Determining Alpha-Smooth Muscle Actin Expression in Embryonic and Mesenchymal Stem Cells of Assorted Mammals Seeded in Collagen Scaffolds In Vitro

By

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ABSTRACT

Healing by contraction is responsible for scarring in adults. Embryos heal by regeneration but the mechanism is unknown. Alpha-smooth muscle actin (α-SMA) is the protein responsible for contraction, thus determining if it is present in embryos which heal by regeneration will further our knowledge about the causes of regenerative healing. This thesis experimentally determined the presence of α-SMA in these cell types by the following procedure. Embryonic and mesenchymal stem cells of various species were cultured and seeded into collagen scaffolds. Contractile behavior was determined by measuring the diameter change of the scaffolds over time. Alpha-smooth muscle actin presence was determined by immunohistochemical evaluation.

This study found that while all the cell types displayed alpha-smooth muscle actin presence in monolayer, not every cell type contracted when seeded into the collagen scaffolds designed to mimic the in vivo environment. Specifically, the embryonic stem cells did not contract. Upon staining, the embryonic stem cell seeded scaffolds and several of the mesenchymal stem cell seeded scaffolds, which did contract, did not stain positive for α-SMA. These results imply that the embryonic scaffolds did not generate actin filament bundles, and that several of the mesenchymal stem cell seeded scaffolds were imaged after α-SMA expression in them ceased.

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Table of Contents

1. Introduction

Determining the expression of alpha-smooth muscle actin (α-SMA) in embryonic and adult stem cells is essential for hypothesizing why an embryo heals by regeneration and an adult heals by contraction. α-SMA is a contractile protein that plays a critical role in adult wound healing. α-SMA containing cells, called myofibroblasts, migrate to the defect area and contract to cinch the wound closed. The result of this healing by contraction is a scar. Scar is dysfunctional tissue that can have adverse effects for the animal. It is known that an embryo heals through regeneration, but unknown if embryonic stem cells express α-SMA.

The goal of this project is to determine if embryonic and adult stem cells contract when placed in an environment mimicking the structure of the extracellular matrix, and if their contraction is due to α-SMA. The hypothesis is that if the embryonic stem cells do not express α-SMA their regenerative capabilities could stem from the lack of this contractile protein. On the other hand, if they do express α-SMA there could be other factors at work. The reason for examining stem cells is because all other cell types are derived from stem cells. Thus if stem cells have the capability for α-SMA, then all the cells differentiated down the line will also have the capability.

2. Background

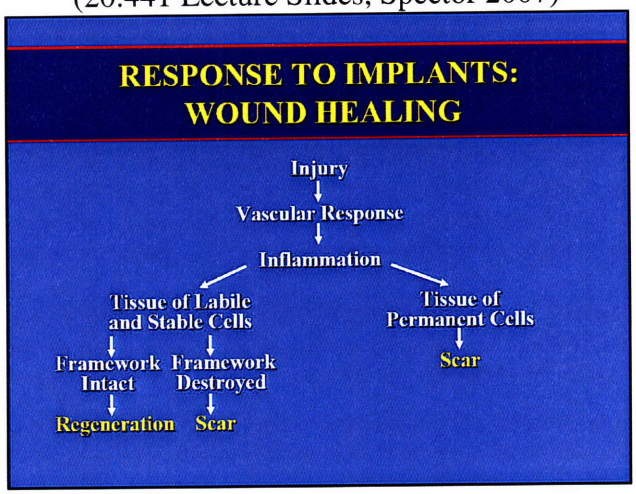
2.1 Organ Structure

Organs are comprised of three tissue layers: epithelia, basement membrane, and stroma. The top two tissue layers, the epithelia and the basement membrane, are capable of regeneration, even in adults. Damage to the stroma, or extracellular matrix, the deepest tissue layer is non-regenerative in adults, but regenerative in embryos.

2.2 Adult Wound Healing

A flow chart for the adult wound healing process is presented in figure 1.

Figure 1: Adult wound healing process
(20.441 Lecture Slides, Spector 2007)



The wound healing process begins with vascularization. In vascularization the wound is clotted and new blood vessels are formed. The cytokines released during vascularization then trigger an inflammatory response where macrophages phagocytose foreign tissues and tissue fragments.

If the injury results in the framework being destroyed this means that the stroma has been damaged. Since the stroma is non-regenerative, a new stroma must be synthesized. The stroma is made of several types of randomly oriented collagen fibers. To synthesize a new stroma, long branching cells called fibroblasts migrate to the defect area and synthesize collagen fibers. A special type of fibroblast called a myofibroblast also migrates into the defect area. Myofibroblasts contain the contractile protein alpha-smooth muscle actin. These cells contract and pinch the wound closed to speed up the recovery process. Although by doing this, myofibroblasts alter the structure of the regenerated stroma. Instead of being randomly oriented, the collagen fibers are aligned along the plane of the wound and directed along the major contraction axis. This is referred to as healing by contraction. The resulting tissue is colloquially known as scar tissue. Scar tissue is structurally different from normal stroma tissue, and as a result is functionally inactive.

If the injury results in the framework being intact this means that the stroma is intact and that the epithelium or basement membrane has been damaged. In this case endothelium cells migrate to the wound area and undergo mitosis. The endothelium cells

also synthesize a new basement membrane. In this scenario, the wound heals by regeneration.

2.3 Embryonic Healing

During the fetal-to-adult transition in nearly all animals, the ability to regenerate degrades, while contraction becomes the major mode of wound closure. Figure 2 shows the transition during a frog's development. The mechanism for fetal regeneration and why it does not continue into adult hood are unknown. It was shown that an adult skin wound healed in a fetal environment still healed by scar formation (Longaker et al). Thus, the hypothesis that the regenerative ability is inherent in the embryonic in vivo environment was proven false. Other studies have examined the role that platelet-derived growth factors, such as, TGFbeta1 play in the wound healing process. TGFbeta1 is a known promoter of myofibroblast activity. One study showed that wounds that healed via regeneration had low levels of TGFbeta1 hypothesizing that the key to regeneration is the impediment of myofibroblast, and thus alpha-smooth muscle actin, activity (O'Kane et al). The aim of this thesis is to discover if embryonic stem cells contain alpha-smooth muscle actin. As previously stated, this protein is responsible for the contraction of myofibroblasts. If embryonic stem cells do not contain this protein it would shed some light on why they do not heal by contraction. If not, it means that there are other forces at work.

