TENDON CELL BEHAVIOR AND REGENERATION

Student Author

Anusorn Mudla is a senior majoring in cellular, molecular, and developmental biology and minoring in chemistry. He has been working in molecular biology in different research labs since freshman year. He started this research under an HHMI summer internship and continued under the DURi internship. Mudla was awarded a scholarship from the Royal Thai Government to study biology in the United States, which fully funded him until his doctoral degree. After graduation, he will continue his passion for research and science with a PhD program in molecular biology at UC San Diego.

Mentor

Sarah Calve is an assistant professor in biomedical engineering and biological sciences. Sarah earned a MS in molecular, cellular and developmental biology and a PhD in macromolecular science and engineering at the University of Michigan. While a postdoctoral fellow at Northwestern's Children's Memorial Hospital, she studied newt limb regeneration. Her research is focused on characterizing how the biomechanical environment regulates tissue assembly during development, wound repair, and regeneration.

Abstract

Tendon injuries have been a challenge for regenerative medicine as current treatments rely mainly on artificial polymers and/or extracellular matrix (ECM), which only mimic the architecture of mature tissue but do not promote regeneration. Moreover, due to the limitation in the number of tendons available in our body, transplantation is restricted. As a result, growing tendons from patients in vitro and transplantation is an appropriate option. Several studies have shown that ECM composition dramatically changes during tissue development, repair, and regeneration. However, the mechanism of ECM action on proliferation and inducing assembly of a tendon is unknown. This study focuses on the influences of ECM and substrate stiffness on tendon proliferation and migration, both behaviorally and molecularly. Mouse Achilles tendons were harvested and grown to obtain primary tendons cells. Cells were plated onto polydimethylsiloxane (PDMS) with the stiffness of 2, 15, 35, and 100 kPa, and coated with different ECMs. We quantified the percent proliferation using EdU incorporation and the migration using time-lapse imaging techniques. RNAs were isolated and performed quantitative polymerase chain reaction (qPCR) for molecular studies. The preliminary results with two-way analysis of variance (ANOVA) ($N = 24$) showed that ECM coating had significant impacts on proliferation ($p < 0.0005$) and migration ($p < 0.0005$), whereas substrate stiffness only affected proliferation ($p < 0.004$) but not migration ($p = 0.540$). The molecular study suggested that ECM and stiffness had no effect on tenascin-C and Sox9 gene expression. We are currently working on repeating the migration and proliferation experiment as well as conducting a qPCR analysis of gene expression of other genes. We believe that by understanding the mechanism of ECM in cell signaling and regulation, we will be able to discover how nature assembles tissue in vivo. This knowledge will be applicable to tissue regeneration and repair, and potentially lead to self-grown tendon transplant.

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Keywords

extracellular matrix, substrate stiffness, tendon injury, tendon cell behavior, tissue engineering, tendon repair, self-grown tendon transplant, time-lapse imaging, tendon regeneration, EdU staining

TENDON CELL BEHAVIOR AND REGENERATION:

Effects of Extracellular Matrix and Substrate Stiffness

Anusorn Mudla, Cellular, Molecular, and Developmental Biology

INTRODUCTION

Tendons are the tough fibrous tissues that connect muscle to bone. For example, the Achilles tendon connects the calf muscle to the heel bone. Tendon injury (tendinopathy) is common and frequently occurs through degenerative diseases or by physical strain, such as in sports. There are approximately 33 million musculoskeletal injuries annually in the United States, and tendons contribute to almost 50% of these injuries (Butler, 1999). In many cases, the tendon is damaged beyond repair and partial or whole replacement is necessary. The current treatments rely mainly on scaffolds, which are predominantly made of artificial polymers or decellularized ECM that mimic but do not promote regeneration. Furthermore, current synthetic replacements, including Dacron grafts, carbon fibers, and Silastic sheets, are unable to adequately restore function for the long term because of their inherent mechanical incompatibility, as well as their tendency to degrade (Iannace, Sabatini, Ambrosio, & Nicolais, 1995). The ideal solution to tendinopathy would be autologous tendon transplantation. However, the availability of viable autograft tissue is limited. The engineering of a functional tendon in vitro would be a potential and appropriate approach to this critical problem.

