The Effects of the Cardiac Extracellular Microenvironment on the Differentiation of Mesenchymal Stem Cells

An honors thesis for the Department of Biomedical Engineering

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Abstract

Mesenchymal stem cell (MSC) cell therapy is a novel treatment for repairing and regenerating cardiac tissue post myocardial infarction (MI). Currently in human clinical trials, the therapy aims to repopulate the infarcted tissue with MSCs and prevent the negative remodeling process associated with MI. However, the treatments are limited by a lack of cell retention and differentiation to cardiomyocytes. Therefore, this study aimed to determine how the extracellular microenvironment, including the composition and elasticity of the extracellular matrix (ECM) and oxygen content, affect the differentiation of MSCs. MSCs were grown on polyacrylamide gels, representing three physiologically relevant stiffnesses, combined with complete ECM composition or a single purified extracellular protein in order to study the effects of the ECM composition and stiffness on MSC differentiation. This method of differentiating MSCs was compared to the standard chemical method for cardiac differentiation: treatment with 5-azacytidine. Then, a disease model was developed to study how changes in stiffness, ECM composition, and oxygen content as a result of MI impact the differentiation of MSCs compared to a healthy cardiac environment. We found that the physiologically healthy stiffnesses tended to promote differentiation when combined with complete ECM. Furthermore, the disease model confirmed the results of the cell therapy clinical trials, where the healthy models better differentiated MSCs compared to the infarct model. However, it was also found that despite hypoxia being a result of MI, it promoted differentiation except when combined with infarct stiffness and infarct ECM composition. Based on these results, MSCs can be differentiated towards cardiomyocytes if given the appropriate environment, which can lead to improved methods of pre-differentiating MSCs prior to injection in cell therapy for more effective MI treatments.

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1. Introduction

1.1 Significance

Since 1918, cardiovascular disease has been the leading cause of death in the United States and is now the leading cause of death in the developed world (Goldwaite, 2009). Out of the many diseases that fit under the umbrella title of cardiovascular disease, myocardial infarction (MI) is the leading cause of heart failure in America (NHLBI, 2008). MI is the buildup of atherosclerotic plaque, which ultimately leads to a coronary artery occlusion (Thygesen et al., 2007). Currently, several treatment methods are used to prevent or treat heart failure. For instance, pharmaceutical approaches include the use of beta blockers and diuretics to aid the heart in blood flow and heart rate control (Goldwaite, 2009). Mechanical devices such as pacemakers have also been engineered to aid in the restoration of healthy cardiac function. While these approaches are effective in improving cardiac function and the patient's quality of life, they also have limitations: pharmaceutical methods rely on patient cooperation and synthetic devices have limited lifetimes, which are different from that of the patient. Mechanical devices also have a higher potential for thrombosis and calcium deposition. Ultimately, both approaches are limited in their inability to repair the infarcted tissue. Thus, native tissue function is not actually regained (Goldwaite, 2009). Therefore, the gold standard for cardiovascular disease treatment is a heart transplant, which restores natural function. However, transplants are still limited as there is a shortage of donor hearts and they require a lifelong regiment of immunosuppressants (AHA Heart Transplant: Statistics, 2007; Goldwaite, 2009).

Since the variety of treatments cannot restore natural pumping function post MI, the morbidity and mortality rates of MI have not decreased (Nugent et al., 2003). Meanwhile, risk factors for cardiovascular disease, such as obesity, continue to increase (Goldwaite, 2009).

Therefore, novel, innovative treatments must be developed for MI in order to alleviate heart failure. The development of a method that would repair damaged cardiac tissue and regenerate healthy tissue function would transform post MI treatment, taking the first steps towards mitigating the long term effects of cardiovascular disease. One such potential treatment is cell therapy, where stem cells are injected into the infarct to repair the damaged tissue and restore native function (Nugent et al., 2003). The therapy has shown promise in early clinical trials. Results show the treatment has potential in preventing the negative remodeling process of the tissue that occurs post MI that ultimately destroys cardiac function. However, the therapy is still limited in its inability to differentiate stem cells and lack of cell retention in the infarct area (Cleland et al., 2005). Thus, cell therapy must be further studied and developed in order to understand the causes of its limitations and improve therapeutic results. *The development of a method that would repair damaged cardiac tissue and regenerate healthy tissue function would transform post MI treatment, taking the first steps towards mitigating the long term effects of cardiovascular disease.*

1.2 Specific Aims and Hypotheses

The overall goal of this project was to determine how the extracellular cardiac environment (extracellular matrix composition, stiffness, and oxygen content) affects the development and differentiation of mesenchymal stem cells (MSCs) and how MSC development changes when the extracellular environment mimics infarcted tissue. In order to study these effects, this project was broken up into three specific aims.

The first specific aim was to assess how the extracellular matrix of healthy fetal, neonatal, and adult hearts impact MSC differentiation grown on stiffnesses of 8kPa, 25 kPa, and 40 kPa. These stiffnesses are representative of different life points, where 8 kPa

approximates the fetal heart ECM stiffness, 25 kPa represents neonatal and adult stiffnesses, and 40 kPa represents the increased stiffness of the heart with age and disease. Rat MSCs were seeded on polyacrylamide gels created for each stiffness with solubilized rat cardiac ECM cross-linked into the gel. Differentiation of MSCs was analyzed for each condition of stiffness and ECM age. In addition, polyacrylamide models of each of the three stiffnesses were also created with purified components of the ECM to analyze what elements of the ECM affect differentiation in MSCs. These components are collagen I, collagen III, collagen V, laminin, and fibronectin. Finally, the ability of these models to differentiate MSCs were compared to the standard chemical method of cardiac differentiation, which uses 5-azacytidine (Miskon et al., 2010). We hypothesized that the younger ECM on the physiologically relevant 25 kPa stiffness would improve differentiation compared to the ECM at higher stiffnesses and would be more effective and precise at guiding differentiation than the 5-azaacytidine method.

The second specific aim is to determine the individual effects of hypoxia, ECM composition, and ECM stiffness on the differentiation of MSCs in an infarct environment. In all conditions, MSCs were seeded onto polyacrylmide gels cross-linked to solubilized adult rat ECM. By varying the concentrations of bis acrylamide to acrylamide in the gels to alter stiffness while maintaining oxygen content and ECM composition at healthy levels the effects of infarct stiffness on MSC differentiation will be determined. To analyze the individual effects of infarct ECM composition against healthy ECM composition, the stiffness and oxygen content were maintained at healthy levels while healthy ECM was doped with Collagen I to create an infarct-like environment. The oxygen content was varied by incubating in normoxic or hypoxic chambers while ECM composition and stiffnesses were maintained at healthy levels. As a control, a model with healthy ECM composition, stiffness, and oxygen content was tested.

Differentiation was observed through histology and Western Blotting. We hypothesized that the control would best promote differentiation as it is most representative of the native, healthy heart, while increasing the stiffness, changing the ECM composition, and using a hypoxic environment would reduce levels of differentiation.

The third specific aim is to determine the synergistic effects of hypoxia, ECM composition, and ECM stiffness on differentiation of MSCs in an infarcted environment. The same methods from Specific Aim I and II were used to create polyacrylamide models, except all variations of having two infarcted elements with one healthy component were tested, as well as a model with all three elements representing infarction. The same control of all elements representing healthy cardiac tissue was used and the models were assayed for differentiation. We hypothesized that increasing the number of elements representing infarction would create an inhospitable environment and result in decreased differentiation.

1.3 Long Term Goals

The long term goal of this research is to develop *in vitro* methods of conditioning MSCs for pre-differentiation to cardiomyocytes in order to improve their effects in cell therapy *in vivo* and to use these methods to develop 2 dimensional disease models. With improved treatment methods for enhanced cell retention and differentiation, the hope is to mitigate long term effects of cardiovascular disease. Using a mimic of the native extracellular environment to differentiate MSCs would be advantageous over current methods that utilize toxic chemicals, such as 5-azacytidine, because it can more specifically direct MSCs to the desired lineage and does not require toxic reagents for therapeutic purposes (Zhang et al., 2009). Furthermore, the disease and healthy models can be used to determine the mechanisms that drive MSC differentiation to cardiomyocytes and shed light on why current cell therapy treatments are failing. It is hoped that

this method of using extracellular matrices and mimicking the microenvironment to predifferentiate MSCs to cardiomyocytes can then be expanded to other body parts to guide stem cell differentiation toward other cell lineages to develop novel disease therapeutics.

2. Background

2.1 Cardiovascular Disease and Changes to the Heart with MI

Out of all the diseases related to cardiovascular disease, MI is the leading cause of death (NHLBI, 2008). Post MI, the affected heart tissue undergoes a negative remodeling process in which the tissue forms a scarred, fibrous region that loses its ability to contract. As a result, the heart loses contractile function and eventually leads to heart failure. The remodeling process begins with a lack of oxygen and nutrients due to a lack of blood flow to the infarcted region causing the cells to undergo necrosis and apoptosis. The necrosis results in rapid swelling by an accumulation of water and electrolytes that rupture the plasma membrane and disrupts cellular organelles to induce an inflammatory response (Krignen et al., 2002). Upon inflammation, cytokines are activated to initiate the pathophysiological changes that direct cardiac remodeling that includes the scarring of the infarct, loss of myocytes by apoptosis and changes in the ECM composition (Guo et al., 2007). Aside from scar formation, the remodeling process expands the area of infarct, causes ventricular dilatation, and thins the ventricular wall (Xu et al., 2005).

The ECM composition changes by increasing collagen production. Despite these changes, the ECM can still maintain native structure of the myocardium but functional ability is decreased depending on the extent of the damages. To compensate for the loss of function, the heart increases blood pressure for increased blood flow. However, the increased pressure increases the stress on the cells, causing the cells to undergo pathological hypertrophy or

dilatation to try to reduce the stress. Ultimately, MI and negative remodeling results in cardiac tissue loss, which limits cardiac function and eventually deteriorates the heart, leading to heart failure (Xu et al., 2005).

2.2 Overview of Cell Therapy

In mature adult hearts, cardiomyocytes cannot self regenerate and the heart lacks a significant storage of precursors or stem cells to restore injured or infarcted areas. The loss of cardiomyocytes results in a fibrous scar with contractile dysfunction. Therefore, in order to restore pump function, stem cell therapy is being developed to repopulate the area with contractile cells. A number of studies, including several clinical trials, have investigated MSC cell therapy, which aims to repair the infarct and regenerate function by injecting MSCs into the injured area (Nugent et al., 2003).

Currently, clinical trials are testing cell therapy to determine its safety and efficacy for preventing the negative repair process. The REPAIR-AMI trial found that injection of bone marrow stem cells resulted in significantly greater left ventricular contractile function compared to the control, suggesting that cell therapy could prevent post infarction heart failure (Gruberg, 2005). Additionally, the STAR-heart study also found increased survival of chronic heart failure patients undergoing cell therapy. The patients had improved quality of life with increased cardiac index exercise capacity and oxygen uptake (Strauer et al., 2006). Results from the studies were statistically significant. After only four months, REPAIR-AMI's found that patients injected with bone marrow stem cells had 48% to 54% improved left ventricular ejection fraction compared to 47% to 50% in the placebo group ($p = 0.021$) (Cleland et al., 2005). However, despite statistical significance, both trials were not clinically significant as benefits were only minimal and transient.

Another study, ASTAMI, found contradictory evidence with better results in the control group, indicating limitations in cell therapy. Specifically, the study found limited MSC differentiation to cardiomyocytes and retention of MSCs in the infarcted area (Cleland et al., 2005). Despite the contradicting results, all of the trials indicated that cell therapy was safe, and with statistically significant data, it still holds promise as new method to prevent heart failure (Wei et al., 2009). Therefore, more research must be done to improve the treatment's ability to prevent negative remodeling of the heart post MI and surpass cell therapy's current limitation in its ability to differentiate and retain injected MSCs.

While cell therapy has shown signs of improved cardiac repair, improved cardiac function, and mostly positive clinical outcomes (Dengler et al., 2011), it is unknown exactly how it affects the infarcted area. While initially thought to work through a direct replacement of cardiomyocytes with differentiated MSCs, some studies now suggest cell therapy works through a paracrine effect (Tan et al., 2010). This theory suggests MSCs create a cytoprotective effect, where the MSCs protect the native cells from toxic signals and chemicals released in the infarct area that initiate the negative remodeling process (Ohnischi et al., 2007). The paracrine effect has been offered as an alternative explanation for how cell therapy works after transient success in clinical trials despite low cell retention and only partial differentiation (Wei et al., 2009).

Furthermore, incomplete differentiation was found *in vitro* by Tan et al., (2010) where their study of sternum-derived bone marrow MSCs exhibited only partial differentiation. The MSCs formed "cardiomyocyte-like-cells" that lacked spontaneous contractions. Despite only partial differentiation in an infarct environment, MSCs still show promise for cell therapy because they have the potential to fully differentiate into cardiomyocytes, as shown by cardiac-like action potentials, rhythmic calcium transients, and inward rectifying potassium currents,

when grown in an appropriate environment (Tan et al., 2010). While cell therapy is a promising new treatment for regenerating function post MI, it is limited in its ability to fully differentiate MSCs to cardiomyocytes, in part because of the inhospitable infarct environment.

2.3 Benefits of using MSCs in Cell Therapy

While other studies have tried different cell types, such as fetal cardiomyocytes (Li et al., 1996), embryonic stem cells (Min et al., 2002), and skeletal muscle satellite cells (Menasche et al., 2001), MSCs have unique characteristics that are beneficial for their use in cell therapy. They are relatively easy to extract from adult bone marrow with little ethical opposition, proliferate extensively, and have the capability to differentiate into a variety of cell lineages, including cells of the endoderm, ectoderm, and endothelium (Jiang et al., 2002). With respect to MI and the cardiac environment, MSCs are especially useful as studies have shown their ability to differentiate into cardiomyocytes if given the proper conditions (Guo et al., 2007). They are genetically stable and can survive in an infarct amongst inflammatory cytokines without initiating an immune response (Lian et al., 2011).