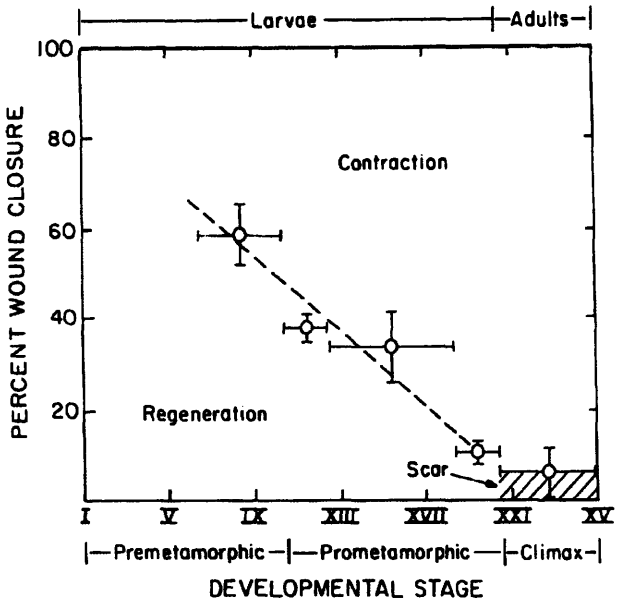


Figure 2: Developmental Wound Healing (20.441 Lecture Slide, Yannas 2007)

3. Experimental Procedure

3.1 Cell Preparation

The following mesenchymal stem cell lines were used in this experiment: pig #1, goat #138, goat #139, goat #140, goat #171, goat #182, goat #316, rat #1, rat #2, rat #3, rat #4, rat #5, rat #6, and rat #7. The following embryonic stem cell lines were used: mouse #1. All cell lines were stored in liquid nitrogen before the experiment. In order to be used for the experiment the cells had to be thawed, cultured, and split until 80% confluency was attained. The following sections will outline the procedures used.

3.1.1 Time Table

The cell preparation time table is described below in table 1.

Table 1: Cell Preparation Time Table

Day	Mouse ESCs	Pig & Rat MSCs	Goat MSCs
1	Thawed		
3	Media Changed		Thawed
6	Split	Thawed	Media Changed
8	Media Changed	Media Changed	Split
10	Split	Split	Media Changed
13	Media Changed	Media Changed	Media Changed
15	Seeded	Seeded	Seeded

The growth of the cell types is described below in table 2.

Table 2: Cell Growth Time Table

Day	Mouse ESCs	Pig & Rat MSCs	Goat MSCs
1	250,000 cells in T-150		
3			250,000 cells in T-150
6	250,000 cells in T-150	250,000 cells in T-150	
8			75,000 cells in 3 layers
10	2 T-150s of 250,000	10e6 cells	

For in-depth cell counts and the cell suspension volumes used in splitting please refer to Appendix B.

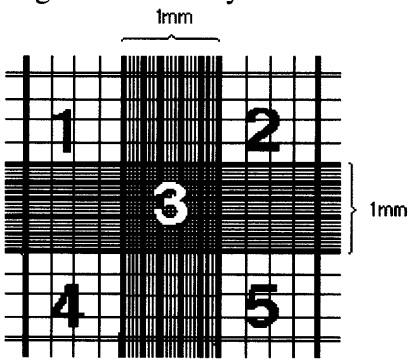
3.1.2 Thawing

The vials were taken out of liquid nitrogen and placed into a 37°C water bath for 40-60 seconds. The defrosted vials were moved into a sterile hood where a drop of expansion media was added to the vial. For the expansion media recipes please refer to Appendix A. After one minute, further expansion media was added on a droplet basis until the vial was full. The solution was then transferred to a 50mL tube, which was spun in the centrifuge for 10 minutes at 1500 RPM and 20°C to obtain a cell pellet. The media was aspirated and the cells were resuspended in 10 mL of expansion media and then counted by the procedure described in section 3.1.3.

3.1.3 Cell Counting

The cells were suspended in 10 mL of expansion media. 100 μL of the cell suspension was collected and diluted with trypan blue with a dilution ratio of 1:2. 15 μL of the diluted sample was mixed and collected in a micropipette tip. The diluted sample was loaded into the sterile hemocytometer. The hemocytometer was placed under a light microscope with the yellow glass filter removed and viewed with the 10x objective lens. Living cells in sections 1,2,3,4, and 5 of the hemocytometer grid, which is depicted in figure 3, were counted.

Figure 3: Hemocytometer Grid



The total cell number was calculated using equation 1.

$$T = \frac{N_C}{N_S} \times D \times 10^4 \times V \quad (1)$$

N_c = # of cells counted, N_s = # of squares counted, D = Dilution factor, V = Volume of media

Using the result from equation 1 and the volume of the cell suspension, the amount of media required to harvest a certain number of cells was ascertained.

3.1.4 Cell Culturing

The number of cells required was placed into a T-150 flask. The flask was then filled with expansion media until the total volume in the flask was 30 mL. The flasks were then placed into an incubator at 37°C and 5.0% CO_2. On Mondays, Wednesdays, and Fridays the media was changed. The media was removed by glass vacuum pipettes. A new pipette was used for each sample from different animals. The flasks were then refilled with 30 mL of fresh media and placed into the incubator.

3.1.5 Splitting

The medium in the flasks was aspirated with a vacuum pipette. The flasks were rinsed with PBS until the bottom of the flask was covered. A Collagenase Type 2 solution was made by placing 75 mg of Collagenase Type 2 into a 50 mL tube and filling the tube with PBS. The Collagenase solution was sterilized by using a vacuum powered 0.22 μm sterile filter. The PBS in the flask was aspirated and 8 mL of the Collagenase solution was added. The flask was then placed into the incubator for 5 minutes. The Collagenase solution was aspirated and placed into a 50 mL tube and 8 mL of trypsin was added to the flask. The flask was again placed into the incubator for 5 minutes. The flask was then placed under a light microscope to verify that the cells were no longer adhering to the flask. 8 mL of expansion media was added to the flask to inactive the trypsin. Using a sterile pipette the complete solution in the flask was transferred into the 50 mL tube containing the Collagenase. The 50 mL tube was centrifuged at 1500 RPM and 20 °C to obtain a cell pellet. The media in the tube was aspirated and the cells were resuspended and counted. After, the cells were centrifuged again and resuspended at the desired seeding density. The cell solution was then transferred to culture flasks and expansion media was added to bring the flasks up to final volume.

3.2 Scaffold and Plate Preparation

The collagen I/III scaffolds were prepared concurrently with the cells to allow immediate seeding once both procedures were complete. The following sections will outline the steps done to prepare the scaffolds and the agarose coated well-plates.

3.2.1 Time Table

The scaffold preparation time table is described below in table 3.