Principle of Tissue Engineering

Tissue engineering uses the combination of cells, engineering materials, and suitable biochemical and physiochemical factors to improve or replace biological functions. The basic principle of tissue engineering (as shown in the Figure 1) is to induce the body to

regenerate tissue by taking advantage of exogenous stimuli. However, due to the complexity of the human system, scientists have attempted to isolate tissues from patients and grow them in vitro. The critical challenge is how to induce proliferation of cells and how to guide cells to assemble to the original tissue before transplanting back to the patients. As a result, scientists need to understand how nature regulates the process of regeneration in the body so we can replicate that in vitro. More precisely, we need to understand how ECM and stiffness influence the assembly of tendon cells so that we can manipulate and employ this knowledge in generating functional tendons in vitro.

Figure 1. Schematic diagram of the basic principles of tissue engineering processes.

Role of Extracellular Matrix During Regeneration and Repair

In humans, scars are usually developed at the site of injuries and prevent regeneration. However, vertebrates such as the newt and other urodele amphibians have retained the ability to completely regenerate injured tissues and lost appendages without scar formation. Moreover, during regeneration, it is thought that multipotent progenitor cells are recruited from differentiated tissues close to the wound site. These stem-like cells give rise to a regenerative blastema that rebuilt tissues complete with muscles, bones, and nerves (Carlson, 1970). However, due to the complexity of the *in vitro* environment, it is unknown how this process is regulated.

A previous study done by Calve, Odelberg, and Simon (2010) demonstrated that components of a dynamic matrix—hyaluronic acid (HA), tenascin-C (TN-C), and fibronectin (FN)—differentially direct cellular behaviors, including DNA synthesis, migration, myotube fragmentation, and myoblast fusion during limb regeneration in a newt. Figure 2 clearly demonstrates the upregulation of TN-C and HA in the regenerating limb in comparison to the intact limb (Calve & Simon, 2012). In addition, another study has reported that HA, which is one component in the ECM complex, is significantly upregulated during muscle hypertrophy. Figure 3 shows the upregulation of HA (red) and TN-C (green) in the Achilles tenectomy mice (Calve & Simon, 2012). Based on these results, we believe that ECM could potentially be a key component in promoting tendon regeneration in mammals by influencing the behavior of tendon cells.

Similar to ECM, substrate stiffness has been reported to regulate cell behavior. A study by Leipzig and Shoichet (2009) demonstrated the importance of substrate stiffness to the proliferation and differentiation of adult neural stem/progenitor cells. This study highlighted the importance of mechanical properties to the success of scaffolds designed to engineer central nervous system tissue (Leipzig & Shoichet, 2009). It has been observed that increasing substrate stiffness increased collective cell migration speed, persistence, and directionality as well as the coordination of cell movements in an epithelial wound-healing assay (Ng, Besser, Danuser, & Brugge, 2012). Furthermore, substrate stiffness was found to influence differentiation of adult neural stem cells (aNSC). aNSCs greatly favored neuronal differentiation on softer gels (~100-500 Pa), whereas harder gels (~1-10 kPa) promoted glial cultures (Saha et al., 2008). Despite the variety of the stiffness optima for different kinds of adherent cells, it is generally true that cell proliferation and differentiation increase with the stiffness of the matrix (Wells, 2008). Nevertheless, there

Figure 2. The transitional matrix infiltrates the satellite cell niche during regeneration in newts. Courtesy of Calve & Simon, 2012.

Figure 3. Hyaluronic acid (HA) and tenascin-C (TN-C) are upregulated during compensatory hypertrophy in the mouse plantaris muscle. Courtesy of Calve, Issac, Gumucio, & Mendias, 2012.

is currently no study on influences of substrate stiffness on mouse primary tendon cell behaviors.

Due to the effects of ECM and substrate stiffness on cell behaviors, the combination of those two factors should influence cell behavior. Indeed, a study by Calve and Simon (2012) demonstrated the effect of stiffness and ECM during limb regeneration in newts. They proposed a model of cooperative biochemical and mechanical

function of the regenerative transitional matrix on cell behavior. In Figure 4, during the regeneration, a stiffness gradient is established ranging from the more proximal, differentiated skeletal muscle (red) to the distal, less stiff muscle with highly expressed TN-C (green). The subfigure on the bottom right illustrates the highly packed intact myofibers, which are surrounded by a rigid ECM network (orange). This environment promotes satellite cells (red nuclei) to fuse with neighboring muscle cells. In contrast, the subfigure on the top right illustrates that the less stiff and less dense matrix environment enhances migration and fragmentation of the myofibers (Calve & Simon, 2012). As muscle cells and tendon cells develop in close proximity within similar environments, it is interesting to study how similar or different tendon cells will respond to ECM and stiffness.