Furthermore, Guo et al. (2007) found that MSCs actually reduce gene expression of the inflammation cytokines and have myogenesis and angiogenesis abilities to reduce negative remodeling post MI. In terms of function, Serraro et al. (2012) found that MSCs have the potential to compensate for cardiomyocyte loss of contractility. The versatility and compatibility of MSCs increases the potential for successful cell therapy for improved cardiovascular disease treatment.

2.4 Overview of Cardiac ECM

The ECM is a natural, three dimensional scaffold composed mainly of proteins secreted by the cells to support and anchor cells. While the composition varies between tissue types, all

ECM is dominated by collagen, fibronectin, and laminin with lesser amounts of elastin, glycosaminoglycans, proteoglycans, and tenascin (Reilly and Engler, 2010; Singelyn et al., 2009). While originally thought to only provide structural support, it is now understood to make significant contributions to cellular and organ function through mechanical and chemical signals. The signals monitor normal developmental and pathological cellular activities, including adhesion, proliferation, migration, and gene expression (Bowers et al., 2010). The tissue specificity of ECM can be utilized in stem cell studies to recapitulate the native environment of the target tissue. Using decellularized ECM in constructs is especially useful as ECM is known to regulate growth, proliferation, and differentiation of cells on a tissue specific dependence. Therefore, it is an ideal material for the growth and development of stem cells as it better mimics the environment of natural, mature tissue compared to the current popular use of a single purified ECM protein. More complex models using a synthetic combination of ECM proteins, such as combining collagen I and fibronectin in one model, has also been developed to show changes in cell behavior based on the synergistic signaling effects. However, these models still do not completely represent the native microenvironment or provide the tissue specific signals of natural ECM (DeQuach et al., 2010; Heng et al., 2004).

Using ECM in constructs replicates how the cells adhere in their natural environment and promotes stem cell differentiation and development according to the specific ECM type. Naturally, ECM provides specific regions for cell binding, such as the Arginine-Glycine-Aspartic Acid (RGD) domains of fibronectin. Once adhered, the entire composition of ECM can dictate cell fate (Reilly and Engler, 2010). Furthermore, the ECM can be modified in constructs by binding growth factors to increase differentiation down a specific lineage (Wells and Discher, 2008). ECM has great potential as a construct material because it most closely recapitulates the

native chemical and biological environment to promote cell adhesion and differentiation down tissue specific lineages *in vitro*.

2.5 Effects of ECM age on MSC differentiation

The ECM composition changes with age in order to dictate cell migration, development, and growth. These changes in ECM composition have a major influence over cardiac function and structure by influencing cell migration, adhesion, and communication. During fetal development, the ECM guides heart development from the heart tube to its standard four chamber structure. For instance, increased ECM synthesis by atrioventricular canal myocytes causes endothelial cells to form the embryonic cushions, which ultimately develop into the primordial heart valves. In addition to ECM synthesis, cells are also impacted by the degradation of ECM, which enhances epithelial-mesenchymal transitions (Bernanke et al., 1984). Furthermore, fibronectin in ECM is found to influence cardiac organogenesis in the fetal stage with closely regulated levels of mRNA, which are not impacted during adult cardiac enlargement (Farhadian et al., 1995). As the heart develops and ages, the ECM also changes in order to provide the cells with the appropriate signals.

At the neonatal stage, the ECM exists as a thin layer in the epicardium that thickens with increased collagen deposition. As the heart develops from the fetal stage to the neonatal stage, it increases the thickness of the ventricular walls and increases tensile strength to adapt to increased functional needs (Manasek et al., 1969; Norris et al., 2008). In addition, increased levels of collagen I, III, and IV, periostin, and fibronectin cause the heart to undergo myocyte and fibroblast proliferation and endothelial cell invasion and differentiation (Bower et al., 2010).

The heart undergoes further development as it ages from neonatal to adult. Myocytes continue to proliferate for a short period after birth and then undergo physiological hypertrophy.

Fibroblast proliferation and the density of vasculature increase as a result of even more heightened levels of collagen I, III, IV, and fibronectin while periostin decreases (Bowers et al., 2010). Upon reaching adulthood, the heart continues to change as it ages with decreased rates of collagen turnover despite high levels of synthesis, implying that it is the synthesized collagen that is quickly degraded (Mays et al., 1991). The many changes in structure and composition of the ECM have differing effects on cells and can potentially be manipulated in ECM-directed cell studies for desired outcomes, such as increased proliferation, migration, or differentiation.

2.6 Effects of ECM composition on MSC development post MI

The ECM composition also plays an important role in MSC adhesion, proliferation, and differentiation post MI (Tan et al., 2010). The collagen that composes most of cardiac ECM provides structural support to promote adhesion and expansion of cells and factors into the determination of cell lineage. Tan et al. (2010) found that while Collagen I and Collagen III are the major components of the ECM, it is actually Collagen V that promotes cell adhesion, proliferation, and differentiation of cardiomyocyte-like-cells to complete cardiomyocytes. Following MI, the composition of cardiac ECM is remodeled with a dramatic increase in collagen I (Pelouch et al., 1994). However, injected MSCs have been found to decrease Collagen I and III levels, which has led to an improved infarct environment. Therefore, a decreased expression of Collagen I and III could potentially protect the tissue from negative remodeling and dilatation (Guo et al., 2007). Thus, the role of ECM composition on MSC cell function must be investigated in order to determine optimal pre-conditioning treatments for cell injection to treat cardiovascular disease.

2.7 Effects of varying Stiffness on MSC development

The ECM composition determines the stiffness of the environment, which in turn impacts the adhesion, proliferation, and differentiation of stem cells. For example, Evans et al. (2009) found that increasing stiffness promotes proliferation and differentiation of embryonic stem cells. Furthermore, Tse et al (2011) found that MSCs have a memory of stiffnesses they previously resided on, as shown by maintenance of plasticity before differentiation down a cell line triggered by a former environment. Therefore, culturing MSCs on a stiffness representative of a cardiac environment prior to injection in cell therapy could precondition the cells for improved efficacy.

In the cardiac environment, tissue stiffness is particularly important to examine as it regulates cellular contractions. Failure to consider the physical properties of the infarct can cause undesired side effects, such as calcified lesions because the increased stiffness resembles a bone environment that can induce osteoblast differentiation. Therefore, the increased stiffness of scarred MI can inhibit the differentiation of MSCs towards cardiomyocytes (Young et al., 2010). Jacot et al. (2008) found that changing the elastic modulus of the substrate impacts the maturation of neonatal rat cardiomyocytes compared to neonatal cardiomyocytes in native, undamaged ECM models which developed normal contractile function. Thus, the altered stiffness of MI could impact the development of injected cells to cardiomyocytes.

In order to study the effects of stiffness, other groups use polyacrylamide gels with cross linked ECM proteins. Variations in stiffness have successfully been modeled *in vitro* by altering the ratio of acrylamide to bisacrylamide (Jacot et al., 2008; Rajagopalan et al., 2004). Polyacrylamide gels are ideal because they are elastic and inert, requiring ligand covalent attachment for cell adhesion with no individual influence on the cells. Therefore, any effect on the cells must be a result of the ligand, allowing for the study of ligand type and surface density

on cellular behavior (Rajagopalan et al., 2004). Additionally, the study by Engler et al. (2006) using polyacrylamide gels found that MSC differentiation is extremely sensitive to the elasticity of the environment. Tissue types vary in environmental stiffness. For example, the muscle environment is much softer compared to bone. Indeed, Engler et al. (2006) found that stiffness alone can determine the differentiation path of MSCs in two dimensions. Therefore combined with ECM from healthy hearts, it should promote differentiation of MSCs to cardiomyocytes through better replication of the native environment.

2.8 Effects of Oxygen on MSC development

Oxygen deprivation is the first significant change post MI due to a lack of blood flow caused by occlusion. MSCs can survive in a hypoxic environment since they are native to bone marrow, which has reduced access to oxygen. However, the lack of oxygen can greatly impact the differentiation of MSCs because oxygen is a crucial stimulus in influencing gene expression and cell lineage. Compared to normoxia, where MSCs express genes for morphogenesis, development, adhesion, and proliferation (Ohnishi et al., 2007), low oxygen levels induce gene expression to maintain stem cell characteristics by increasing proliferation and reducing apoptosis (van Oorschot et al., 2011). At low oxygen content, MSCs enter a survival mode where they expend most of their energy for growth and proliferation rather than differentiation (van Oorschot et al., 2011). While it is known that hypoxia prevents MSC differentiation, it is unclear if it is the main reason for partial differentiation of MSCs in an infarct.

2.9 Current Standards for Cardiomyocyte Differentiation with 5-Azacytidine

One of the current methods for MSC differentiation to cardiomyocytes is the addition of 5-azacytidine, a demethylating agent, to the culture medium for about 24-48 hours. While the exact mechanism of differentiation to cardiomyocytes is unknown, 5-azacytidine is believed to

activate specific phenotypic genes by random hypomethylation of DNA cytosine. Wakitani and Saito (1995) were one of the first to suggest using 5-azacytidine to pre-differentiate MSCs to a myogenic lineage based on their findings that 5-azacytidine causes MSCs to differentiate into myogenic cells. Their ability to differentiate MSCs to myocytes led to the possibility of cardiomyocyte differentiation with 5-azacytidine.

Based on Wakitani and Saito's experiments, many groups aimed to optimize MSC differentiation with 5-azacytidine but yielded inconclusive results (Liu et al., 2003). However, Miskon et al. (2009) detected beating cells after combining the 5-azacytidine method with a suspension treatment prior to seeding on tissue culture plates with ECM proteins adsorbed to the surface. They found that after three weeks of culture, beating could be induced by adding acetylcholine or any other chemical reagent and after four weeks, the cells would be synchronously. Thus, they concluded that 5-azacytidine with guiding factors such as single ECM proteins differentiate MSCs to cardiomyoctes. While this offers an *in vitro* method for differentiating MSCs, the clinical potential of 5-azacytidine is limited by its toxicity.

2.10 Summary

Based on previous studies, MSCs have potential for cell therapy as they can differentiate into cardiomyocytes to replace cells lost to MI and can block the cytotoxic signals to prevent further negative remodeling in the infarct. However, undifferentiated MSCs have had limited success in clinical trials and have potential to differentiate down a variety of pathways. Therefore, differentiation is not specific to cardiomyocytes as would be desired. However, predifferentiating MSCs by mimicking the native environment *in vitro* could improve the efficacy of cell therapy by pre-determining the cells and conditioning them to a cardiac

environment prior to injection. If these methods work, then ECM-stiffness model can be applied to other tissue types to improve other therapies.

3. Materials and Methods

3.1 Variation of ECM Age on MSC Differentiation

3.1.1 Polyacrylamide (PAAm) gels

i. Glass Slide Activation: 9 mm x 9 mm glass cover slips (Chemglass Scientific) were activated to create binding sites for the PAAm gel. The glass slides serve as the foundation for the *in vitro* models and allow for easier handling. For each model, a glass cover slip is flame activated to increase hydrophilicity and 0.1M NaOH (Acros) was smeared on the flame activated side in a chemical hood. After the NaOH dried, 3-aminopropyltrimethoxy (Sigma-Aldrich) was applied with Q-tips. When dry, the cover slips were individually placed in 24 well plates and washed twice for 8 and 5 minutes, respectively, with distilled deionized (ddi) water on an orbital shaker. The water was aspirated and 0.5% glutaraldhyde (Sigma-Aldrich) was added to each well plate to cover the slide. After 30 minutes, the glutaraldehyde was aspirated and the glass cover slips underwent 3 five minute washes with ddi water. The slides were stored in ddi water at 4ºC until ready for use (Wang and Pelham, 1998).

ii. Casting Polyacrylamide Gels with ECM: By changing the amount of cross-linking between 40% acrylamide (AAM) to 2% bis acrylamide, polyacrylamide gels can be cast to a specific stiffness. Gels were cast at 0.05%, 0.1%, and 0.2% Bis acrylamide ratios to represent 8 kPa, 20 kPa, and 40 kPa, respectively in order to represent fetal elasticity (8 kPa), neonatal and adult elasticity (20 kPa), and damaged or aged elasticity (40 kPa) (Berry et al., 2006). The recipes listed in Table 1 were followed to create each stiffness. The desired bis acrylamide

percentage was determined by performing tensile tests on different percentages of bis acrylamide to AAm gels and developing a graph of Young's modulus, which exponentially increases with increasing bis acrylamide concentration. The exponential relationship is determined by the linear elastic modulus of each gel up to 25% strain (Jacot et al., 2008). For this experiment, solubilized cardiac ECM or individual ECM component proteins were cross linked into the PAAm gels at all three stiffnesses using acrylic acid *N*-hydroxysuccinimide (NHS) ester to create covalent linkages between amine groups. Fetal, neonatal, and adult cardiac ECM from Sprague Dawley rats were studied along with purified collagen I, III, V, fibronectin (Fn), and Laminin (Ln). Brain and kidney ECM from Sprague Dawley rats were also used as negative controls. The volume of protein added was based on concentration for 400µg per 500 µL. To prevent NHS from hydrolyzing, which is common at basic pH, HCl is added lower the pH to 6.6-7.1. As the amount of protein and HCl added varies based on stock concentrations of the proteins, the final step is to add ddi water to bring the total volume to 0.5 mL. TEMED and 10% ammonium persulfate (APS) were added to cross link AAm to bis AAm.