Table 3: Scaffold Preparation Time Table

Day	Procedures
1	Slurry Prepared, Freeze Dried
3	Baked Molds
6	Punched Scaffolds
10	Prewet Scaffolds
13	Agarose Plates Made
15	Crosslinked and Seeded

3.2.2 Slurry Preparation

200 mL HCl solution at 0.001N was prepared in the following way. 50 μL of 6N HCl was added to 3 mL of dH_2O to make 0.1N HCl. 2 mL of 0.1N HCl was added to 198 mL dH_2O to make 200mL of 0.001N HCl. The solution was then placed upon a magnetic stirrer and 6N HCl was added on a droplet basis until the pH of the solution was 3. 1 g of Biogide collagen powder was added to the stirring solution. To keep the pH at 3, 100 μL of 6N HCl was added. This solution was then blended at 4 °C and 15,000 rpm for 90 minutes. To keep the pH at 3, 50 μL of 6N HCl was added. The solution was then blended again in the same way as before. The solution was split between four 50 mL tubes, which were then centrifuged at 1500 rpm for 20 minutes. The solution was then poured in 16 mL plastic molds and the bubbles were removed with a spatula. The molds were then ready for freeze drying.

3.2.3 Freeze Drying

In order to achieve 120 μm pores, the molds were placed in a freeze drying machine with the settings described in table 4.

Table 4: Freeze Drying Cycle

Thermal Treatment			Freeze Condenser Vacuum				Drying Cycle Steps			
T (°C)	t (min)	R/H	Freeze (°C)	t (min)	Condenser (°C)	Vac (mTorr)	T (°C)	t (min)	R/H	Vac (mTorr)
15/-15 /-15	10/30 /240	H/R/H	-15	20	-60	200	-5	1200	H	200

Secondary Drying			
Set Point (°C)	T (°C)	t (min)	Vac (mTorr)
40	20	30	200

3.2.4 Baking

The molds were taken out of the freeze drier and baked at 110 °C for 24 hours.

3.2.5 Punching

Using an 8 mm punch, 169 scaffolds were punched out of the molds.

3.2.6 Pre-wetting

The 8 mm scaffolds were placed in 100% reagent alcohol at a volume of 4-5 mL per scaffold. This solution was placed on the rocker for one day. The scaffolds were then placed in 80% reagent alcohol for 30 minutes on the rocker, and then in 50% reagent alcohol for 30 minutes on the rocker. Afterwards, they were rinsed twice in sterile water. The scaffolds were left in sterile water for 5 days until all the air was removed, which was signified by the fact that the scaffolds were no longer floating in solution.

3.2.7 Cross-linking Collagen-GAG Scaffolds by Carbodiimide Treatment

For 169 scaffolds and a 1:1:5 (EDAC:NHS:COOH) ratio the EDAC and NHS amounts were calculated by equations 2 and 3 respectively.

$$\#\text{Scaffolds} \times \frac{\text{g collagen}}{\text{scaffold}} \times \frac{\text{mol COOH}}{\text{g collagen}} \times \frac{\text{mol EDAC}}{\text{mol COOH}} \times \frac{\text{g EDAC}}{\text{mol EDAC}} = \text{g EDAC} \qquad (2)$$

$$\#\text{Scaffolds} \times \frac{\text{g collagen}}{\text{scaffold}} \times \frac{\text{mol COOH}}{\text{g collagen}} \times \frac{\text{mol NHS}}{\text{mol COOH}} \times \frac{\text{g NHS}}{\text{mol NHS}} = \text{g NHS} \qquad (3)$$

The calculated amounts were 0.0156 g EDAC and 0.0094 g NHS. These amounts were dissolved in 169 mL of dH_2O, which was calculated by the rule of 1 mL of dH_2O per scaffold. This solution was then placed through a sterile filter. The pre-wet scaffolds were then placed into the filtered EDAC/NHS solution for 30 minutes at room temperature. After 30 minutes, the scaffolds were transferred to 50 mL tubes. The scaffolds were then rinsed twice in PBS and placed on the rocker for 1 hour. After 1 hour, the PBS was removed and the scaffolds were rinsed twice with dH_2O. The scaffolds were then stored in dH_2O at 4°C.

3.2.8 Agarose Plate Preparation

4 g of Seaplaque agarose was added to a flask of 100 mL of dH_2O. The opening of the flask was covered with aluminum foil and the flask was placed on a magnetic stirrer. The flask was then autoclaved (water added to autoclave bin) on the setting for Liquid #2. After the autoclave cycle, the door was opened and the solution was allowed to cool to 50-60 °C. Under the sterile hood, the 24-well plates were coated with 1.5 mL of liquid agarose solution per well. The well-plates were then placed in sterile bags and put in the cold room overnight.

3.3 Seeding

The following sections outline the steps done to seed the cells on the scaffolds and place them in the agarose coated well-plates. All the steps were done on day 15.

3.3.1 Scaffold Preparation

One scaffold was placed in each agarose coated well. Care was taken to make sure the scaffolds were completely flat and that the scaffold was not contorted in the well. The excess moisture in the well, brought by the wet scaffolds, was wicked away with filter paper.

3.3.2 Cell Preparation and Seeding

One million cells were to be seeded on each side of a scaffold for a total of two million cells per scaffold. To ensure the best chance of absorption by the scaffold, the

volume of 1 million cells was limited to 20 μL. Since there were 9 scaffolds per sample, 18 million cells in 360 μL was required for each sample. For a safety buffer we used 20 million cells in 400 μL.

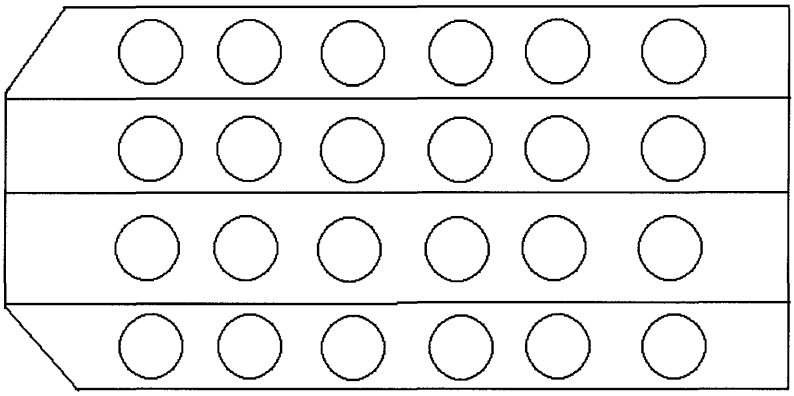
The total number of cells in each sample was determined by the cell counting procedure mentioned in section 3.1.3. Please refer to Appendix B for cell counts and cell suspension volumes. A volume containing 20 million cells was placed in a 15 mL tube. This tube was then centrifuged at 1500 rpm for 10 minutes to obtain a cell pellet. The media from the tube was aspirated and the pellet was resuspended in 400 μL of expansion media. From this solution, 20 μL was micropipetted and placed on one side of the scaffold. After 10 minutes, the scaffold was turned over and another 20 μL was added to the other side. After 10 minutes, 1 mL of media was added. This was done for every sample and scaffold.