Previous Unsuccessful Tendon Constructs

An attempt to engineer functional tendons has been previously made, but they did not completely resemble the properties of mature functional tendons. Instead, they displayed an ultrastructural morphology similar to those of neonatal rat Achilles tendons. This finding suggested that under the right conditions, adult tendon fibroblasts rebuild tendon morphology by recapitulating the embryonic state (Calve et al., 2004). However, in order to fully generate functional tendons in vitro for clinical use, we need to examine the exact role of mechanics and biochemistry in the assembly and organization of tendons. Therefore, by studying how ECM and stiffness influence the behavior of tendon cells in vitro, we would potentially promote tendon cells to self-assemble in the way the nature does in vivo.

MATERIALS AND METHODS

Acquisition of Primary Tendon Cells

Achilles tendons were harvested from newly weaned mice (about 21 days of age). The tendon tissues were first kept in DPBS solution while removing any remaining muscle tissue. Tendons were then sterilized by briefly washing with 70% ethanol. Achilles tendon cells were dissociated by placement in 2% Type II collagenase in F-12 medium.

Figure 4. Model of cooperative biochemical and mechanical function of the regenerative transitional matrix on cell behavior. Courtesy of Calve & Simon, 2012.

Figure 5. Formation of 3-D self-assembling tendon construct. Methods: Primary rat Achilles tendon fibroblasts were plated at a density of 200 cells/mm² onto 35-mm dishes coated with 2.0 mg/cm² lamin and fed with high serum medium until the cells became confluent before changing to low serum medium. Adapted from Calve et al., 2004.

The solution was placed in a reciprocal shaking bath at 37°C for 3 hours, with vigorous shaking every 30 minutes to facilitate breakdown of ECM. After the tissue was dissociated, the cells were pelleted by centrifugation at 1,000 rpm for 5 minutes, and the supernatant was removed by aspiration. The cells were resuspended with tendon growth medium (20% fetal bovine serum [FBS] in F-12 medium and 5% antibiotic-penicillin streptomycin), plated onto 35-mm tissue culture dishes, and incubated at 37°C. The tendon cells would gradually dissociate from the tendon fibers that remained undigested. After 5 days, the medium was aspirated and replaced with new fresh medium. The cells were trypsonized and passaged onto new dishes when they were 60% confluent. Otherwise, the medium was changed every 2 days. The primary cells would yield sufficient numbers at passage 4 (P4) or 5 (P5).

Figure 6. Summary diagram of experimental methods.

Preparation of Culture Plates

For the behavioral study, a 48-well cell culture plate was covered with a thin layer of polydimethylsiloxane (PDMS, Sylgard 527, Dow Corning) substrate, silicone-like material (thickness ~3 mm) that has a stiffness of 2, 15, 35, and 100 kPa. The plate was sterilized with 70% ethanol before coating with the extracellular matrixes. The plate was left overnight in a laminar flow hood to allow ethanol to evaporate. Then the wells were washed with DPBS before adding coating solution. With 48-well plates, we were able to duplicate the experiment.

The commercially available extracellular matrixes—fibronectin (FN), gelatin (GL) or type I collagen, tenascin-C (TN-C), and laminin (LM) or type VI collagen—were diluted in the F-12 medium at the

concentration of 1µg/cm², except laminin at 2 µg/cm². Each well of the plate was covered with 0.3 ml of ECM solution. The plate was incubated at 37°C for 24 hours. For molecular study, 12-well culture plates were used with a similar coating method. The ECM solution was aspirated out and washed with DPBS right before plating the cells.

ECM	Company	Host	µg/cm ²
Fibronectin	Millipore	Human plasma	1
Gelatin	Millipore	Porcine	TBD
Laminin	Millipore	Mouse cell line	2
Tenascin-C	Millipore	Human cell line	1

Table 1. ECMs used to coat PDMS.

Proliferation Study–EdU Incorporation

5-ethylnyl-2’-deoxyuridine (EdU) is a thymidine analogue in which a terminal alkyne group replaces the methyl group in the 5 position of the pyrimidine ring. Figure 7a shows the structure of EdU. When cells undergo the cell cycle, EdU is incorporated into DNA during DNA replication. The terminal alkyne group exposed in the major groove of the DNA helix can readily react with an organic azide via the click reaction (Figure 7c). In our study, we measured the presence of EdU incorporation as an indirect measurement of cell proliferation (Salic & Mitchison, 2008).