Table 1. ECM Differentiation Experiment PAAm Gel Recipe			
Ingredients	0.05%	0.10%	0.20%
40% Acrylamide	125	125	125
2% Bis acrylamide	17.5	25	50
10X PBS	50	50	50
ECM/Protein	a	a	a
TEMED	1	1	1
1N HCl	x	y	z
NHS (5 mg/mL)	40	40	40
10% APS	2.5	2.5	2.5
ddi H_20	b	c	d

Table 1. Recipe for polyacrylamide gels where the percentages listed represent the percent of bis acrylamide. The 0.05%, 0.10%, and 0.20% correlate to approximately 8 kPa, 25 kPa, and 40 kPa, respectively. The volume of ECM protein is written as a and must be calculated based on the concentration of ECM for 400 µg. The volume of 1N HCl (x, y, z) should be added for a pH of 6.6-7.1. The volume of water (b, c, d) should be calculated for a total volume of 0.50 mL.

To create the gels, the activated glass slides were air dried in a sterile biological hood. 10µL of each PAAm solution was pippetted onto each slide and an non-activated slide was

placed on top. After polymerization (~30 minutes), the non-activated cover slip was removed with a razor and washed three times with sterile 1X PBS (Jacot et al., 2008; Rajagopalan et al., 2004). 4 gels per condition were created, such that 2 gels were saved for histology, 1 gel was used for cell lysis for protein isolation and Western blotting, and 1 gel was used for RNA isolation. The gels were stored for a maximum of 24 hours in sterile 1X PBS at 4ºC.

3.1.2 Isolation, perfusion decellularization, and solubilization of ECM

i. Isolation and Decellarization of ECM: Using decellularized rat hearts best represents the native myocardial ECM since it maintains biological and chemical qualities that are unique to individual hearts (Singelyn et al., 2009). Hearts from adult, neonatal, and fetal Sprague Dawley rats were isolated according to our approved Tufts University IACUC protocol. To isolate the adult hearts, the rats were euthanized using CO_2 asphyxiation followed by diaphragm perforation. The rib cage was cut open to access the heart, which was then cleaned of fat and connective tissue. The first three arterial branches after the aortic arch were ligated with a 6-0 nylon suture (Ethicon) and cut distal to the suture. The descending aorta was cut about 1 centimeter from the arch, and the caval and pulmonary veins, pulmonary artery, thoracic aorta, and vena cava were transected to remove the heart from the chest cavity and placed in 1X PBS.

Next, the descending aorta was sutured around 1.8 mm aortic cannula for retrograde coronary perfusion decellerization. The perfusate consisted of 1% sodium dodecyl sulfate (SDS) (Invitrogen) in ddi water for 36 hours or until visible cellular material was removed. Then, the heart was washed with ddi water for 15 minutes followed by a Triton-X 100 (Sigma-Aldrich), and another wash with ddi water. Finally, the heart was washed with PBS for 48 hours with solution changes every 12 hours (Ott et al., 2008).

To obtain neonatal rat hearts, the pups were euthanized by decapitation. A sternotomy was performed and the heart was forced out of the chest wall by pinching the shoulder blades together. The hearts were placed in 1X PBS for initial blood removal before decellularization. To obtain the fetal pups, an anesthetized pregnant dam (100mg/kg ketamine and 10 mg/kg xylazine) underwent a hysterectomy in order to remove the fetuses from the placenta. Then, the pups underwent a similar decapitation and sternotomy method as the neonatal pups.

Instead of perfusion, fetal and neonatal hearts were decelled by diffusion and convection by soaking in 0.05% SDS and 0.1% SDS, respectively. They were placed in conicals on an orbital for 24 hours or until no more visible cellular material was detected. Since the hearts were much thinner than adult hearts, perfusion was unnecessary. Upon completion of the SDS treatment, the hearts were rinsed three times with 1X PBS.

ii. Solubilizing Decelled Hearts: The hearts were solubilized in a solution of pepsin in 0.1 M HCl (Figure 1). To solubilize the adult hearts, the left ventricle was dissected and sonicated in ddi water at 30% for 30 seconds with a Branson 450 Digital Sonifier (Branson, Danbury, CT). As the fetal and neonatal hearts are much smaller, sonication was unnecessary. All samples were frozen in ddi water at -20ºC and lyophilized for 24-48 hours. The adult hearts were solubilized in 1 mg/mL pepsin in 0.1M HCl with a 10:1 tissue to pepsin ratio to achieve 10 mg/mL ECM solutions. The fetal and neonatal hearts were solubilized in 0.1 mg/mL pepsin in 0.1M HCl for a concentration of ~2 mg/mL. The hearts were digested by the pepsin/HCl solution on a stir plate for 48 hours. Upon

Figure 1. Fetal, neonatal, and adult Sprague Dawley rat hearts were lyophilized and solubilized with pepsin in 0.1M HCl

completion, the solution was neutralized with 1N NaOH prior to incorporation into PAAm gels or frozen at -20ºC for storage.

3.1.3 Cell Culture

i. Cell Line: MSCs were purchased from Texas A&M National Stem Cell Repository, which are derived from Lewis rats. According to Texas A&M, culture conditions were varied between strain differences of rats for optimal isolation and expansion to maintain multipotency (TAMHSCCOM, 2009). Prior to seeding the MSCs on the *in vitro* models, the elasticity of the MSCs was maintained for greatest potential to differentiate into cardiomyocytes when seeded (TAMHSCCOM, 2009). The MSCs were used between passages 8-10.

ii. Culture Medium: The MSCs will be given complete culture medium (CCM) for MSCs with 20% Fetal Bovine Serum (FBS) (Gibco) and 1% 10,000 U/mLPenicillin/Streptomycin (Gibco) in α-Minimum Essential Medium (αMEM) without ribonucleosides or deoxyribonucleosides (Gibco). The CCM was supplemented with 2% L-Glutamine (200 mM in .85% NaCl) (Gibco), added separately during each feeding. The media is kept at 4°C and warmed to 37°C when fed to MSCs (TAMHSCCOM, 2009). Media was changed approximately every two days.

iii. Cell Culture Specifications: MSCs were seeded at ~10,000 cells per gel. They were cultured for 7 days in an incubator maintained at atmospheric (20%) O_2, 37°C, and 5% CO_2. The gels were transferred to new 24 well plates after 24 hours in order to minimize paracrine signaling from MSCs adhered to TCPS.

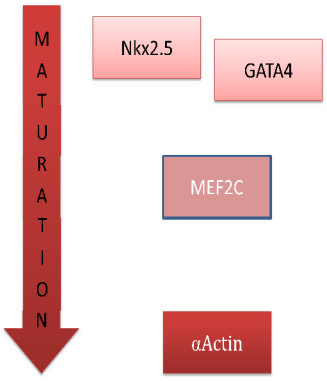

Figure 2. Progression of expression of cardiac differentiation markers where Nkx2.5 and GATA4 indicate early cardiogenesis while αActin signifies a mature cardiomyocyte.

3.1.4 Differentiation Analysis

i. Immunohistochemistry: Transcription factors Nkx2.5 and GATA4 are common indicators of early cardiac differentiation

while Mef2C and αActin are regularly used to indicate further downstream cardiac commitment (Figure 2) (Beltrami et al., 2003).

Immunohistochemistry was performed at room temperature by fixing with 4% paraformaldehyde for 20 minutes. The samples were permeated with 0.1% TritonX for 10 minutes after washing the paraformaldehyde with 1X PBS. After 3 1X PBS rinses, the samples were blocked with 5% donkey serum in 1% bovine serum albumin (BSA) for 30 minutes. Primary antibodies were applied at a 1:200 concentration in 1% BSA for 1 hour. After primary antibody incubation, the gels underwent three 5 minute 1X PBS rinses and the appropriate secondary antibodies were administered at a 1:400 concentration in 1% BSA for 1 hour in the dark. Hoechst nuclear stain was administered with the secondary antibody incubation at 1:10,000 concentration or upon 3 X 5 minute 1X PBS washes of the secondary antibody, the gels were set with Vectashield hardmount with DAPI (Vector Labs, Philadelphia, PA). Fluorescent images were obtained using an Olympus IX81 microscope (Olympus Americas, Center Valley, PA).

ii. Image Analysis and Quantification: Histology was quantified using a custom built algorithm (pipeline) developed by Joshua Resnikoff on CellProfiler software (Broad Institute, Cambridge, MA). Briefly, the background was filtered out twice and cells were identified by the blue dapi nuclar signal. The "propagate" algorithm determined the edges of the cells in order to measure the intensity of the red (TRITC) and green (GFP) signals. The intensities of each cell per image was averaged and plotted.

iii. Cell Lysis for Protein Purification and Quantification: In order to purify the proteins from the cells, the gels were moved into new 24-well plates to prevent collection of MSCs adhered to the tissue culture plastic of the well, and the cells were removed from the gels by

incubating with 0.05% Trypsin for two minutes. Trypsin was deactivated with an FBS quench (50µL/well). The solution was centrifuged at 1000g and 20°C for 5 minutes and the FBS-trypsin solution was aspirated from the pellet. The pellet was resuspended in 0.1-0.5 mL lysis buffer, consisting of 50% 2X NP40, 2.5% 40X sodium deoxycholate, 1% 100X sodium orthovanadate, 0.1% 1000X aprotinin, 0.1% 1000X pepstatin, 0.1% leupeptin, and 46.2% ddi water. After resuspension, the solution was vortexed for 1 minute and then put on a rotisserie for 15 minutes at 4ºC. The solution was spun down at 13000 rpm for 15 minutes at 4ºC. The supernatant was collected as the cell lysate and the pellet was saved. Both were stored at -20ºC for future use.

A BCA protein assay was performed on the cell lysate to determine total concentration of protein. The Pierce® BCA Protein Assay Kit instructions for products 23225 and 23227 microplate instructions were followed with a sample to working reagent (WR) ratio of 1:20. Briefly, 10 µL of sample was pippetted into a 96 well plate for replicates of 3. 200 µL of BCA WR was added to each well and mixed thoroughly on an orbital. The plate was incubated for 30 minutes at 37ºC and absorbance was measured on a microplate readerat 562nm. The absorbance was compared to a standard curve of BSA in lysis buffer to determine protein concentration.

Table 2. SDS-PAGE Gels		
Ingredients	10% Resolving Gel	
water	9.96	mL
1M Tris pH 8.8	13.5	mL
10% SDS	360	µL
30% Acrylamide	12	mL
10% APS	360	µL
TEMED	36	µL
Ingredients	3% Stacking Gel	
water	11.04	mL
1M Tris pH 6.8	1.875	mL
10% SDS	150	µL
30% Acrylamide	1.95	mL
10% APS	150	µL
TEMED	15	µL

Table 2. Recipe for SDS-PAGE gels run for Western Blotting. Based on the range of sizes for the proteins of interest (35 kPa- 72 kPa), 10% resolving gels were created with a 3% stacking gel. The recipe created 4 gels with a 1.5 mm depth and 10 lanes.

iv. Western Blotting: Based on the

BCA protein assay, 10% SDS-PAGE gels were run with the highest possible concentration of protein in 48μL running buffer samples, consisting of the cell lysate, 3:1 4X sample buffer: 2M DTT, and ddi water. Upon mixing the samples, they were placed on a heat block for 5 minutes at 100ºC and quickly spun down. The samples were loaded into 1.5 mm 10% SDS-PAGE gels (Table 2) with a rainbow marker. The gels were run in running buffer (10% 10X Tris/Glycine/SDS buffer) at constant 200V until the bands ran off of the gel. Then, the proteins were transferred to nitrocellulose paper in transfer buffer (10% 10X CAPS pH 11, 10% methanol). The transfer was run at constant 100V for 1-1.5 hours.

The blots were probed for Nkx2.5, GATA4, MEF2C, and α-actin. They were blocked in 5% milk in tris buffered saline and tween 20 (TBST) for an hour at room temperature and then incubated in 1/500 concentration of primary antibodies in 5% milk in TBST for 1 hour at room temperature. After 3 X 5 minute rinses, HRP-conjugated secondary antibodies were applied at a concentration of 1/1000 in 5% milk in TBST for 1 hour. The blots were developed using ECL reagents, Syngene software, and Syngene G:Box developer. All of the blots were normalized to β-actin with a primary concentration of 1/1000 and secondary concentration of 1/2000. Western blots were quantified using ImageJ analysis (NIH, Bethesda, MD).

3.2 Infarcted ECM on MSC Differentiation

3.2.1 Polyacrylamide gels

The same methods as the variation on ECM age on MSC differentiation experiment were used to activate glass slides and create polyacrylamide gels. However, only adult ECM was used and 25 kPa and 50 kPa gels were made according to the recipe in Table 3 in order to compare the effects of healthy and infarcted cardiac ECM. The stiffnesses were chosen since average

stiffness of healthy cardiac tissue is about 20 kPa while infarcted tissue has a stiffness of about 50 kPa (Berry et al., 2006).

Table 3. Infarct Experiment PAAm Gel Recipes				
Ingredient	0.10%	0.30%	0.1% Doped	0.3% Doped
40% Acrylamide	125	125	125	125
2% Bis Acrylamide	25	75	25	75
10X PBS	50	50	50	50
ECM (10mg/mL)	31	31	20	20
Collagen I (10mg/mL)	0	0	11	11
TEMED	1	1	1	1
1N HCl	a	b	c	d
NHS (1mg/mL)	5	25	5	25
10% APS	2.5	2.5	2.5	2.5
ddi H_20	w	x	y	z

Table 3. Recipe for polyacrylamide gels with listed percentages indicating the percent of bis acrylamide. 0.10% and 0.3% correlate to approximately 25 kPa and 50 kPa, respectively. The doped conditions indicate the addition of purified collagen I to the ECM solution. The volume of HCl (a, b, c, d) was determined to decrease the pH to 6.6-7.1. The amount of water (w, x, y, z) was calculated to bring the total volume to 0.50 mL.

