPa gels, depending on their extent of topological
modification. Areas of the pattern that have not been irradiated maintain their original
modulus, but because the overall gel may become more easily deformed by cell
contraction, the stiffness may decrease.

In conclusion, our gel systems should be considered a valuable tool in the study of
cardiac disease. Traditionally, studies in this subject (and many others) have not
expanded much beyond culturing cells on TCPS. Though TCPS has its benefits, it falls
short of exhibiting many characteristics of the cardiac environment in vivo. Because our
gels have degrees of stiffness more comparable to that of heart tissue and have
topographic directional cues, they are more appropriate for these types of studies. With
these gel platforms, we can investigate hypotheses that involve questions about platform
moduli, substrate, topology, and cell shape that cannot be otherwise investigated on
TCPS alone. Eventually, these gels may be the new standard for culturing NRVMs in attempts to more accurately research cardiac disease progression and characteristics.

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Supplementary Materials

Cell Aspect Ratio vs. Pattern Aspect Ratio

Data courtesy of Ciera N. Dolechek

S1) Cell aspect ratio as it varies on different patterns and moduli. Everything to the left of the arrows is significantly different from the infinitely long pattern.
S2) Alignment index of f-actin fibers in cells plated on gels with 2, 5, and 10 µm wide channels. The data are normalized to the alignment index of cells on TCPS (TCPS = 1). Lines connect data that are significantly different (levels vary noticeably) and the stars indicate a pattern that has a significant difference in alignment between the two moduli. These data are less conclusive than the data comparing the aspect ratios of the rectangular patterns.
<table>
<thead>
<tr>
<th>MYH6 Expression</th>
<th>MYH7 Expression</th>
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<tbody>
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<td><img src="image1" alt="Graph" /></td>
<td><img src="image2" alt="Graph" /></td>
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- **a)** MYH6 gene expression
- **b)** MYH7 gene expression

<table>
<thead>
<tr>
<th>MYH6:MYH7 Expression</th>
<th>NPPA Expression</th>
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<tr>
<td><img src="image3" alt="Graph" /></td>
<td><img src="image4" alt="Graph" /></td>
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- **c)** MYH6:MYH7 gene expression ratio
- **d)** NPPA (ANF) gene expression

<table>
<thead>
<tr>
<th>COL1A1 Expression</th>
<th>CCN2 Expression</th>
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<tr>
<td><img src="image5" alt="Graph" /></td>
<td><img src="image6" alt="Graph" /></td>
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- **e)** COL1A1 gene expression
- **f)** CCN2 (CTGF) gene expression
S3) Gene expression levels of all nine genes studied on the 10kPa and 35kPa gels with channeled (infinite aspect ratio) patterns. All expression levels have been normalized to the expression level of TCPS. The X-axis lists the types of gel patterns (channels and smooth) and the Y-axis measures the fold change of the gene expression level compared to TCPS. These data are less conclusive than those previously shown comparing the gene expression levels on patterns with varying aspect ratios. Statistical analysis has not yet been performed on the above data.
**Gene Primers** (custom synthesized by Invitrogen)

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<th>Reverse</th>
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