Tendon cells were plated onto each well at subconfluent density = 5x10³ cells/cm², and EdU was added at the concentration of 50 µM. The cells were cultured at 37°C for 48 hours before staining. In the process of staining, fluorescent molecules (Alexa Fluor [AF-488]; Fig 8b, green) bind to an EdU molecule, which can be used as an indicator for cells that undergo DNA replication, or in other words, cell proliferation. We costained the cells with DAPI, which is a blue fluorescent dye that stained the nucleus of the cells, indicating the total number of cells. We took 5 pictures at 50x from 5 different positions of each well.

We analyzed the pictures using Image-Pro Plus Imaging Software Version 7.0.0.591, developed by Media Cybernetics. The principle of Image-Pro Plus Imaging Software was that cells that were stained with AF-488 and DAPI would appear bright on the images in contrast to a dark background. When we set up the threshold for the brightness to distinguish the background from the cells, the program automatically counted the total number of cells in the image.

Figure 7. Use of EdU to detect cell proliferation.

Figure 8. Outputs of cell counting using Image-Pro Plus—the total number of cells (DAPI) and the number of proliferating cells (AF-488).

Figure 8 shows the quantification of the total number of cells in an image (DAPI) and the number of proliferating cells (AF-488). We combined the number of cells from each position of the same well and used it as the $N = 1$ in the statistical analysis. As a result, we had $N = 2$ for each ECM and stiffness combination, and the total was $N = 24$. The percent proliferation was calculated using the following equation.

$$\text{Percent Proliferation} = \frac{\text{Number of cells proliferated (AF-488)}}{\text{Total number of cells (DAPI)}} \times 100$$

Figure 9. Migratory tracts of tendon cells using manual track option of Image-Pro Plus.

Migration Study: Time-Lapse Imaging

Tendon cells were plated similarly to the proliferation study and incubated at 37°C for 6 hours to allow cells to adhere to the substrate. The plate was then placed on a computer-controlled Leica DMI6000 live-cell microscope, which can maintain temperature at 37°C and concentration at 5% CO₂.

throughout the time-lapse imaging period. We took pictures at 100x from 3 positions of each well every 30 minutes for 24 hours (total = 48 pictures/position). We used Image-Pro Plus Imaging Software to merge 48 pictures in sequence to generate a time-lapse imaging video. We manually tracked the nucleus movement of 4 cells from each position (total = 12 cells per well). The software yielded the total distance of each cell, and we used the average of 12 cells in that well as $N = 1$ in the statistical analysis.

Molecular Study: qPCR Analysis for Gene Expression and Differentiation

To determine how the combination of ECM and stiffness controls gene expression, we performed a quantitative polymerase chain reaction (qPCR) or a real-time PCR targeting for tendon (scleraxis, tenomodulin), bone (runx2, osterix), cartilage (sox9, aggrecan), and adipocyte (LPL, PPAR γ) genes. The list of genes and primers is shown in Table 2. All primers were tested with RNA isolated from each tissue type to confirm the specificity.

Tendon cells were plated onto 12 well plates at the density of 4.5×10^4 cells/cm². The plates were incubated at 37°C for 48 hours. RNA was isolated using a NucleoSpin RNA kit from Macherey-Nagel. We used a Thermo Scientific NanoDrop 2000 Spectrophotometer to quantify the amount of RNA concentration in each sample. We used 200 ng of RNA and reverse-transcribed it into cDNA using Bio-RAD iScript Reverse Transcription Supermix. We performed qPCR using 3 μ g of cDNA and Bio-RAD SsoAdvanced Universal SYBR Green Supermix and operating with Bio-RAD CFX96 real-time C1000 Touch Thermal Cycler.

To avoid difficulty with normalizing target gene expression in overloaded tissues with typical housekeeping genes, we calculated a relative copy number using the linear regression of efficiency method (Rutledge & Stewart,

2008). The current comparative CT method relies on several assumptions, including (1) approximately equal amplification efficiencies between target and reference genes, and (2) a constant amplification efficiency close to 1 in the exponential phase of PCR (Heid, Stevens, Livak, & Williams, 1996) that are not generally true in qPCR reaction. Our analysis, on the other hand, is based upon a four-parameter sigmoidal model that captures the dynamics of amplification efficiency for each individual reaction (Liu & Saint, 2002). We used a custom MATLAB R2012a program developed by Benjamin Seelbinder using the linear regression of efficiency method (Figure 10) to analyze the data.