3.2.2 Isolation, Perfusion Decellularization, and Solubilization of ECM

i. Isolation, decellularization, and solubilization of ECM: Adult ECM was obtained by perfusion decellularization and solubilized as described above in section 3.1.

ii. Creating Infarct-like ECM: The solubilized adult ECM was doped with collagen I in order to create an infarct-like model as seen in Table 3. Based on literature, the major change in ECM composition during the negative remodeling process post MI is a doubling of collagen concentration. Collagen I and III compose over 90% of myocardial collagen with collagen I comprising about 80% of that amount (McCormick et al., 1994; Pelouch et al., 1994; Thomas et al., 2000). Therefore, the infarct mimic ECM consisted of the solubilized healthy ECM doped with appropriate amount of collagen I.

3.2.3 Cell Culture

i. Cell source: MSCs were purchased from Cell Applications, Inc. The cells were used at passage 8-10.

ii. Cell Medium: The same medium was used as in the variation on ECM age on MSC differentiation experiment.

iii. Cell Culture Specifications: In normoxic environments, MSCs were seeded at ~10,000 cells per gel. In hypoxic environments, MSCs were seeded at ~3,780 or ~10,0000 cells per gel. For normoxic conditions, MSCs were cultured in an incubator maintained at atmospheric (20%) O_2, 37°C, and 5% CO_2. Hypoxic conditions of the infarct experiment were cultured in a hypoxic incubator with oxygen levels at 5% and 37°C. The MSCs were cultured for 10 days before fixation.

3.2.4 Differentiation Analysis

Differentiation was analyzed and quantified on Day 7 and 10 by immunohistochemistry and CellProfiler using the same methods as described above. Western blots were also run for cells collected on Day 10.

3.3 5-Azacytidine MSC Differentiation Experiment

3.3.1 Precoat 24 Well TCPS Plate with ECM Proteins

Prior to cell seeding, gelatin, collagen I, or neonatal ECM was adsorbed to 24 well TCPS plates to determine if proteins improved 5-azacytidine differentiation methods. The concentrations of Collagen I, gelatin, and neonatal ECM for adsorption were 200 μg/mL, 200 μg/mL, and 500 μg/mL, respectively. Enough of each solution to cover the bottom was pippetted into each well and allowed to air dry overnight in a sterile biological hood. They were gently rinsed with sterile 1X PBS.

3.3.2 Cell Culture

i. Cell Line: For this experiment, we used different MSCs from prior experiments. MSCs were purchased from Cell Applications, Inc and expanded to passage 4-6.

ii. Cell Media: Two different media were used for the three trials of the 5-azacytidine experiment. In Trial 1, the differentiation medium consisted of 300 µL L-ascorbic acid phosphate magnesium salt n-hydrate (Sigma-Aldrich), 0.025 µg/mL human basic fibroblast growth factor containing DMEM, and 1% 10,000 U/mLPenicillin/Streptomycin (Gibco). In Trial 2 and 3, the medium consisted of 10%, 5%, or 2% FBS and 1% 10,000 U/mLPenicillin/Streptomycin (Gibco). In both trials, 10µM 5-azacytidine was added to the differentiation medium for the first 24 hours. Media was changed every 3 days.

iii. Cell Seeding: The protocol developed by Miskon et al. (2010) was followed for cell seeding. They used two methods of seeding: suspension and monolayers. Cells were seeded at about 27,000 MSCs/well.

For the suspension method, MSCs were removed from culture flasks using a 2 minute incubation at 37ºC with 0.25 % Trypsin. The Trypsin was quenched with FBS and the cells were spun down at 200X g for five minutes at room temperature. After aspirating the Trypsin, the cells were resuspended in differentiation medium containing 5-azacytidine. Three different suspension methods were used. In the first trial, the cell solution was subject to the hang drop method for two hours. This method consisted of creating 200 µL drops of solution on Petri dishes, inverting the dish and incubating at 37ºC. Then the drops were collected, additional differentiation media with 5-azacytidine was added, and seeded on the precoated 24 well plates. The second trial, cells resuspended in 2 mL per media condition with 5-azacytidine were placed

in 50 mL conical. They were rotated on a rotisserie in the incubator for two hours before seeding on the pre-coated plates. The caps were lightly screwed onto the conical for oxygenation. The final trial placed 0.50 mL resuspended cells in 5-azacytidine in 1.2 mL Cryogenic tubes. The tubes were placed in 37ºC incubator with the caps lightly screwed on to allow for oxygenation. Every ten minutes the caps were screwed tightly and the tubes were inverted to maintain cell suspension in the medium. After two hours the cells were seeded onto the pre-coated plates.

The monolayer method immediately seeded the cells into the 24 well plates after resuspension in respective media.

iv. Controls: Several controls were run in order to examine the effects of protein adsorption and differentiation medium. In both trials, MSCs were also seeded on TCPS with no protein adsorbed to the surface. The differentiation medium was given to a control of MSCs without 5-azacytidine, while another set of control MSCs were given MSC media (20% FBS, 1% Pen/Strep, αMEM). Finally, cardiomyocytes isolated from neonatal rats were given differentiation medium without 5-azacytidine or myo medium (1% Pen/Strep, 10% Horse serum, 2% FBS in DMEM) and fibroblasts with fibroblast medium (15% FBS, 1%Pen/Strep in DMEM) were seeded for a negative control.

3.3.3 Differentiation Analysis

The MSCs were monitored daily and changes in morphology were noted. Literature has suggested that MSCs undergoing cardiac differentiation change their morphology to become more elongated and stick like (Zhang et al., 2009). Representative images were taken of each condition using phase contrast microscopy on an Olympus IX81 microscope (Olympus Americas, Center Valley, PA).

Immunohistochemistry was also performed to assay for Nkx2.5 and GATA4 using the same methods as previously stated.

3.4 Statistical Analysis

For the first MSC differentiation experiment based on ECM age, two way ANOVAs were used to determine statistical significance between each condition. A p-value < 0.05 indicated significant differences between conditions. If significance was found, the Holm-Sidak method was used to perform pairwise multiple comparisons within groups. $p<0.05$ indicated significance. For the infarct experiments, two way ANOVAs were performed while maintaining one of the three conditions constant. All variations of holding a condition constant were run and $p <0.05$ indicated significance between conditions. The Holm-Sidak method was used to determine significance amongst experimental conditions with a $p<0.05$ indicating significance.

4. Results

4.1 Variation of ECM Age on MSC Differentiation

4.1.1 Immunohistochemistry

The differentiation of MSCs towards cardiomyocytes was determined qualitatively with immunohistochemistry. All conditions were probed for cardiac markers Nkx2.5, GATA4, MEF2C, and αActin. Nkx2.5 and GATA4 were probed on the same gel, while the further downstream markers, MEF2C and αActin, were stained on a separate gel. Representative images of each condition are shown in Figures 3-4. Background stains can be attributed to defects in the gel, incomplete washing of secondary antibodies, and drying out of the gel prior to imaging.

After one week, all cardiac ECM conditions stained positively for Nkx2.5 and GATA4 with the 40kPa condition having the weakest signal (Figure 3). Nkx2.5 was detected with TRITC, which tended to wash out the GFP signal of GATA4. As a result, there was lower visibility on the overlaid images. The fluorescence for both conditions was translocated to the nucleus, as seen in the overlaid images as violet. Nkx2.5 and GATA4 are expected in the nucleus because they are coupled transcription factors (Durocher et al, 1997). The collagen conditions also stained positively for Nkx2.5 and GATA4 at all stiffnesses with collagen I and collagen V staining stronger than collagen III (Figure 3). Fibronectin stained positively with a stronger signal at 8 and 25 kPa while minimal signal was observed in all stiffnesses of laminin (Figure 4). Brain and kidney ECM also stained minimally, except kidney at 8 kPa and brain at 25 kPa (Figure 4). The control of MSCs on TCPS also expressed Nkx2.5 and GATA4, but both signals were not nuclear specific (Figure 4).

After one week, fewer conditions expressed MEF2C and αActin than Nkx2.5 and GATA4 (Figure 5-6) with a greater number of conditions staining positive for MEF2C than αActin. In the ECM conditions, only the 8 and 25 kPa conditions showed expression. MEF2C was detected using TRITC, which tended to overpower the GFP signal of αActin in the overlaid images. MEF2C was punctuated in the nucleus but was also detected in the cytoplasm, especially in the 8 kPa conditions. In the collagen conditions, only collagen III had strong expression of MEF2C at 25 kPa. Fibronectin had a stronger nuclear stain at 8kPa but a stronger cytoplasmic signal in the 40 kPa condition. Little signal was detected in all laminin conditions. Kidney and brain ECM were negative for both MEF2C and αActin, and little expression was detected in the TCPS negative control.

Figure 3. Representative images of MSCs grown on the complete ECM or collagen PAAm gels labeled with Nkx2.5 (red) and GATA4 (green). The columns represent stiffnesses of 8 kPa, 25 kPa, and 40 kPa from left to right with the ECM protein content represented in rows. All images are labeled with Dapi nuclear stain. Scale bar = 100 um

Figure 4. Representative images of MSCs grown on 8 kPa, 25 kPa, and 40 kPa (left to right columns) with fibronectin, laminin, kidney ECM, or brain ECM (rows). MSCs are labeled with Nkx2.5 (red), GATA4 in (green) and Dapi nuclear stain. MSCs on tissue culture polystyrene (TCPS) is also depicted. Strong background signals are a result of defects in the gel and drying out of the gel prior to imaging. Scale bar = 100 um

Figure 5. Representative images of MSCs grown on PAAm gel models of 8 kPa, 25 kPa, and 40 kPa (columns) with fetal ECM, neonatal ECM, adult ECM, collagen I, collagen III, or collagen V. Immunohistology shown for MEF2C (red) and alpha actin (green). All images are labeled with Dapi nuclear stain. Scale bar = 100um

Figure 6. Representative images of immunohistology of MEF2C (red,) and alpha actin (green) for MSCs grown on PAAm gels. Conditions with stiffnesses of 8 kPa, 25 kPa, and 40 kPa (columns) with fibronectin, laminin, kidney ECM, and brain ECM (rows) are depicted. The TCPS control is also shown. scale bar = 100 um

4.1.2 Quantification of Immunohistochemistry for MSC Differentiation

In order to analyze MSC differentiation to cardiomyocytes quantitatively, a pipeline, developed by Joshua Resnikoff in CellProfiler, was used to filter out the background and measure the intensity of each signal per cell as detected by the Dapi nuclear stain. Data points which were clearly unfiltered background artifact were removed. Generally, most conditions had stronger expression at the cardiac physiological stiffnesses of 8 and 25 kPa. Concurrent with the histological images, αActin expression was much more widespread between conditions and the other three cardiac markers were found to have about 10 fold higher intensity.

A limit of the intensity measurements was punctuated expression in the nucleus. Since the TRITC images tended to wash out the GFP images, it's possible that a lower intensity was measured for GATA4 and αActin. Additionally, since protein expression was found in the nucleus, the overlaid image color was violet, which could have interfered with detection of TRITC and GFP.

For all four proteins probed, statistically significant interactions were found between the ECM protein conditions and stiffnesses ($p<0.05$). Furthermore, statistically significant results were obtained when comparing stiffnesses within the same ECM protein composition (Figure 7-10). Most notably, the biologically relevant stiffnesses (8 kPa and 25 kPa) tended to have higher expression of the cardiac differentiation markers. Significance could have been gained or lost in some conditions due to variability in intensity measurements or high background in the images.

In the GATA4 intensity measurements, 25 kPa had one of the highest expressions, which was rivaled by brain ECM at 8 and 40 kPa (Figure 8). GATA4 has been identified in the central nervous system, but its role has not been thoroughly characterized (Agnihotri et al., 2009). There was also notably high expression of αActin in brain at 25 kPa; however, this is likely a

result of excessive background from the damaged gel that could not be removed with the pipeline (Figure 10).

For all cardiac expression markers, expression in the collagen conditions tended to rival that found in the ECM conditions, especially for Nkx2.5 and MEF2C. The collagen was purchased for cell experiments and was thus purified and optimized for cell adhesion. Contrastingly, all of the ECM conditions were isolated and solubilized in the lab with little purification. Therefore, more cells tended to adhere to the collagen conditions as a result of the greater number of available binding sites. The optimized binding between the protein and cell could have resulted in a greater influence in differentiation because the cell had more exposure to the protein. In addition, with a greater number of cells bound, there would be an increased level of paracrine signaling to guide differentiation.