Unfortunately, we did not obtain 20 million cells from any rat sample. We decided to seed one scaffold for rat #2 and rat #7, and four scaffolds for rat #4. Please refer to Appendix B for exact cell counts.

3.3.3 Well-Plates

The final assembly of the well plates is as follows. Well-plate #1 contained: 6 samples of goat #171, goat #140, goat #139, and goat #138. Well-plate #2 contained: 6 samples of goat #316 and goat #182, and one sample of rat #2 and rat #7. Well-plate #3 contained: 3 samples of goat #138, goat #139, goat #140, goat #171, goat #182, goat #316, pig #1, and mouse #1 grown in myogenic media. Well-plate #4 contained: 4 samples of rat #4, 6 samples of pig #1 and mouse #1, and 6 control samples which were non cell seeded scaffolds placed in the different types of media. The controls were as follows: goat/pig expansion media, rat expansion media, mouse expansion media, no media, goat/pig myogenic media, and mouse myogenic media. For a graphical depiction of a well-plate please refer to figure 4.

Figure 4: 24 well-plate



3.4 Cells on Scaffold

The cells were to remain on the scaffolds for two weeks. On days 15, 17, 20, 22, 24, 27, and 29 the media was changed and the scaffold contraction was measured. The scaffold contraction was defined as the change in the diameter of the scaffold. To measure the scaffold diameter, the wells containing the scaffolds were placed over a sheet of paper with circles of various diameters printed on it. The scaffolds in the wells were lined up with the circle that was the closest to the diameter of the scaffold and that was defined as the diameter of scaffold. If the scaffold was oval shaped, the circles that were closest to the width and height of the scaffold were written down. The effective diameter was then calculated by equation 6, requiring the results of equations 4 and 5 as inputs.

$$Q = \frac{(\pi \cdot a \cdot b)}{4} \tag{4}$$

$$P = 2 \cdot \pi \cdot \left[\frac{1}{2} \cdot \left(a^2 + b^2\right)\right]^{\frac{1}{2}} \tag{5}$$

$$D = \frac{\left(1.55 \cdot Q^{.625}\right)}{P^{.2}} \tag{6}$$

a = width diameter, b = height diameter

The contraction data is presented in Appendix C, and the graphical representation of the contraction data is presented in the results section.

3.5 Post-Experiment

The goal of the post-experiment methods was to determine if the macroscopically observed contraction was due to the presence of alpha-smooth muscle actin. To determine the presence of α-SMA, the scaffolds would be sectioned and stained with a dye that would turn red in the presence of α-SMA. The stained sections would then be imaged under a microscope.

3.5.1 Tissue Processor and Paraffin Embedding

On day 29, exactly two weeks after beginning the scaffold measurements, the scaffolds were ready to be placed in the tissue processor to be embedded in paraffin. To prepare the scaffolds for the tissue processor the scaffolds had to be placed in paraformaldehyde to kill the cells and effectively "freeze" the cells in the scaffold. The media from all the wells was aspirated and replaced with DBS. The scaffolds were taken out of the well-plates and put into individual tubes containing 4% paraformaldehyde for three hours. After three hours, the scaffolds were taken out of the paraformaldehyde solution and placed in individual cassettes. The cassettes were loaded into the tissue processor for 19 hours to embed the scaffolds in paraffin. The cycle for the tissue processor is described below in table 5.

Table 5: Tissue Processor Cycle

Solution	Time (min)	Temperature (°C)
70% Ethanol	10	Room Temperature
80% Ethanol	90	Room Temperature
95% Ethanol	90	Room Temperature
95% Ethanol	90	Room Temperature
100% Ethanol	90	Room Temperature
100% Ethanol	90	Room Temperature
100% Ethanol	90	Room Temperature
Xylene	90	Room Temperature
Xylene	90	Room Temperature
Xylene	90	Room Temperature
Paraffin	180	58
Paraffin	180	58

On day 31, the scaffolds were removed from the cassettes. The scaffolds were placed on the bottom of a mold and paraffin was dispensed into the mold. The cassette was then placed on top and more paraffin was dispensed. The scaffolds were placed on the bottom to minimize the amount of paraffin that would have to be shaved off before sections could be taken. The cassettes being molded to the paraffin allowed fixation to the microtome. The molds were then placed on a cooling surface for 45 minutes before being placed into a freezer.

3.5.2 Sectioning

In order to section a sample, the sample was taken out of the freezer and removed from the mold. The jaws of the microtome were tightened onto the cassette part of the sample to provide fixation. In order to create the 6 μm samples necessary for staining, the microtome was set to advance 6 μm on each rotation. Four to six slides were created per sample with 3 to 4 sections on each slide. The slides were placed on the slide warmer for 30 minutes before being stored away. The following samples had sections created on days 34 and 36: goat #138-1, goat #139-1, goat #140-1, goat #171-2, goat #182-1, goat #316-1, pig #1-1, mouse #1-1, rat #2-1, rat #4-1, and rat #7-1. In addition to provide positive and negative controls for the stains, goat aorta was also sectioned.

3.6 Staining

The staining process consisted of pretreatment, staining, and counterstaining and mounting. All of the aforementioned steps took place on day 41.

3.6.1 Pretreatment

The slides were deparaffinized and rehydrated by being subjected to the following treatment described below in table 6.

Table 6: Pretreatment Procedure

Solution	Time	Number of Rinses
Xylene	5	2
100% EtOH	3	2

95% EtOH	2	2
80% EtOH	1	1
TBS	2	2

3.6.2 Staining

The slides were loaded onto the cover plates and placed into the DAKO Autostainer. As previously mentioned, goat aorta provided the positive and negative controls for the stain. The autostainer performed the following steps described in table 7 to stain the slides for α-SMA.

Table 7: DAKO Autostainer steps

Steps
Rinse (TBS + Tween)
Add 0.1% Protease – 45 min
Rinse (TBS + Tween)
End. Enzyme Block: H_2O_2 – Peroxidase blocking solution – 10 min
Rinse (TBS + Tween)
Pretreatment Serum Block with 5% goat serum – 30 min
NO RINSE
Anti-Alpha SMA (Primary antibody or negative mouse control) – 30 min
Rinse (TBS + Tween)
Second. Reagent: Biotin Conjugate – 15 min
Rinse (TBS + Tween)
Tertiary Reagent: Extravidin-Peroxidase – 15 min
Rinse (TBS + Tween)
Switch
Substrate: AEC 10 – 10 min

3.6.3 Counterstaining and Mounting

After the DAKO autostainer completed its program, the slides were removed and placed into TBS. The slides were then counterstained with Mayers Hematoxylin for 1.5 minutes. Afterwards, the slides were placed under running tap water for 3 minutes. The slides were then coverslipped with Faramount aqueous mounting media. The coverslipped slides were allowed to dry for several days.