Statistical Analysis

The IBM SPSS 21.0 statistic software package was used to perform two-way ANOVA, followed by Bonferroni's post-hoc test ($\alpha = 0.05$ and $N = 24$) on the effect of ECM and stiffness.

RESULTS

Preliminary results from the proliferation study are shown in Table 3 and Figure 11. In terms of ECM, tenascin-C yielded the highest percent proliferation (average = 36%) and laminin the lowest (22%). Fibronectin and gelatin resulted in similar percent proliferation (31% and 33%, respectively). In term of stiffness, substrate with 2 kPa stiffness yielded the highest percent proliferation of 35% on average. If we only considered the effect of stiffness alone, we found that the percent proliferation decreased as the stiffness increased (from 35% to 24%).

On average laminin, fibronectin, tenascin-C, and gelatin yielded migratory distances of 533, 498, 425, and 286 μ m, respectively. However, if we only considered the effect of the substrate stiffness, on average the migratory distance was similar across every stiffness given a particular ECM.

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'	bp	Tissue	Source
<i>Scleraxis</i>	AACACCCAGCCCAACAGAT	GGCTCCGTGACTCTTCCAG	169	Tendon	(1)
<i>Temomodulin</i>	TGTACTGGATCAATCCACTCT	GCTCATTCTGGTCAATCCCCT	114	Tendon	(2)
<i>Tenascin-C</i>	GGGGTCTCTGCGTACAGGGC	AGCGCCGGTCTCTGCAGTCTT	165	Tendon	(1)
<i>Collagen 1a2</i>	CCAGCGAAGAACTCATACAGC	GGACACCCCTTCTACGTTGT	105	Tendon/bone	(1)
<i>Runx2</i>	GACTGTGGTTACCGTCATGGC	ACTTGGTTTTTCATAACAGCGGA	84	Bone	(2)
<i>Osterix</i>	AGCGACCACTTGAGCAAACAT	GCGGCTGATTGGCTTCTTCT	121	Bone	(3)
<i>Sox9</i>	AGTACCCGCATCTGCACAAC	ACGAAGGGTCTCTTCTCGCT	88	Cartilage	(2)
<i>Aggrecan</i>	CCCAGGATAAAACCAGGCAG	CGGCCAAGGGTTGTAAATGG	105	Cartilage	(2)
<i>LPL</i>	AAGCTGGTGGGAAATGATGTGG	CCGTTCTGCATACTCAAAGTTAGG	198	Adipocytes	(3)
<i>PPARγ</i>	GGAAGACCACTCGCATTCCTT	GTAATCAGCAACCATTGGGTCA	121	Adipocytes	(2)

Table 2. Primers use for qPCR.

Figure 10. Output from MATLAB custom program used in qPCR analysis.

	Proliferation	Migration
ECM	$p < 0.0005$	$p < 0.0005$
Stiffness	$p = 0.003$	$p = 0.540$
ECM*Stiffness	$p = 0.223$	$p = 0.748$

Table 3. Summary of p-values from two-way ANOVA from behavioral study.

For the molecular study, we only finished performing qPCR for tenascin-C and Sox-9 genes. The results are shown in Figures 14 and 15. The relative fluorescent unit (RFU) data measured from the thermal cycler were converted to relative fluorescent intensity (F0) by the MATLAB program. F0 information from gelatin (denatured type I collagen) coated on 2 kPa stiffness was missing because of the experimental errors. Since two-way ANOVA indicated no significant differences in both ECM and stiffness, we did not proceed to statistical analysis for Bonferroni's post-hoc test at this point.

DISCUSSION

According to the summary of p-values from proliferation and migration (shown in Table 3), we found that ECM significantly influenced the proliferation and migration ($p < 0.0005$). However, the stiffness only significantly affected the

proliferation ($p = 0.003$) but not the migration ($p = 0.540$). The combination of ECM and stiffness neither influenced the proliferation ($p = 0.223$) nor the migration ($p = 0.748$).