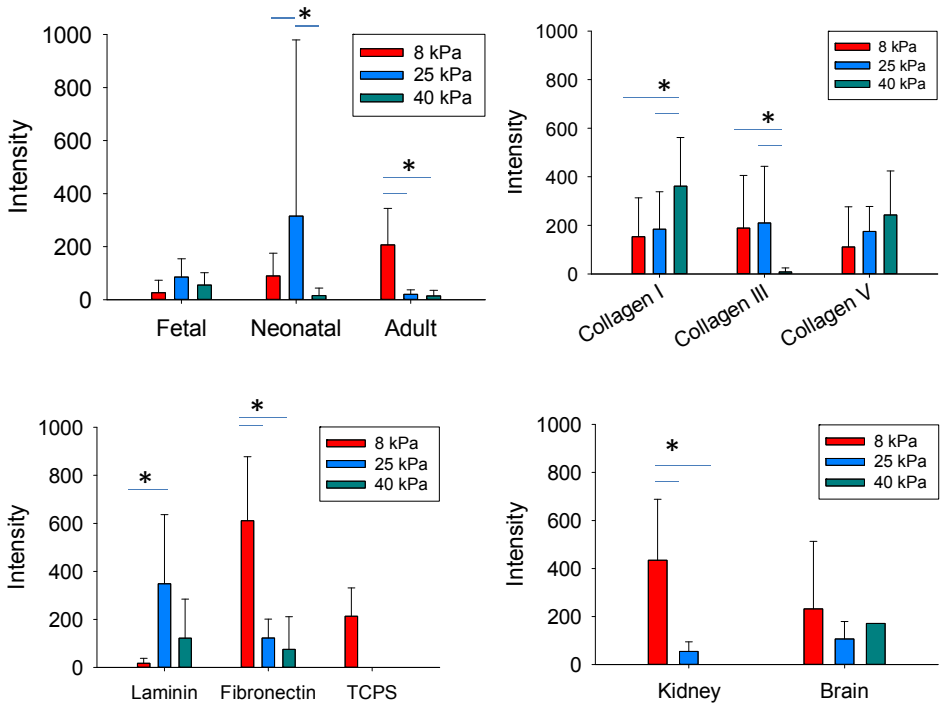

Figure 7. Quantification of the intensity of Nkx2.5 fluorescence based on immunohistology. Conditions are grouped by their protein content and arranged by stiffnesses of 8 kPa (red), 25 kPa (blue), and 40 kPa (green). * signifies statistical significance ($p<0.05$)

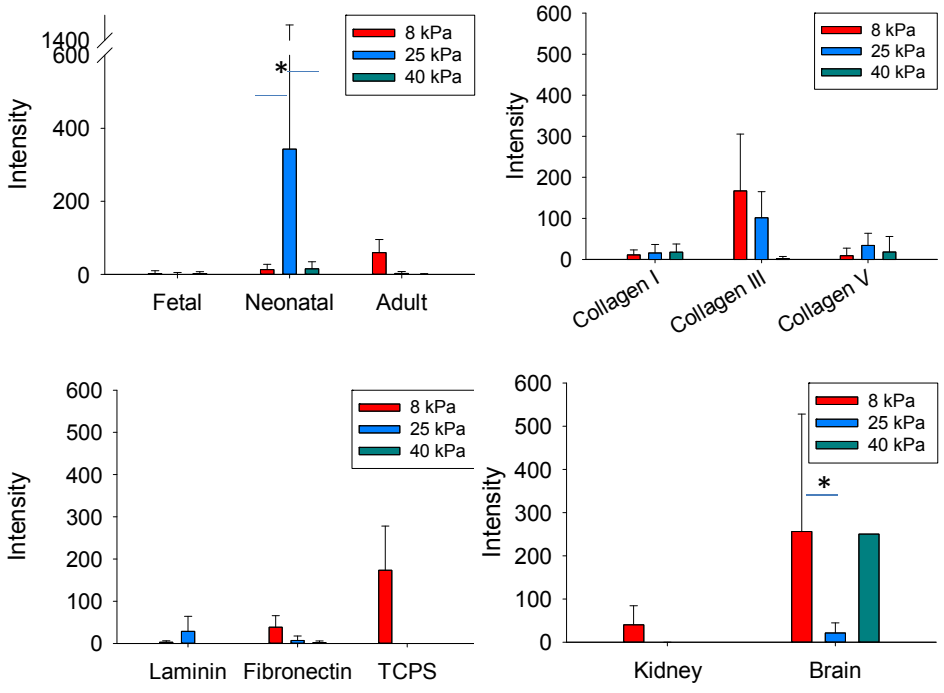

Figure 8. Quantification of the intensity of GATA4 fluorescence of immunohistology images. Conditions are grouped by protein content and arranged by stiffnesses of 8 kPa (red), 25 kPa (blue), and 40 kPa (green). (* signifies $p<0.05$).

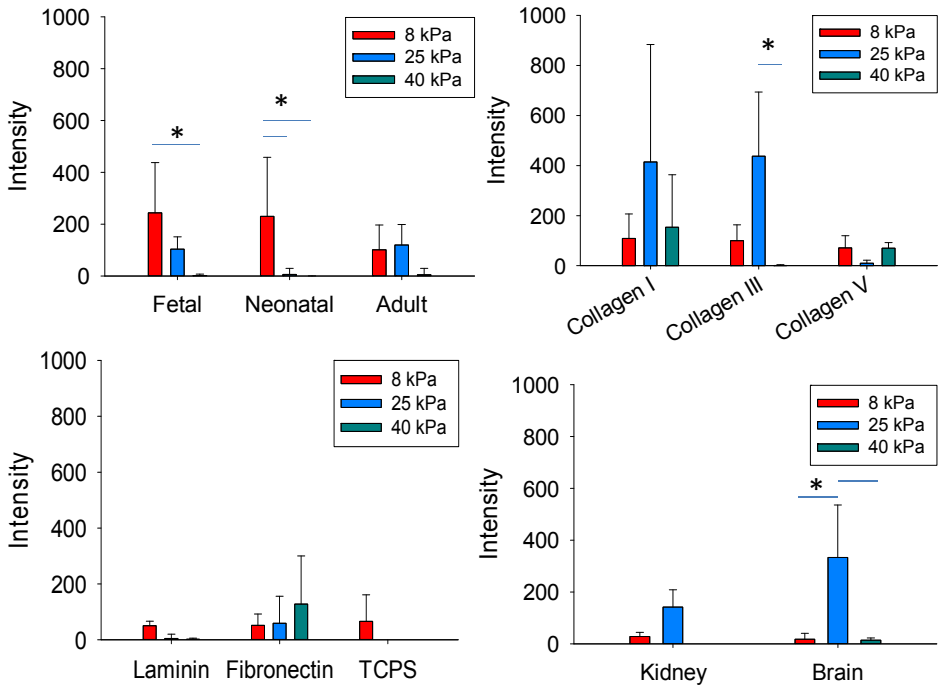

Figure 9. Quantification of MEF2C fluorescence based on immunohistology. Conditions are grouped by protein content and arranged by stiffnesses of 8 kPa (red), 25 kPa (blue), and 40 kPa (green). (* signifies $p < 0.05$)

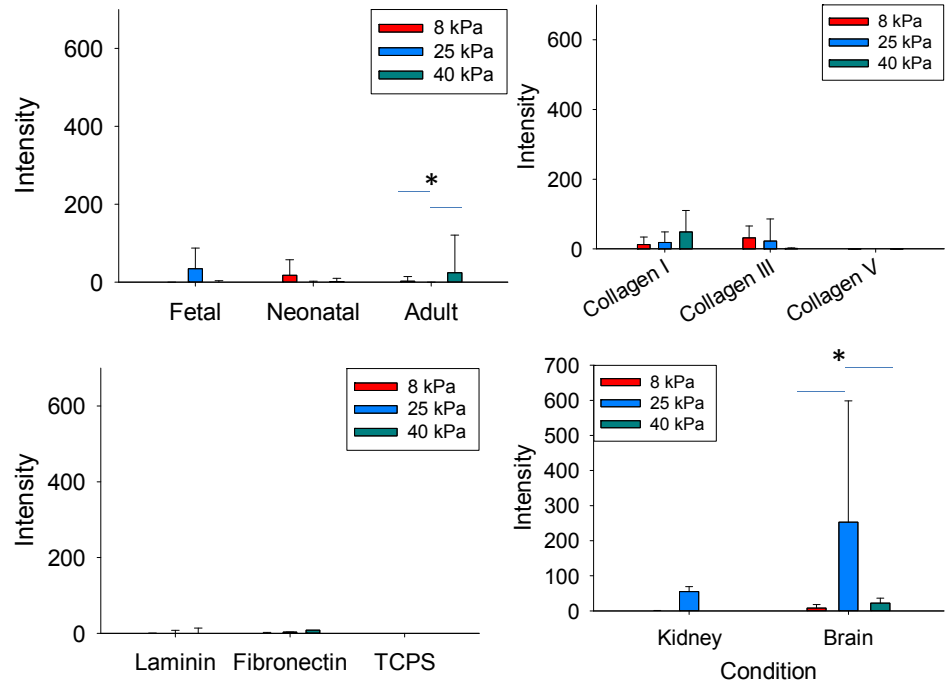

Figure 10. Quantification of alpha actin fluorescence from immunohistology images. Conditions are arranged by protein with stiffnesses of 8 kPa (red), 25 kPa (blue), and 40 kPa (green).

4.1.3 Western blotting for cardiac markers concurred with immunohistochemistry results

Western blotting was used as another method to quantify differentiation of MSCs towards cardiomyocytes. The blots were quantified in ImageJ and normalized to β-actin expression. Due to restrictions of blot size and the number of conditions, only 1 sample per condition was run to analyze trends between ECM protein content and stiffness on MSC differentiation. Figure 11 shows the fold change of the protein of interest (Nkx2.5, GATA4, MEF2C, or αActin) with respect to βActin. Samples with no expression of the protein of interest were not plotted, such as laminin and fibronectin for Nkx2.5.

MEF2C tended to have higher expression at 25 kPa. As expected from histology results, αActin generally had the lowest expression but tended to have highest expression at 25 kPa. Fetal ECM at 8 kPa had the greatest fold change in Nkx2.5 and GATA4. However, this was most likely a result of low βActin concentration rather than a high expression of GATA4 or Nkx2.5. Low βActin expression is most likely due to limited isolation of protein during cell lysis or interference by trypsin and FBS in the BCA assay. Remnants of trypsin and FBS would have indicated a higher concentration of protein than actually available for gel loading. These complications could have affected all conditions; therefore, the ratio of Nkx2.5/GATA4 was analyzed for all conditions containing both Nkx2.5 and GATA4 (Figure 12). The Nkx2.5 ratio to GATA4 should be close to 1 since they are coupled transcription factors (Durocher et al., 1997). However, since Nkx2.5 is found to regulate GATA4, it would not be surprising to have a higher concentration of Nkx2.5 (Riazi et al., 2009). With the major exception of Adult ECM at 40 kPa, the ratio of Nkx2.5 to GATA4 tended to be higher at 8 or 25 kPa, especially in collagen V, which is thought to be the major signal for cardiac differentiation (Tan et al., 2009). The high ratio at 40 kPa Adult ECM could also be a result of low GATA4 expression as seen in Figure 11B. While

these results are preliminary, differentiation still tended to be stronger at 25 or 8 kPa in comparing all conditions, in conjuncture with the quantified immunohistochemistry data.

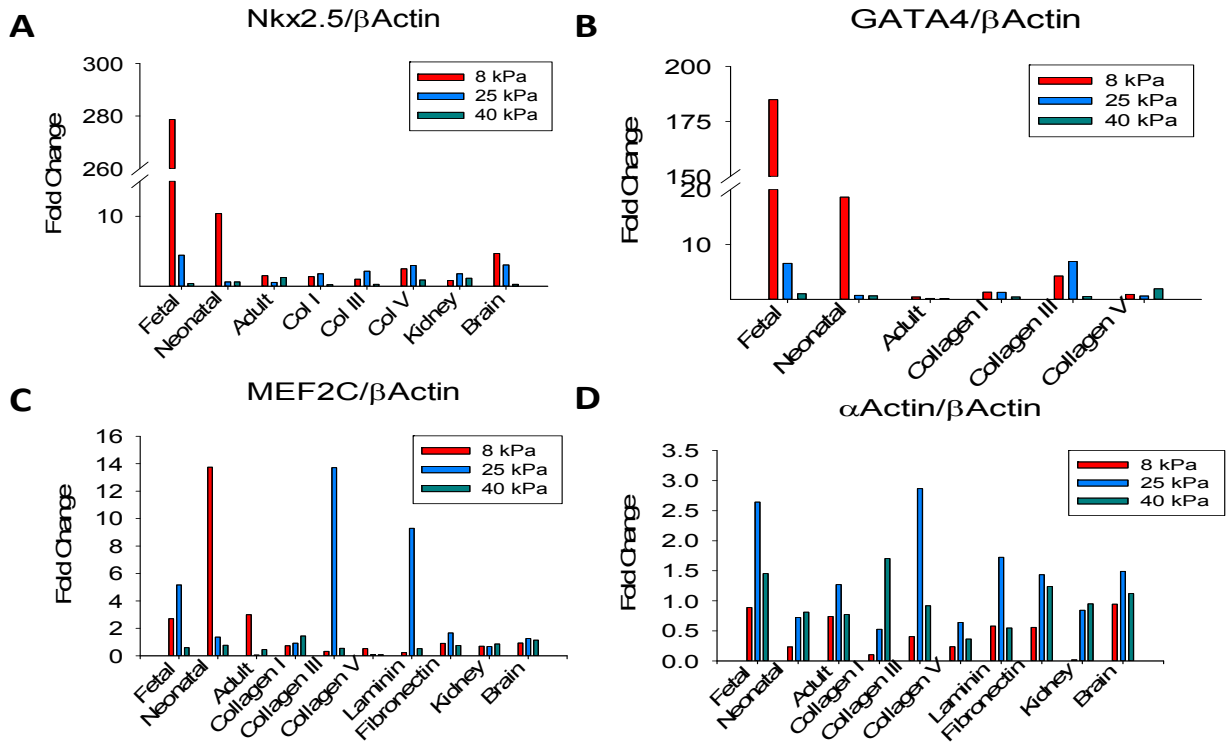

Figure 11. Quantification of Western Blots normalized to beta actin. Parts A-D organize the conditions by protein content and arranged by stiffnesses of 8 kPa (red), 25 kPa (blue), and 40 kPa (green). A. Illustrates the fold change of Nkx2.5 to beta actin of all conditions except fibronectin and laminin, which was not detected in the Western blots. B. Depicts the fold change of GATA4 normalized to beta actin. All conditions not shown did not have expression of GATA4. C. Depicts fold change of MEF2C normalized to beta actin. D. Depicts fold change of alpha actin expression normalized to beta actin.