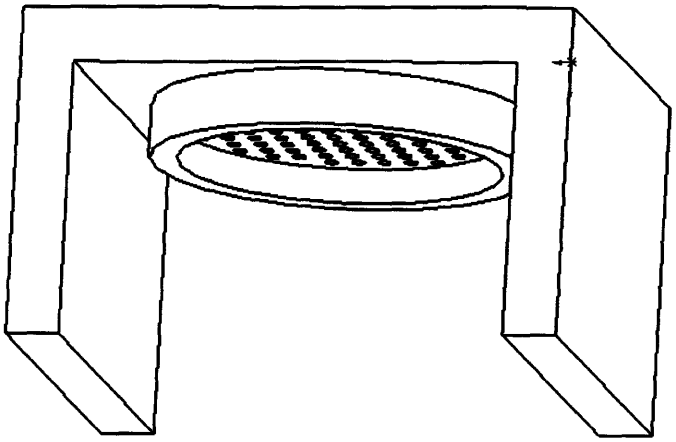
3.7 Imaging

The coverslipped slides were placed under a light microscope connected to a computer. The slides were viewed under the 10x and 40x objective lens and images were captured using computer software.

4. Scaffold Strainer

The draining of the fluid from the 50 mL tubes to rinse the scaffolds in the cross-linking procedure outlined in section 3.2.7, was accomplished by tipping the tube and using tweezers to prevent the scaffolds from sliding out. This process was not only time consuming but arduous as the scaffolds would perpetually slide past the tweezers and into the beaker below. Thus, I designed a scaffold strainer that could be placed into the tube. The scaffold strainer is pictured below in figure 5.

Figure 5: Scaffold Strainer



The outer diameter of the hollow cylinder is 27.94 mm so it will fit snugly into the inner diameter of the 50 mL tube. The strainer holes are 1 mm to prevent the scaffolds from slipping through them, since the scaffolds are 8 mm in diameter. The depth of the hollow cylinder is only 4 mm so as to not go deep enough into the tube to come in contact with the scaffolds resting on the sides of the tubes during insertion of the strainer. The two flaps are greater than the outer diameter of the tube to allow the user to hold the strainer in place with two fingers.

The other design being considered was a screw on strainer. This design was scraped as it would have been more time consuming to screw the cap on and off since there were several tubes to do and several rinses per tube.

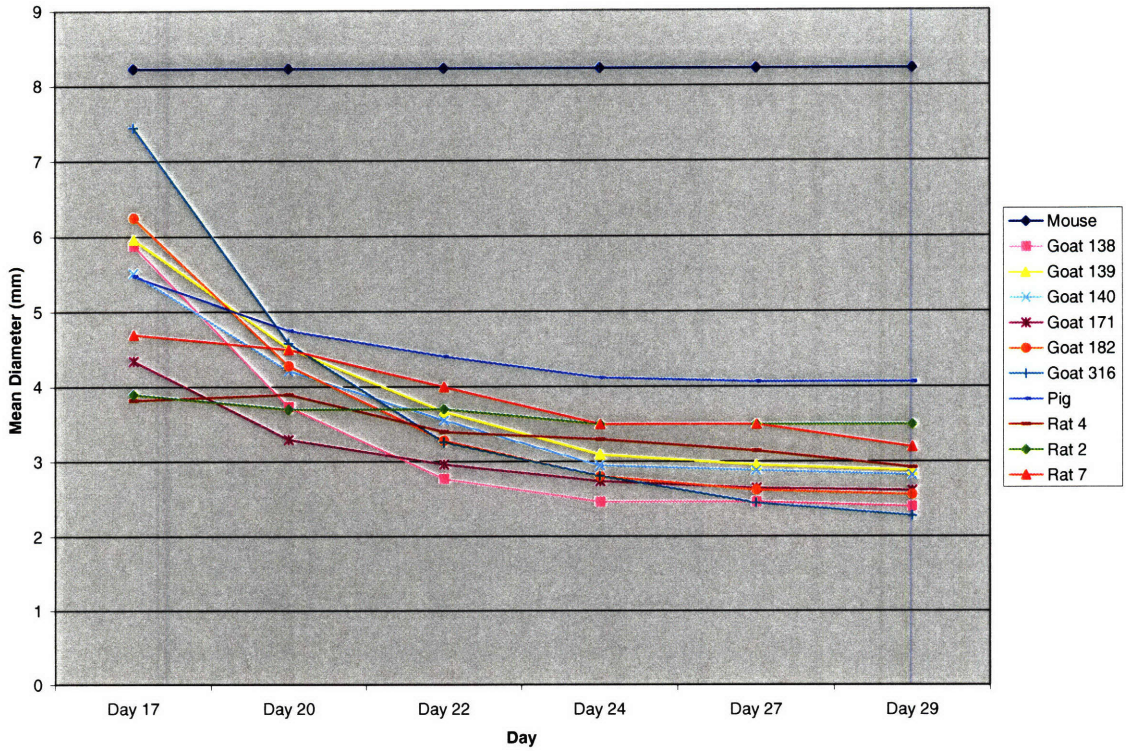
5. Results

In order to determine if contraction occurred and if the contraction was due to α-SMA, the pertinent results from the experiment are the scaffold diameters and the images obtained from the stain.

5.1 Contraction Data

The scaffold diameters were measured by the procedure outlined in section 3.4. The cells in myogenic media will not be considered, since they were being cultured for a separate experiment. Figure 6 displays the scaffold diameters versus time, excluding day 15 when all scaffolds were assumed to be 8 mm. Each data point represents an average diameter for the samples of that animal.

Figure 6: Mean Scaffold Diameter (mm) vs. Time (day)



Although not displayed for clarity's sake, it should be noted that the diameters of every control sample remained constant.

The time constants for the data sets displayed above were determined by fitting a 4^{th} degree polynomial curve to the data using matlab. The time constant was defined by the time taken for the curve to reach 63% of its final value. The time constants are presented below in table 8.

Table 8: Time Constants

Cell Type	Time Constant (Days)
Mouse	N/A
Goat 138	2.025
Goat 139	2.502
Goat 140	2.485
Goat 171	2.11
Goat 182	2.28
Goat 316	2.212
Pig	2.383
Rat 2	3.287
Rat 4	3.389
Rat 7	3.389

Since the goats and rats differed in their average time constant, it was necessary to determine if the animal type was a statistically significant factor on the time constant. The null hypothesis was that there would be no effect of animal type on the time constant. To test the null hypothesis, an unpaired t-test was run to compare the two population means. The results are below in table 9.

Table 9: Unpaired t-test: Scaffold Diameter with Goat and Rat Animal Types as Factors

	Mean Diff.	DF	t-Value	P-Value
Goat, Rat	-1.086	7	-9.177	<.0001

As can be seen in the unpaired t-test analysis above, the animal type was a statistically significant factor on the time constant. This is because the P-value is much smaller than the 5% significance level.