Regarding the ECM coating, the Bonferroni's post-hoc test suggested that laminin yielded a significantly lower percent proliferation in comparison to all other ECMs ($p < 0.005$). However, the differences in percent proliferation among fibronectin, collagen, and tenascin-C were not significant ($p > 0.05$). In terms of migration, Bonferroni's post-hoc test suggested that collagen yielded the significantly lowest total migration distance ($p < 0.05$). In addition, there was significant difference between laminin and tenascin-C ($p = 0.039$). Nevertheless, the differences between laminin and fibronectin were not significant ($p > 0.05$), similar to the comparison between fibronectin and tenascin-C ($p = 0.308$). In the case of stiffness, the Bonferroni's post-hoc test suggested that only 100 kPa substrate yields significant difference to other stiffness ($p < 0.05$). The migration result was consistent with the two-way ANOVA, confirming that substrate stiffness had no effect on tendon cell migration.

The two-way ANOVA results from the molecular study of tenascin-C and Sox9 showed that ECM and stiffness did not influence the expression of these genes ($p > 0.05$). The indifference in these two genes might be due to several factors. First, this was the first time we performed this experiment. Therefore, some unknown experimental errors

may have occurred. Second, the cells might not yet respond to ECM and substrate stiffness upon 48 hours of culturing. Third, these two genes might not, in nature, be regulated by ECM and/or stiffness. In addition, the p-values that were higher than 0.05 might be due to a small sample size ($N = 1$ for each condition). Nevertheless, we need to repeat the experiment to obtain consistent data and to have a larger sample size, as well as compare to other gene targets.

Figure 11. Results from the proliferation study.

Figure 12. Fluorescent staining of tendon cells, DAPI (blue) indicates nucleus of cells; AF-488 (green) indicates proliferated cells.

Figure 13. Results from migration study.

Since there was no significant difference in the effect of substrate stiffness on migration of tendon cells, we were concerned about the accuracy of measurement. The problem with manually tracking the process was that we could only track the nuclei of the cells. In fact, the nuclei might not be the center of the cells. In addition, we could not track all cells in the picture at that particular position because some of the cells went out of frame during imaging. Therefore, the cells we chose might not be representative of that condition. Furthermore, the quantity of pictures was varied because of the thickness of PDMS. Currently, we have tried to control the thickness of PDMS to 1 mm, and we are still in the process of testing. To minimize the variation in the cell tracking process, we are trying to obtain tendon cells from transgenic mice in which the tendon cells are GFP+, because this means we can use the imaging software to automatically track all cells in at that position. In this way we can analyze all the cells in each condition more accurately, because the program can track the center of the mass. In our lab now, we already have green myoblasts (muscle cells), which are fluorescently green and can be automatically tracked using Image-Pro Plus Imaging Software. Figure 16 shows the picture of fluorescently green myoblasts which are available in our lab.

Another problem that we recently took into consideration was the effect of high serum concentration. We have been using 20% FBS in tendon growth medium. Due to the structure of each ECM, they might have the ability to bind to growth factors in the serum differently. Therefore, it led to difference in cell behavior. The results we observed could have been altered by the serum effect. To overcome this problem, we are now using Gibco Advanced Medium in which we do not need to add FBS, and we can culture cells more than 48 hours without changing media, while maintaining cell viability.

Figure 14. The relative fluorescent intensity of the tenascin-C gene; the height of the bar indirectly represents the amount of TN-C mRNA in each sample.

Figure 15. The relative fluorescent intensity of the Sox9 gene; the height of the bar indirectly represents the amount of mRNA in each sample.

Figure 16. Primary myoblast cells harvested from Pax3 GFP transgenic mice. These cells were tested to be trackable using Image-Pro Plus.

	Tenascin-C	Sox9
ECM	p = 0.736	p = 0.599
Stiffness	p = 0.274	p = 0.786

Table 4. Summary of p-values from two-way ANOVA Analysis from molecular study.

Lastly, we aim to finish the molecular study by testing gene expression for all different primers. The problem with the molecular study was the amount of RNA, because the NanoDrop machine was not sensitive to low RNA concentration. Therefore, we could not be certain when the amount of RNA was lower than 10 ng/μl. However, in order to obtain large amounts of RNA, we needed to plate cells at high concentration. This could potentially interfere with how the cells behave and express their genes. Therefore, we are optimizing the concentration of cells during plating. In addition, we plan to test the effect of incubation time in culturing the tendon cells before isolated RNA to make sure that cells have time to respond to ECM and stiffness.

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