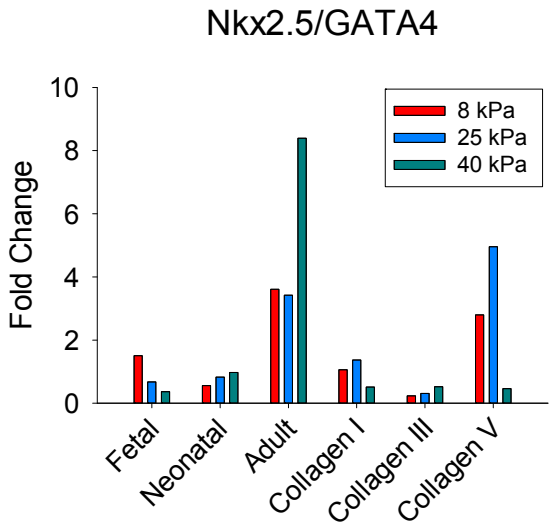

Figure 12. Quantification of Western blot results comparing the Nkx2.5 to GATA4 expression of all blots that detected both proteins. The conditions are arranged by protein content and arranged by stiffnesses of 8 kPa (red), 25 kPa (blue), and 40 kPa (green).

4.2 Infarcted ECM on MSC Differentiation

4.2.1 Immunohistochemistry

To detect MSC differentiation towards cardiomyocytes, each condition was stained for Nkx2.5 and GATA4 (Figures 13-15). Differentiation was assayed on day 4 and 10 of Trial 1 and day 7 and 10 of Trial 2. On day 4, only hypoxic conditions showed indication of differentiation with strong signals of Nkx2.5. All conditions indicated differentiation with expression of GATA4. GATA4 was detected with TRITC, which tended to overpower the GFP Nkx2.5 signal. The signal was also centralized to the nucleus, indicated by the violet color in the overlaid images of Dapi, TRITC, and GFP. Models with normal ECM composition and stiffness tended to have the strongest signal in both hypoxia and normoxia. All conditions showed stronger expression of GATA4 and Nkx2.5 by day 10 in both trials. The signal also tended to spread to the cytoplasm while still maintaining a strong nuclear stain on day 10.

Figure 13. Immunohistology of MSCs grown on PAAm gels representing all 8 of the healthy, partially infarcted, or completely infarcted conditions from Day 4 of Trial 1. Expression of Nkx2.5 (green) and GATA4 (red) are shown and call conditions show Dapi nuclear stain. The images are arranged by oxygen content with stiffness and ECM content labeled. Healthy stiffness indicates 25 kPa gels and infarct stiffness represents 40 kPa gels. Healthy ECM means solubilized adult ECM while infarct ECM is solubilized Adult ECM doped with Collage I. Scale bar = 100um

Figure 14. Depicts immunohistology of MSCs grown on PAAm gels representing healthy or infarct stiffness and healthy or infarct ECM on Day 7 of Trial 2. Nkx2.5 is shown in green and GATA4 in red. All nuclei are stained with Dapi. Images are arranged by oxygen content and labeled with infarct (50 kPa) or healthy (25 kPa) stiffness and infarct (adult ECM doped with collagen I) or healthy (adult ECM) ECM. Scale bar = 100 um

Figure 15. Depicts immunohistology of Nkx2.5 (green) and GATA4 (red) for MSCs grown on the 8 infarct conditions on Day 10 of Trial 2. All images are stained with Dapi nuclear stain. The images are arranged by oxygen content and labeled with stiffness (healthy = 25 kPa; infarct = 50 kPa) and ECM content (infarct = adult ECM doped with collagen I; healthy = adult ECM). Scale bar = 100 um

4.2.2 Quantification of immunohistochemistry for MSC differentiation in an infarct environment

In order to quantify MSC differentiation, the images from histology were analyzed using the CellProfiler pipeline on Day 10 of both trials. In both trials normal ECM content and stiffness in normoxia had the greatest intensity of GATA4 and Nkx2.5 (Figure 16). Hypoxic conditions tended to have a higher GATA4 and Nkx2.5 expression. In Trial 1, hypoxic conditions promoted differentiation fairly evenly across all stiffness and ECM composition conditions and were about equivalent to healthy stiffness, healthy ECM composition of normoxia. A similar trend was found in Trial 2 except in the completely infarcted model, which had a lower expression of Nkx2.5 and GATA4. This could be an aspect of the younger passage

MSCs used in Trial 2 or the different hypoxic incubator used, despite both incubators being set at 5% CO_2.

Two way ANOVAs were run for all of the conditions while keeping one condition constant. For example, stiffness and ECM composition were compared while oxygen content was held constant. All variations of maintaining a condition constant were performed and the Holm-Sidak method was used for all pairwise multiple comparison procedures. Based on the results, there was statistical significance between the conditions, most notably between hypoxia and normoxia (Figure 16). However, neither ECM composition nor stiffness had greater statistical impact on differentiation within a level of oxygen.

Figure 16. Depicts quantification of Nkx2.5 and GATA4 expression from immunohistology images. Conditions are labeled in the order of stiffness, ECM composition, and oxygen content where N= normal/healthy and I = infarct. For stiffness N = 25

kPa and I = 50 kPa. Normal ECM composition is composed of solubilized adult rat ECM while infarct ECM indicates solubilized adult ECM doped with collagen I. Infarct oxygen content is 5% O_2 while healthy oxygen content is 20%. A. Quantification of images from Day 10 of Trial 1. B. Quantification of images from Day 10 of Trial 2. (* indicates $p < 0.05$)

4.2.3 Western blotting to confirm immunohistochemistry results of MSC differentiation

Western blotting was used as an alternative method for semi-quantitative analysis of differentiation. The blots were probed for Nkx2.5 and GATA4. The intensity of expression was quantified on ImageJ and normalized to βActin. Results are displayed in Figure 17. While we aimed to load equal concentrations of each sample, Figure 17A shows a clear inequality of protein per sample because βActin should have similar expression if the same protein concentration is loaded. This is most likely a result of a predicted higher protein concentration compared to actuality in the BCA assay because remnants of trypsin and FBS could interfere with absorption patterns. However, the expression of Nkx2.5 and GATA4 showed the same trend as compared to the quantified histology: the completely healthy condition tended to have the highest rate of differentiation. The trend of higher Nkx2.5 and GATA4 expression in all hypoxia conditions except the completely infarcted model was also observed (Figure 17B and C).

The ratio of Nkx2.5/GATA4 was also examined since they are coupled transcription factors. The completely healthy condition tended to have a higher Nkx2.5 expression as well as the normal stiffness, normal ECM composition and hypoxic environment. A higher Nkx2.5 expression might be normal because Nkx2.5 regulates GATA4 (Riazi et al., 2009). Surprisingly, the completely infarcted condition also had a similar Nkx2.5/GATA4 ratio but had a large standard deviation.

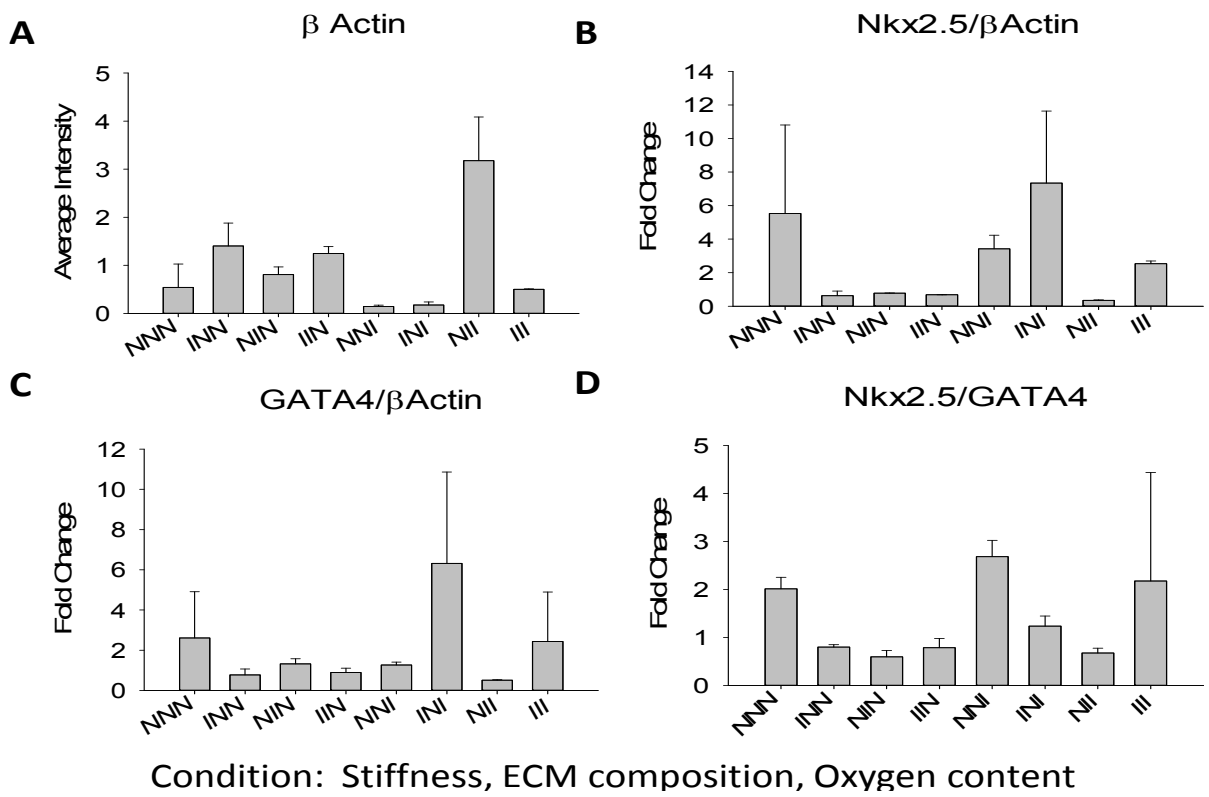

Figure 17. Quantification of Western Blotting of all 8 models of the infarct experiment from Day 10 of Trial 1. Conditions are listed as stiffness, ECM composition, and oxygen content where N = normal/healthy and I = infarct. For stiffness N = 25 kPa and I = 50 kPa. Normal ECM composition is composed of solubilized adult rat ECM while infarct ECM indicates solubilized adult ECM doped with collagen I. Infarct oxygen content is 5% O_2 while healthy oxygen content is 20%. A. Average intensity of beta actin. B. Fold change of Nkx2.5, normalized to beta actin. C. Fold change of GATA4 normalized to beta actin C. Ratio of Nkx2.5 to GATA4 expression, both of which were normalized to beta actin.

4.3 The effect of 5-Azacytidine on MSC Differentiation

4.3.1 Changes in MSC morphology to indicate differentiation

Three trials were run in an attempt to differentiate MSCs to cardiomyocytes using 5-azacytidine. In the first trial using the hanging drop suspension method, little to no cells were collected after the two hour suspension treatment. Representative images of the MSCs in the 5-azacytidine differentiation media and differentiation media without 5-azacytidine are shown in Figure 18. In this figure, the cells in the suspension treatment were determined to be dead based on their balled up morphology. However, the morphology of the cells in the monolayer treatment looked promising as initially they appeared to be adopting the "ball- or stick-

morphology" indicative of myotube-cell differentiation (Liu et al., 2003). However, most of the cells died after 1 week and the trial was terminated.

Figure 18. Representative images of MSCs from Day 5 of Trial 1 organized by suspension or monolayer treatment. Only conditions grown in differentiation media are shown as the other conditions showed similar results. 5-azacytidine treatments are shown in columns 1 and 3 while differentiation medium with no 5-azacytidine treatment is shown in columns 2 and 4. The adsorbed protein conditions are labeled on the left. Scale bar = 100 um

Based on the results of Trial 1, we adjusted the differentiation media in hopes of preventing cell death by adding 10% FBS and modified the suspension method for increased cell collection. While more cells survived the suspension treatment, the addition of serum to the media resulted in greater proliferation, which inhibits differentiation (van Oorschot et al, 2011) (Figure 19). Therefore, the trial was terminated after a week for excessive cell proliferation.

Figure 19. Representative images from Trial 2 of MSCs treated with and without 5-azacytidine and differentiation media with 10% FBS are shown. Adsorbed protein conditions are listed on the left and the images are separated by suspension or monolayer treatment. Scale bar = 100um

For the third trial, lower percentages of FBS were used in hopes of preventing rapid proliferation. Differentiation media with 5%, 2%, and 0% serum were used. We found that higher levels of serum correlated with greater proliferation, where 0% FBS resulted in cell death. MSCs became over-confluent at the end of 2 weeks and were fixed before reaching the 3 week point where Miskon et al. (2010) observed beating cells (Figure 20).

Figure 20. Representative images of Trial 3 depicting MSCs grown with 2% serum differentiation media with or without 5-azacytidine. The adsorbed protein conditions are shown on the left. Images are arranged by suspension or monolayer methods. Conditions not shown had similar results in terms of MSC morphology and proliferation. Scale bar = 100 um

4.3.2 Immunohistochemistry

MSCs in Trial 3 were fixed and stained for Nkx2.5 and GATA4. Based on Miskon et al.'s (2010) results of highest differentiation rates found on gelatin, only the gelatin and ECM adsorbed conditions were stained and TCPS was stained as a control (Figure 21). The complete ECM adsorbed condition was stained since Miskon et al. did not use this condition. Compared to the positive control of cardiomyocytes, MSCs were not completely differentiated. Similar signals were detected in MSCs grown with differentiation media without 5-azacytidine as

compared to those with the 5-azacytidine treatment, especially for Nkx2.5 images. This could be a result of the treated TCPS, where the adsorbed ECM proteins influenced differentiation to a greater extent than 5-azacytidine.