To determine how the cell type and the day affected the scaffold diameter, and how the interaction of cell type and day affected the scaffold diameter a two-way analysis of variance (ANOVA) test was run using StatView software.

For our two-way ANOVA there were several null hypotheses being tested at the same time. Null hypothesis 1: there is no difference in scaffold diameter between day 17, day 20, day 22, day 24, day 27, and day 29. Null hypothesis 2: there is no difference in scaffold diameter between any of the cell types. Null hypothesis 3: there is no interaction between cell type and day. The significance level for α, the probability of rejecting the null hypothesis when the null hypothesis is actually true, was chosen to be 5%. Thus, any P-value smaller than α is said to be statistically significant.

The ANOVA test had an 8 x 6 between-subjects design, meaning that there were 8 animals tested and 6 samples per animal. This between-subjects design was necessary, because a singularity would occur if the number of samples was not constant among the animals considered. Thus, the animals considered for statistical analysis were: Mouse, Goat #138, Goat #139, Goat #140, Goat #171, Goat #182, Goat #316, and Pig. The ANOVA results are presented below in table 10.

Table 10: ANOVA for Goats, Pig, Mouse

ANOVA Table for Diameter
Row exclusion: edkarenscaffold.svd

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Cell Type	7	693.133	99.019	676.966	<.0001	4738.764	1.000
Day	5	253.753	50.751	346.969	<.0001	1734.845	1.000
Cell Type * Day	35	79.412	2.269	15.512	<.0001	542.917	1.000
Residual	240	35.104	.146				

As can be seen in the ANOVA analysis above, the cell type, diameter, and cell type by day interaction are all statistically significant. Meaning that there were real effects on diameter based on the cell type in the scaffold, the day the measurement was taken, and the interaction between cell type and day.

Since there were more than two categories for day and cell type, a Fisher's PLSD is necessary to determine where the differences lie. The Fisher's PLSD tables for cell type and day are presented below in tables 11 and 12 respectively.

Table 11: Fisher's PLSD effect Cell Type

Fisher's PLSD for Diameter
Effect: Cell Type
Significance Level: 5 %
Row exclusion: edkarenscaffold.svd

	Mean Diff.	Crit. Diff.	P-Value	
Mouse, G138	4.946	.178	<.0001	S
Mouse, G139	4.386	.178	<.0001	S
Mouse, G140	4.577	.178	<.0001	S
Mouse, G171	5.130	.178	<.0001	S
Mouse, G182	4.599	.178	<.0001	S
Mouse, G316	4.429	.178	<.0001	S
Mouse, Rg	3.751	.178	<.0001	S
G138, G139	-.559	.178	<.0001	S
G138, G140	-.369	.178	<.0001	S
G138, G171	.184	.178	.0422	S
G138, G182	-.347	.178	.0002	S
G138, G316	-.517	.178	<.0001	S
G138, Rg	-1.194	.178	<.0001	S
G139, G140	.190	.178	.0359	S
G139, G171	.744	.178	<.0001	S
G139, G182	.212	.178	.0194	S
G139, G316	.043	.178	.6347	
G139, Rg	-.635	.178	<.0001	S
G140, G171	.563	.178	<.0001	S
G140, G182	.022	.178	.8073	
G140, G316	-.147	.178	.1035	
G140, Rg	-.825	.178	<.0001	S
G171, G182	-.531	.178	<.0001	S
G171, G316	-.701	.178	<.0001	S
G171, Rg	-1.379	.178	<.0001	S
G182, G316	-.169	.178	.0615	
G182, Rg	-.847	.178	<.0001	S
G316, Rg	-.678	.178	<.0001	S

Table 12: Fisher's PLSD effect Day

Fisher's PLSD for Diameter
Effect: Day
Significance Level: 5 %
Row exclusion: edkarenscaffold.svd

	Mean Diff.	Crit. Diff.	P-Value	
1, 2	1.439	.154	<.0001	S
1, 3	2.125	.154	<.0001	S
1, 4	2.490	.154	<.0001	S
1, 5	2.601	.154	<.0001	S
1, 6	2.658	.154	<.0001	S
2, 3	.686	.154	<.0001	S
2, 4	1.051	.154	<.0001	S
2, 5	1.162	.154	<.0001	S
2, 6	1.219	.154	<.0001	S
3, 4	.366	.154	<.0001	S
3, 5	.476	.154	<.0001	S
3, 6	.533	.154	<.0001	S
4, 5	.111	.154	.1571	
4, 6	.167	.154	.0331	S
5, 6	.057	.154	.4699	

Due to the difference in time constants between goats and rats, it was necessary to run an ANOVA to determine the effect of cell type and day on diameter. This ANOVA analysis is different than the one conducted above since the cell type is now confined to the animal groups of rat and goat. These animal groups will not be considered on an individual sample basis, but on a species basis. Meaning that all goat samples are collapsed into a category and all rat samples are collapsed into a category. The ANOVA is given below in table 13.

Table 13: ANOVA for Goats and Rats

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Animal Group	1	.015	.015	.045	.8315	.045	.055
Day	5	67.220	13.444	40.141	<.0001	200.706	1.000
Animal Group * Day	5	24.062	4.812	14.369	<.0001	71.845	1.000
Residual	240	80.380	.335				

5.2 Imaging data

The images from the stains were obtained by the procedure outlined in section 3.7. The positive control stained for α-SMA, and the negative control did not stain for α-SMA, indicating the stain was valid. No negative stains from any samples stained for α-SMA. The results from the positive stains are listed below in table 14.

Table 14: Results of Stain

Cell Type	Positive Stain
Mouse	No
Goat 138	No
Goat 139	No
Goat 140	No
Goat 171	Yes
Goat 182	Yes
Goat 316	No
Pig	Yes
Rat 2	No
Rat 4	Yes
Rat 7	Yes

In an experiment run concurrently, 10,000 cells from the samples used in this experiment were placed in monolayer and stained for α-SMA after 1 day. The results from this immunohistochemical evaluation were that all positive stains stained positive for α-SMA.

6. Discussion

As previously mentioned, the results from the concurrent experiment run in monolayer reveal that all the cell types stained positive for α-SMA This signifies that all the cell types used in this experiment have the capability to express α-SMA. Taking this observation and applying it to the results we obtained from the experiment discussed in this paper raises some interesting questions. Firstly, if all the cell types can express α-SMA why did the mouse embryonic stem cell seeded scaffold not contract? Secondly, why did several goat and rat mesenchymal stem cell seeded scaffolds that contracted not

stain positive for α-SMA? The answers to these two questions will be discussed in detail below.