Figure 21. Representative images of immunohistology from Trial 3 MSCs on day 10 of only the suspension method. Expression of Nkx2.5 (green) and GATA4 (red) are shown and all conditions display Dapi nuclear stain. The first three columns depict MSCs while the last two columns are the positive controls of isolated native neonatal cardiomyocytes. The adsorbed proteins are arranged by rows and labeled on the left. The different media treatments are arranged by columns and labeled above the images. Only suspension images are displayed because these conditions were more likely to cause differentiation than the monolayer treatments based on prior studies. Scale bar = 100 um

5. Discussion

The goal of this study was to analyze the differentiation of MSCs to cardiomyocytes when cultured in a mimic of the extracellular environment. We created healthy and infarct 2-dimensional models of varying stiffnesses and cardiac ECM content to drive differentiation. These results were compared to accepted chemical methods of differentiating MSCs with 5-azacytidine to determine the efficiency of our method. The 2-dimensional models infused with ECM proteins and varying stiffnesses allowed us to investigate the effects of the different

components of the extracellular environment on MSC differentiation. Then, by creating disease models, the impact of changes to the extracellular environment as a result of MI on MSC differentiation was studied.

5.1 Variation of ECM Age on MSC Differentiation

Differentiation of MSCs based on the extracellular matrix elasticity (Engler et al., 2006) and ECM composition (Tan et al., 2010; Bowers et al., 2010) have previously been studied independently. Since the mechanical and elastic properties of the heart change with development (Engler et al., 2006; Reilly et al., 2010), we designed a model incorporating the biologically relevant elasticities of the heart with solubilized ECM at different age points. Differentiation of MSCs was qualitatively determined with immunohistochemistry by expression of Nkx2.5, GATA4, MEF2C, and αActin.

Qualitatively, all conditions induced expression of Nkx2.5 and GATA4. There was little to no signal for the further downstream cardiac developmental markers, MEF2C and αActin, but the naturally derived ECM gels at 8 kPa and 25 kPa had the most prominent expression. Collagen III at 25 kPa and fibronectin at 40 kPa also had strong expression of MEF2C. As expected, Nkx2.5 and GATA4 expression was strongly translocated to the nuclei (Arminin et al., 2009; Liu et al., 2003) with lower expression in the cytoplasm. MEF2C (Nadruz et al*.,* 2005) and cytoplasmic αActin were unsurprisingly more commonly found in the cytoplasm. The presence of MEF2C in the cytoplasm supports previous results of a FAK-mediated MEF2C activation (Nadruz et al*.,* 2005). The presence of αActin in the cytoplasm supports its functional role in muscle contraction (Nowak et al., 1999). Immunohistochemistry illustrated the presence of early cardiac specific transcription factors but showed limited expression of mature cardiac markers, suggesting that after 1 week, cardiogenesis began but was not fully developed.

Differentiation was quantified using a CellProfiler pipeline, which selected cells by a Hoechst nuclear Dapi stain, automatically expanded the area to approximate the size of the cell, and measured the intensity of GFP and TRITC fluorescence. The quantitative data correlated with conclusions drawn from histological observations and confirmed the presence of the cardiac marker with ECM protein composition and stiffness. In the models using complete ECM protein composition, there tended to be greater expression at physiologically relevant stiffnesses of 8 or 25 kPa compared to 40 kPa for Nkx2.5, GATA4, MEF2C, and αActin with statistical significance ($P<0.05$) in Nkx2.5 for adult ECM at 8 kPa and neonatal ECM at 25 kPa. GATA4 expression had statistical significance at neonatal ECM 25 kPa while MEF2C had significance at neonatal ECM at 8 kPa and fetal ECM at 8 kPa compared to their respective 40 kPa conditions. Finally, αActin had significance at 8 kPa and 40 kPa over 25 kPa. Therefore, when elasticity is combined with substrate composition to model the extracellular environment, MSC differentiation is not strictly dependent on the individual factor but rather a synergistic effect. Otherwise, a decreasing linear trend would have been observed with increasing stiffness at the fetal condition because 8 kPa most accurately represents fetal elastic properties (Engler et al., 2006). Similarly, the adult and neonatal conditions should have both peaked at 25 kPa.

Contrary to our hypothesis, individualized ECM protein models expressed similar levels of intensity for the four cardiac markers. Using major ECM proteins, such as collagen, to represent the ECM environment is not uncommon in differentiation studies (Tan et al, 2010). However, our results show that individual proteins tend to optimize differentiation at different stiffnesses compared to complete ECM models. This may be a result of increased adhesion on the purified single ECM protein gels. The individual proteins were purchased for cell studies, where they are processed for optimized cell interactions. Thus, with a higher percentage of the

gel covered in bound MSCs, there could be a greater amount of paracrine signaling for cardiac differentiation.

The results of quantifying immunohistochemistry were confirmed through Western Blotting. An increase in Nkx2.5 and GATA4 were seen at the lower, physiologically relevant stiffnesses and MEF2C and αActin were expressed at lower rates. While the results for cardiac differentiation markers were normalized to βactin, the variation of βactin in each sample could still have skewed the results of cardiac marker expression, such as at 8 kPa fetal ECM. Therefore, the Nkx2.5/GATA4 ratio was determined.

As expected, the ratio of Nkx2.5/GATA4 was close to one in all conditions since Nkx2.5 regulates GATA4. Therefore, we can deduce that cardiogenesis has been initiated in the MSCs, while low expression of MEF2C and αActin illustrate that the cells have not yet reached maturity. While 40 kPa adult ECM had the highest ratio, it may have been an effect of the low expression of GATA4. Furthermore, there was difficulty in loading equal concentrations of protein across all samples as seen by varying levels of βactin despite protein concentrations determined by BCA analysis. Despite low protein expression and sample sizes of 1, a similar trend was observed with respect to the quantified histology with higher expression in the complete ECM conditions and some high expression in the collagen only conditions.

5.2 Infarcted ECM on MSC Differentiation

After studying the effects of healthy ECM composition and stiffnesses, we aimed to determine how changes to the ECM and stiffness as a result of MI impacted MSC differentiation. MI causes a negative remodeling process that results in increased collagen deposition and scar formation that increases the stiffness of the ECM (Young et al., 2010; Pelouch et al., 1994). With blockage of the coronary artery, the microenvironment also turns hypoxic (Ohnishi et al.,

2007). In order to determine individual and synergistic effects of the changes in ECM and stiffness, we created 8 models with different combinations of healthy and infarct ECM, oxygen content, and stiffness. After 4, 7 and 10 days, differentiation was analyzed by immunohistochemsitry to detect the presence of Nkx2.5 and GATA4.

Based on the histological images, all conditions induced cardiogenesis. The experiment was repeated twice where Day 4 and 10 were analyzed for differentiation in the first trial and the second trial examined Day 7 and 10. Both trials showed differentiation at all time points and qualitatively, the fluorescent intensity increased with later time points. By Day 10, both Nkx2.5 and GATA4 was highly expressed in the control of all conditions representing a healthy cardiac extracellular environment. This suggests that with time the MSCs will develop into more mature cardiomyocytes as predicted for the healthy cardiac environment.

On Day 4, only the hypoxic conditions stained positively for Nkx2.5 and GATA4. This trend of higher fluorescence in hypoxic conditions compared to normoxic continued through the duration of the trial with intensities reaching similar levels to the model with all healthy components. While initially unexpected, these results can be explained by the nature of MSCs. Derived from the bone marrow, MSCs are native to a hypoxic environment and have been shown to thrive under such *in vitro* conditions (Das et al., 2010). Additionally, a hypoxic environment is critical to cardiogenesis during fetal development (Patterson and Zhang, 2010). Therefore, a variety of studies have actually studied using hypoxia as a means of guiding MSC differentiation (Das et al., 2010; van Oorschot et al., 2011). While our results show a higher fluorescence to indicate differentiation in hypoxic environments compared to normoxic, it is important to note that fluorescence tended to decrease for both GATA4 and Nkx2.5 in the condition with most factors representing infarction as shown in the Figure 16. Therefore, the combination of infarct

stiffness, infarct ECM composition, and hypoxia most likely causes a negative synergistic effect to prevent MSC differentiation, which confirms observations in current cell therapy clinical trials (Cleland et al., 2006).

In Trial 2, the fluorescence was more translocated to the nucleus compared to Trial 1. Since Nkx2.5 and GATA4 are transcription factors, they are expected to be active in the nucleus. Therefore, their translocated expression indicates that the proteins are expressed in the correct location for functionality to promote cardiac differentiation. While the cause of this is unclear, it could be a result of different cell lines used as MSCs from Texas A&M were used in Trial 1 while MSCs used from Cell Apparatus was used in Trial 2. It is important for future studies to determine where Nkx2.5 and GATA4 are truly being expressed as they are only functional in the nuclei.

The quantitative histology was confirmed by Western Blotting with similar trends of Nkx2.5 and GATA4 expression. The completely healthy model tended to have the highest expression of both GATA4 and Nkx2.5. As seen in histology, the hypoxic conditions, excluding the completely infarcted model, also had higher expression of Nkx2.5 and GATA4, further indicating that hypoxic environments can promote cardiac differentiation. While the results were not statistically significant, when taken in tandem with the quantified histology, they clearly show a trend of cardiac differentiation, where the completely infarcted condition has one of the lowest abilities while the completely healthy model had the highest ability.

5.3 The effect of 5-azacytidine on MSC differentiation

Prior studies have successfully differentiated MSCs to cardiomyocytes with spontaneous beating (Miskon et al., 2010; Wakitani et al., 1995; Zhang et al., 2009; Song et al., 2003). After three trials of differentiating MSCs using 5-azacytidine, we were unable to carry out the

experiment to three weeks, the experimental time point where differentiated MSCs were observed to beat (Miskon et al., 2010). Instead, we had difficulty in mimicking the suspension method with the resources available in our lab and despite replication of Miskon et al.'s (2010) differentiation media, the cells did not survive in serum free media. Low to zero serum in the media is desired because high serum tends to promote proliferation and prevent differentiation.

Despite a lack of beating, we performed immunohistochemistry to determine if early cardiogenesis had occurred. Based on the images, Nkx2.5 and GATA4 were present with a weak fluorescent signal after 10 days. However, the fluorescence was not translocated to the nuclei as expected when comparing to the positive control. It is unclear as to why the signal was detected in the cytoplasm as Nkx2.5 and GATA4 are coupled transcription factors that work in the nucleus.

Additionally, it appears that adsorbed ECM proteins influence MSC differentiation along with 5-azacytidine, corroborating results of Miskon et al. (2010). Little signal was detected from MSCs grown on TCPS while the strongest signal was found on MSCs grown on complete ECM protein composition. Differentiation is likely due to a synergistic effect. In comparing the 5-azacytidine treatment in terms of ease, viability, and differentiation potential to the PAAm gel models, it can be concluded that differentiation of MSCs using 2-dimensional models of the extracellular environment is more efficient than 5-azacytidine.

5.4 Conclusions

The 2-dimensional PAAm model with crosslinked solubilized ECM is an effective method of differentiating MSCs towards cardiomyocytes as shown by the expression of early cardiac differentiation markers. This system can be further expanded to create *in vitro* disease models of MI. As hypothesized, the biologically relevant stiffnesses combined with complete

ECM induced differentiation better than the elevated stiffness. Furthermore, by creating controls of models with single ECM proteins, differentiation appears to be influenced by the strong presence of collagen I in cardiac ECM as well as collagen V and fibronectin. Post MI, the model was still able to promote differentiation towards cardiomyocytes; however, compared to normal, healthy ECM conditions, models with elements representing infarction had decreased levels of differentiation. Overall, it was more effective to re-create the natural cardiac microenvironment to promote differentiation compared to using 5-azacytidine treatments. Therefore, the PAAm gel models of the cardiac extracellular environment have potential to serve as a means of pre-differentiating MSCs for cell therapy by addressing the current limitations in MI therapeutics.

6. Future Directions

As more is learned and understood about the extracellular matrix, the potential to differentiate MSCs solely based on creating the organ-specific microenvironment expands to improve MSC cell therapy treatments and ultimately, mitigate myocardial infarction. Therefore, further studies in the short and long term should be explored. Short term studies should focus on improving the limitations of this study, especially in quantifying differentiation and optimization of the ECM protein infused PAAM gel model. Long term experiments should focus on determining the viability of the model at longer time points and enhance for cell therapy.

First, greater analysis using similar experimental conditions should be run to improve quantification methods of differentiation. Firstly, Western blotting must be improved by improving the cell lysis process for accurate loading. While Western blots provides a good semi-quantitative analysis of differentiation, RT PCR could be used for a more accurate and precise measurement of a variety of cardiac differentiation markers. Finally in the short term, real

infarct ECM at several time points, such as 1 week, 2 week, and 1 month after infarction, should be used to analyze the impact of complete infarcted ECM on MSC differentiation. The variety of time points would allow analysis of how the different levels remodeling impact differentiation.

Before use at a therapeutic or large scale, the PAAm model needs to be optimized for MSC growth. Adhesion of MSCs to the gels must be optimized as a great limitation to the study was a low number of cells found on the PAAm gels. Since MSCs only bind to the ECM proteins infused in the cell, the decellularization and solubilization method may need to be optimized for improved cell adhesion. Compared to purified ECM proteins, the harsh pepsin digestion may inactivate some of the proteins for adhesion or incorporation into the PAAm gels could limit exposure of binding sites at the surface of the gel. Therefore, alternative methods of incorporating ECM could also be studied, such as imprinting.

Next, longer duration studies should be performed to determine longevity of differentiation. After one week, there was only an indication of early cardiac differentiation with expression of Nkx2.5 and GATA4 while the later markers of MEF2C and αActin were limited. Therefore, longer studies should be performed to determine if MSCs will ever differentiate to mature cardiomyocytes with contractile capabilities or if they will de-differentiate, revert back to stem cells, or differentiate towards a different lineage.

Finally, in accordance with the long term goals of the study, the PAAm gel model and use of ECM for differentiation should be expanded to other cell or tissue types. Alternative cell types could be used to determine if this method is effective such as induced pluripotent stem cells or embryonic stem cells. Furthermore, the different cell types can be combined with different ECM

to differentiate cells to a variety of tissue types. Therefore, the PAAm-ECM model can be used for a variety of cell therapy treatments and developed into different disease models.