The mouse seeded scaffold was the only scaffold in the experiment to not display contraction. The mouse seeded scaffold was the only embryonic stem cell seeded scaffold in the experiment. In a recent paper it was determined that the initiation of actin polymerization in muscle cells requires a strong filament nucleator (Chereau et al). Specifically, the protein leiomodin was identified as a strong filament nucleator. It is plausible that the mouse embryonic stem cells had not yet expressed a protein to act as an actin filament nucleator, and thus were not able to construct actin filament bundles. Without these actin filament bundles, it would be impossible for the cells to contract.

Every cell seeded scaffold contracted except for the mouse, but only Goat #171, Goat #182, Pig, Rat #4, and Rat #7 stained positive for α-SMA. A plausible explanation for this observation is that by the time the samples were ready to be embedded in paraffin the α-SMA expression was finished in the scaffolds that did not stain. The samples were embedded two weeks after being seeded with cells. After two weeks, it is plausible that the maximum amount of contraction possible was achieved. No longer being able to contract the already collapsed pores, the cells were no longer provided with the mechanical stimulus needed to continue expressing α-SMA. Additionally, the cells could pool in the collapsed pores and compete for nutrients. Not being able to obtain the nutrients they needed the cells expressing α-SMA could have died. Although, DNA content tests were not run so it is impossible to determine the amount of cells present at any given time. The data reinforces the idea that the maximum contraction was achieved. From figure 6, we see that the in the last three days the contraction versus time begins to plateau. The fisher's PLSD for the effect of day on diameter in goat, pig, and mouse confirms this. Between days 4 and 5, and 5 and 6 the null hypothesis holds. Meaning that the there is no statistically significant effect of day on the diameter for those days. The fisher's PLSD for the goat and rat animal types confirms this as well. Between days: 3 and 4, 4 and 5, and 5 and 6, the null hypothesis holds. The fact that some stained for α-SMA speaks to the amount of variability in the experiment. The size of the pores, the amount of cells, the exact amount of media supplied, the samples themselves, are all variables that are impossible to keep constant, and the fact that some scaffolds that

contracted stained positive for α-SMA can be attributed to differences in such variables between the samples.

On a side note, the images obtained show the stained region was limited to the periphery of the scaffold section. While the reason for this behavior is unknown, it has been documented in other research and thus reinforces the validity of our results (Vickers et al).

In regards to the time constants, from table 8 it is clearly visible that the goats and rats display different average time constants. From the unpaired t-test we find that there the variation of the time constant with regards to animal type is statistically significant. From this fact we would expect that the effect of animal type, in this case being goat and pig, would be statistically significant on scaffold diameter. Unexpectedly, we find from the ANOVA that the null hypothesis holds for the effect of animal type on diameter. Meaning that there is no statistically significant difference for the effect of goat or rat on scaffold diameter.

7. Future Work

The results of the stain run in this experiment are for one sample of the animals mentioned. To gain a fuller understanding, it would be necessary to section and stain all samples.

In future experiments it would be necessary to test the DNA content of the scaffolds at various instances in time. This would allow us to determine the number of cells present at any given moment. With this knowledge we could test the hypothesis that there are fewer cells as time progresses due cell death caused by pooling. It would also be necessary to run stains at different points in time. This would allow us to determine if alpha-smooth muscle actin expression took place at any time during the experiment not just at the end of the experiment.

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Appendix A: Expansion Media Recipes

Mouse ESC

Media: alpha-MEM, 7.5% Newborn calf serum, 2.5% Fetal calf serum (FBS), 1% P/S

445 mL	alpha-MEM
37.5 mL	Newborn calf serum
12.5 mL	Fetal calf serum (FBS)
5.0 mL	Pen/Strep

Pig & Goat MSC

Media: LG-DMEM, 10% FBS, 1% P/S, 10ng/mL bFGF*

450 mL	LG-DMEM
45 mL	FBS
5 mL	Pen/Strep

*Add 1 μL of bFGF per 1 mL of media using 0.22 μm sterile filter

Rat MSC

Media: LG-DMEM, 20% FBS, 1% antibiotic/antimycotic, 10ng/mL bFGF*

412.5 mL	LG-DMEM
82.5 mL	FBS
5 mL	Antibiotic/Antimycotic

*Add 1 μL of bFGF per 1 mL of media using 0.22 μm sterile filter

Appendix B: Cell Counts and Cell Suspension Volumes

Mouse ESC

Day 1: 1.1e6 cells counted. 2.27 mL required for 250,000 cells.
Day 6: 100.4e6 cells counted. 50 µL required for 250,000 cells.
Day 15: 130.4e6 cells counted. 3.07 mL required for 20e6 cells.

Pig #1

Day 15: 36.4e6 cells counted. 5.49 mL required for 20e6 cells.

Goat MSC #138

Day 3: 1.16e6 cells counted. 2.15 mL required for 250,000 cells
Day 8: 12.8e6 cells counted. 60 µL required for 75,000 cells.
Day 15: 45.2e6 cells counted. 4.42 mL required for 20e6 cells.

Goat MSC #139

Day 3: 720,000 cells counted. 3.47 mL required for 250,000 cells
Day 8: 11.3e6 cells counted. 66 µL required for 75,000 cells.
Day 15: 43e6 cells counted. 4.65 mL required for 2e6 cells.

Goat MSC #140

Day 3: 600,000 cells counted. 4.20 mL required for 250,000 cells
Day 8: 10e6 cells counted. 75 µL required for 75,000 cells.
Day 15: 29.8e6 cells counted. 6.71 mL required for 20e6 cells.

Goat MSC #171

Day 3: 820,000 cells counted. 3.05 mL required for 250,000 cells.
Day 8: 8.8e6 cells counted. 85 µL required for 75,000 cells.
Day 15: 24.4e6 cells counted. 8.20 mL required for 20e6 cells.

Goat MSC #182

Day 3: 560,000 cells counted. 4.46 mL required for 250,000 cells.
Day 8: 11e6 cells counted. 68 µL required for 75,000 cells.
Day 15: 26.25e6 cells counted. 11.4 mL required for 20e6 cells.

Goat MSC #316

Day 3: 800,000 cells counted. 3.125 mL required for 250,000 cells.
Day 8: 13.3e6 cells counted. 56 µL required for 75,000 cells.
Day 15: 39e6 cells counted. 5.12 mL required for 20e6 cells.

Rat #1

Day 15: 2.12e6 cells counted.

Rat #2

Day 15: 2.32e6 cells counted. 8.62 mL required for 2e6 cells.

Rat #3

Day 15: 550,000 cells counted.

Rat #4

Day 15: 8.5e6 cells counted. 9.4 mL required for 8e6 cells.

Rat #5

Day 15: 700,000 cells counted.

Rat #6

Day 15: 550,000 cells counted.

Rat #7

Day 15: 3.25e6 cells counted. 6.15 mL required for 2e6 cells.

Appendix C: Contraction Data

Mouse	Day 15 3/19/2008	Day 17 3/21/2008	Day 20 3/24/2008	Day 22 3/26/2008	Day 24 3/28/2008	Day 27 3/31/2008	Day 29 4/2/2008
Sample 1	8	8	8	8	8	8	8
Sample 2	8	8	8	8	8	8	8
Sample 3	8	7.9	7.9	7.9	7.9	7.9	7.9
Sample 4	8	8.5	8.5	8.5	8.5	8.5	8.5
Sample 5	8	8.5	8.5	8.5	8.5	8.5	8.5
Sample 6	8	8.5	8.5	8.5	8.5	8.5	8.5
Goat 138							
Sample 1	8	6	3.6	2.9	2.3	2.3	2.3
Sample 2	8	5.3	2.7	2.5	2.3	2.3	2.0
Sample 3	8	6	5	2.7	2.5	2.5	2.5
Sample 4	8	5.5	3.6	3.1	2.5	2.5	2.5
Sample 5	8	6.5	3.7	2.8	2.5	2.5	2.5
Sample 6	8	6	3.8	2.7	2.5	2.5	2.5
Goat 139							
Sample 1	8	6.5	4	3	2.5	2.7	2.7
Sample 2	8	5.5	4.2	4.2	3.2	3.2	3
Sample 3	8	5.5	3.9	3.4	3.2	2.7	2.7
Sample 4	8	6.3	6.3	4	3.2	2.7	2.5
Sample 5	8	6	4	3.5	3.2	3.2	3
Sample 6	8	6	4.7	3.9	3.4	3.4	3.4
Goat 140							
Sample 1	8	6	4.9	3.8	3.2	3.1	2.9
Sample 2	8	5.8	4.1	3.2	2.7	2.7	2.5
Sample 3	8	5.5	3.7	3	2.7	2.7	2.7
Sample 4	8	5.5	3.9	3.4	2.9	2.9	2.9
Sample 5	8	5	4	3.7	2.9	2.9	2.9
Sample 6	8	5.3	4.7	4.2	3.4	3.2	3.2
Goat 171							
Sample 1	8	4	3.2	3	2.7	2.7	2.7
Sample 2	8	4.4	3.2	2.9	2.7	2.3	2.3
Sample 3	8	4.4	3.2	3	2.7	2.5	2.5
Sample 4	8	4.4	3.5	3.2	3.2	3.4	3.2
Sample 5	8	4.2	3.2	2.7	2.7	2.3	2.3
Sample 6	8	4.7	3.5	3	2.7	2.7	2.7
Goat 182							
Sample 1	8	6.5	4.4	2.7	2.5	2.5	2.5
Sample 2	8	6	3.9	3.1	2.5	2.5	2.3
Sample 3	8	6.6	5.0	3.6	3.1	2.5	2.3
Sample 4	8	6	3.7	2.9	2.3	2.3	2.3
Sample 5	8	7	4.2	3.4	2.9	2.7	2.7
Sample 6	8	5.4	4.5	4	3.5	3.2	3.2
Goat 316							
Sample 1	8	7.1	3.7	2.9	2.7	2.3	2.3
Sample 2	8	7.6	5.8	3.7	3.2	2.7	2.7
Sample 3	8	7.5	6.0	3.9	3.2	2.3	2.0

Sample 4	8	7.5	4.2	3.4	2.9	2.3	2.3
Sample 5	8	7.5	4.2	3.2	2.7	2.7	2.3
Sample 6	8	7.5	3.6	2.5	2.3	2.3	2.0
Pig							
Sample 1	8	5.5	4.7	4.1	3.8	3.8	3.8
Sample 2	8	5.4	5.0	4.4	4.2	4.2	4.2
Sample 3	8	5.3	4.2	4.2	4	4	4
Sample 4	8	5.5	5	4.7	4.5	4.2	4.2
Sample 5	8	5.5	5	4.7	4.2	4.2	4.2
Sample 6	8	5.7	4.7	4.2	4	4	4
Goat 138 Myo							
Sample 1	8	6.8	3.9	2.7	2.5	2.1	2.1
Sample 2	8	6.7	3.2	2.7	2.7	2.3	2.3
Sample 3	8	6.8	4	3.5	3.5	3.5	3.5
Goat 139 Myo							
Sample 1	8	7.1	4.2	3.2	3.2	2.9	2.9
Sample 2	8	7.3	4.2	4	3.7	3.5	3.5
Sample 3	8	7.4	4.2	3.7	3.7	3.4	3.2
Goat 140 Myo							
Sample 1	8	6.8	4.2	3.7	3.5	3.2	3.2
Sample 2	8	6.8	4.2	3.2	2.9	2.7	2.7
Sample 3	8	6	4.2	3.7	3.4	3.4	3.2
Goat 171 Myo							
Sample 1	8	6.5	4.2	3.5	3	2.7	2.7
Sample 2	8	5.9	4.2	3.5	3	2.7	2.7
Sample 3	8	6	4.2	3.5	3.5	3.5	3.5
Goat 182 Myo							
Sample 1	8	8.2	4.7	3.7	3.2	3.2	2.9
Sample 2	8	7.5	4.5	3.5	3.2	3.2	3
Sample 3	8	7.5	3.9	2.7	2.5	2.5	2.5
Goat 316 Myo							
Sample 1	8	8.2	4.5	3.5	3	2.7	2.7
Sample 2	8	8.2	4.5	3.7	3.5	3.5	3.2
Sample 3	8	7.9	5	4	3.5	3.5	3.5
Mouse Myo							
Sample 1	8	8	8	8	8	8	8
Sample 2	8	8	8	8	8	8	8
Sample 3	8	8	8	8	8	8	8
Pig Myo							
Sample 1	8	6	4.7	4.5	3.6	3.7	3.7
Sample 2	8	5.9	5	4.4	4.2	4.5	4.5
Sample 3	8	5.7	4.7	4.1	4.1	3.7	3.7
Rat 4							
Sample 1	8	4.5	3.7	3.2	3	2.7	2.7
Sample 2	8	3.5	3.7	3.2	3.2	3.2	3
Sample 3	8	3.2	4	3.5	3.5	3.2	3
Sample 4	8	4.1	4.2	3.7	3.5	3.5	3
Control							

G exp	7.9	7.9	7.9	7.9	7.9	7.9	7.9
R exp	8	8	8	8	8	8	8
M exp	5.8	5.4	5.8	5.8	5.8	5.8	5.8
None	7.5	7.5	7.5	7.5	7.5	7.5	7.5
G myo	8	8	8	8	8	8	8
M myo	8	8	8	8	8	8	8
Rat 2							
Sample 1	8	3.9	3.7	3.7	3.5	3.5	3.5
Rat 7							
Sample 1	8	4.7	4.5	4	3.5	3.5	3.2