7. Appendix

7.1 Statistical Analysis for the MSC Differentiation Experiment

Two way ANOVAs were run for the data obtained by quantifying the intensity of Nkx2.5, GATA4, MEF2C, and αActin from the MSC differentiation experiment. The two variables were the ECM protein type and stiffness. SigmaPlot was used to perform the analysis where $p < 0.05$ was considered to have statistically significant difference of means. The Holm-Sidak method was used to perform pairwise multiple comparisons within groups in conditions with significance.

Nkx2.5

Two Way Analysis of Variance Thursday, April 12, 2012, 12:13:36 PM

Data source: Nkx data in Anovas

General Linear Model

Dependent Variable: Intensity

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
ECM Protein	9	2855119.766	317235.530	10.581	<0.001
Stiffness	2	490395.990	245197.995	8.179	<0.001
ECM Protein x Stiffness	18	8675677.126	481982.063	16.077	<0.001
Residual	839	25153509.088	29980.345		
Total	868	38700236.205	44585.526		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of ECM Protein depends on what level of Stiffness is present. There is a statistically significant interaction between ECM Protein and Stiffness. (P = <0.001)

Power of performed test with alpha = 0.0500: for ECM Protein : 1.000

Power of performed test with alpha = 0.0500: for Stiffness : 0.947
Power of performed test with alpha = 0.0500: for ECM Protein x Stiffness : 1.000

Least square means for ECM Protein :

Group	Mean	SEM
Adult	93.482	31.276
Brain	170.005	60.758
Col I	232.868	17.944
Col III	135.369	25.981
Col V	176.386	21.957
Fetal	58.545	13.666
Fn	269.642	24.467
Kidney	162.978	44.440
Ln	149.479	33.890
Neonatal	139.954	19.615

Least square means for Stiffness :

Group	Mean	SEM
8.000	210.975	14.368
25.000	158.993	14.584
40.000	106.643	22.818

Least square means for ECM Protein x Stiffness :

Group	Mean	SEM
Adult x 8.000	238.139	46.276
Adult x 25.000	28.388	57.716
Adult x 40.000	13.919	57.716
Brain x 8.000	232.131	40.811
Brain x 25.000	106.492	39.723
Brain x 40.000	171.392	173.148
Col I x 8.000	153.246	43.287
Col I x 25.000	183.951	24.011
Col I x 40.000	361.408	21.153
Col III x 8.000	188.309	49.984
Col III x 25.000	209.432	44.707
Col III x 40.000	8.366	39.723
Col V x 8.000	111.331	54.754
Col V x 25.000	174.782	15.186
Col V x 40.000	243.045	33.322
Fetal x 8.000	35.042	17.765
Fetal x 25.000	85.224	29.695
Fetal x 40.000	55.369	21.990
Fn x 8.000	610.767	36.915
Fn x 25.000	122.487	16.661
Fn x 40.000	75.671	61.217
Kidney x 8.000	434.331	65.444
Kidney x 25.000	54.603	86.574
Kidney x 40.000	-4.938E-013	77.434
Ln x 8.000	17.211	48.023
Ln x 25.000	309.326	61.217
Ln x 40.000	121.898	65.444
Neonatal x 8.000	89.248	34.630
Neonatal x 25.000	315.246	33.957
Neonatal x 40.000	15.367	33.322

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **ECM Protein**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Col I vs. Fetal	174.323	7.729	<0.001	Yes
Fn vs. Fetal	211.097	7.532	<0.001	Yes
Col V vs. Fetal	117.841	4.556	<0.001	Yes
Fn vs. Adult	176.160	4.436	<0.001	Yes
Fn vs. Neonatal	129.688	4.136	0.002	Yes
Col I vs. Adult	139.386	3.866	0.005	Yes
Fn vs. Col III	134.273	3.762	0.007	Yes
Col I vs. Neonatal	92.914	3.495	0.019	Yes
Neonatal vs. Fetal	81.409	3.405	0.025	Yes
Col I vs. Col III	97.499	3.088	0.072	No
Fn vs. Ln	120.163	2.875	0.135	No
Fn vs. Col V	93.256	2.837	0.147	No
Col III vs. Fetal	76.824	2.617	0.259	No
Ln vs. Fetal	90.934	2.489	0.343	No
Kidney vs. Fetal	104.433	2.246	0.543	No
Col I vs. Ln	83.390	2.175	0.598	No
Col V vs. Adult	82.904	2.169	0.591	No
Fn vs. Kidney	106.664	2.103	0.640	No
Col I vs. Col V	56.482	1.992	0.725	No
Brain vs. Fetal	111.460	1.790	0.864	No
Fn vs. Brain	99.637	1.521	0.968	No
Col I vs. Kidney	69.890	1.458	0.977	No
Kidney vs. Adult	69.496	1.279	0.994	No
Neonatal vs. Adult	46.472	1.259	0.994	No
Col V vs. Neonatal	36.432	1.237	0.994	No
Ln vs. Adult	55.997	1.214	0.994	No
Fn vs. Col I	36.774	1.212	0.992	No
Col V vs. Col III	41.017	1.206	0.991	No
Brain vs. Adult	76.523	1.120	0.994	No
Col III vs. Adult	41.887	1.030	0.997	No
Adult vs. Fetal	34.937	1.024	0.996	No
Col I vs. Brain	62.863	0.992	0.996	No
Col V vs. Ln	26.907	0.666	1.000	No
Kidney vs. Col III	27.609	0.536	1.000	No
Brain vs. Col III	34.636	0.524	1.000	No
Kidney vs. Neonatal	23.024	0.474	1.000	No
Brain vs. Neonatal	30.051	0.471	1.000	No
Ln vs. Col III	14.110	0.330	1.000	No
Brain vs. Ln	20.527	0.295	1.000	No
Col V vs. Kidney	13.408	0.270	1.000	No
Ln vs. Neonatal	9.525	0.243	1.000	No
Kidney vs. Ln	13.499	0.242	0.999	No
Neonatal vs. Col III	4.585	0.141	0.999	No
Col V vs. Brain	6.381	0.0988	0.994	No
Brain vs. Kidney	7.027	0.0934	0.926	No

Comparisons for factor: **Stiffness**

Comparison	Diff of Means	t	P	P<0.050
8.000 vs. 40.000	104.332	3.869	<0.001	Yes
8.000 vs. 25.000	51.982	2.539	0.022	Yes
25.000 vs. 40.000	52.350	1.933	0.054	No

Comparisons for factor: **Stiffness within Adult**

Comparison	Diff of Means	t	P	P<0.05
8.000 vs. 40.000	224.220	3.031	0.008	Yes
8.000 vs. 25.000	209.751	2.835	0.009	Yes
25.000 vs. 40.000	14.470	0.177	0.859	No

Comparisons for factor: **Stiffness within Brain**

Comparison	Diff of Means	t	P	P<0.05
8.000 vs. 25.000	125.639	2.206	0.081	No
40.000 vs. 25.000	64.900	0.365	0.919	No
8.000 vs. 40.000	60.739	0.341	0.733	No

Comparisons for factor: **Stiffness within Col I**

Comparison	Diff of Means	t	P	P<0.05
40.000 vs. 25.000	177.457	5.545	<0.001	Yes
40.000 vs. 8.000	208.162	4.321	<0.001	Yes
25.000 vs. 8.000	30.705	0.620	0.535	No

Comparisons for factor: **Stiffness within Col III**

Comparison	Diff of Means	t	P	P<0.05
25.000 vs. 40.000	201.066	3.362	0.002	Yes
8.000 vs. 40.000	179.944	2.818	0.010	Yes
25.000 vs. 8.000	21.122	0.315	0.753	No

Comparisons for factor: **Stiffness within Col V**

Comparison	Diff of Means	t	P	P<0.05
40.000 vs. 8.000	131.714	2.055	0.116	No
40.000 vs. 25.000	68.263	1.864	0.121	No
25.000 vs. 8.000	63.451	1.117	0.264	No

Comparisons for factor: **Stiffness within Fetal**

Comparison	Diff of Means	t	P	P<0.05
25.000 vs. 8.000	50.182	1.450	0.380	No
25.000 vs. 40.000	29.854	0.808	0.663	No
40.000 vs. 8.000	20.327	0.719	0.472	No

Comparisons for factor: **Stiffness within Fn**

Comparison	Diff of Means	t	P	P<0.05
8.000 vs. 25.000	488.280	12.056	<0.001	Yes
8.000 vs. 40.000	535.096	7.485	<0.001	Yes
25.000 vs. 40.000	46.816	0.738	0.461	No

Comparisons for factor: **Stiffness within Kidney**

Comparison	Diff of Means	t	P	P<0.05
8.000 vs. 40.000	434.331	4.284	<0.001	Yes
8.000 vs. 25.000	379.728	3.499	<0.001	Yes
25.000 vs. 40.000	54.603	0.470	0.638	No

Comparisons for factor: **Stiffness within Ln**

Comparison	Diff of Means	t	P	P<0.05
25.000 vs. 8.000	292.115	3.754	<0.001	Yes
25.000 vs. 40.000	187.428	2.092	0.072	No
40.000 vs. 8.000	104.687	1.290	0.198	No

Comparisons for factor: **Stiffness within Neonatal**

Comparison	Diff of Means	t	P	P<0.05
25.000 vs. 40.000	299.879	6.303	<0.001	Yes
25.000 vs. 8.000	225.999	4.660	<0.001	Yes
8.000 vs. 40.000	73.880	1.537	0.125	No

Comparisons for factor: **ECM Protein within 8**

Comparison	Diff of Means	t	P	P<0.05
Fn vs. Fetal	575.725	14.053	<0.001	Yes
Fn vs. Neonatal	521.519	10.303	<0.001	Yes
Fn vs. Ln	593.556	9.799	<0.001	Yes
Fn vs. Col I	457.521	8.042	<0.001	Yes
Fn vs. Col V	499.436	7.563	<0.001	Yes
Fn vs. Brain	378.636	6.881	<0.001	Yes
Fn vs. Col III	422.457	6.799	<0.001	Yes
Fn vs. Adult	372.628	6.295	<0.001	Yes
Kidney vs. Fetal	399.289	5.888	<0.001	Yes
Kidney vs. Ln	417.120	5.139	<0.001	Yes
Kidney vs. Neonatal	345.084	4.661	<0.001	Yes
Brain vs. Fetal	197.089	4.428	<0.001	Yes
Adult vs. Fetal	203.097	4.097	0.002	Yes
Kidney vs. Col V	323.000	3.785	0.005	Yes
Kidney vs. Col I	281.085	3.582	0.011	Yes
Brain vs. Ln	214.920	3.410	0.020	Yes
Adult vs. Ln	220.928	3.313	0.028	Yes
Kidney vs. Col III	246.022	2.988	0.078	No
Col III vs. Fetal	153.268	2.889	0.102	No
Brain vs. Neonatal	142.883	2.670	0.183	No
Kidney vs. Brain	202.200	2.622	0.200	No
Adult vs. Neonatal	148.891	2.576	0.217	No
Col I vs. Fetal	118.204	2.526	0.237	No
Col III vs. Ln	171.098	2.468	0.263	No
Kidney vs. Adult	196.192	2.448	0.265	No
Fn vs. Kidney	176.436	2.348	0.320	No
Col I vs. Ln	136.035	2.104	0.498	No
Brain vs. Col V	120.800	1.769	0.765	No
Adult vs. Col V	126.808	1.769	0.745	No
Col III vs. Neonatal	99.062	1.629	0.826	No
Neonatal vs. Fetal	54.206	1.393	0.932	No
Adult vs. Col I	84.893	1.340	0.939	No
Brain vs. Col I	78.885	1.326	0.930	No
Col V vs. Fetal	76.289	1.325	0.915	No

Col V vs. Ln	94.120	1.292	0.910	No
Neonatal vs. Ln	72.036	1.217	0.921	No
Col I vs. Neonatal	63.998	1.154	0.924	No
Col III vs. Col V	76.978	1.038	0.942	No
Adult vs. Col III	49.829	0.732	0.987	No
Brain vs. Col III	43.821	0.679	0.984	No
Col I vs. Col V	41.915	0.601	0.981	No
Col III vs. Col I	35.064	0.530	0.973	No
Fetal vs. Ln	17.831	0.348	0.980	No
Col V vs. Neonatal	22.083	0.341	0.929	No
Adult vs. Brain	6.008	0.0974	0.922	No

Comparisons for factor: **ECM Protein within 25**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Neonatal vs. Fetal	230.023	5.099	<0.001	Yes
Neonatal vs. Fn	192.759	5.096	<0.001	Yes
Neonatal vs. Adult	286.858	4.284	<0.001	Yes
Neonatal vs. Brain	208.754	3.995	0.003	Yes
Neonatal vs. Col V	140.464	3.776	0.007	Yes
Ln vs. Adult	280.938	3.339	0.035	Yes
Ln vs. Fetal	224.103	3.294	0.039	Yes
Neonatal vs. Col I	131.295	3.157	0.061	No
Ln vs. Fn	186.839	2.945	0.116	No
Neonatal vs. Kidney	260.643	2.803	0.171	No
Ln vs. Brain	202.834	2.779	0.177	No
Col V vs. Fetal	89.558	2.685	0.223	No
Col I vs. Fetal	98.727	2.585	0.280	No
Col I vs. Adult	155.563	2.489	0.343	No
Col III vs. Adult	181.044	2.480	0.341	No
Col V vs. Adult	146.394	2.453	0.352	No
Ln vs. Kidney	254.724	2.402	0.383	No
Col V vs. Fn	52.295	2.320	0.442	No
Col III vs. Fetal	124.208	2.314	0.435	No
Ln vs. Col V	134.545	2.133	0.584	No
Col I vs. Fn	61.464	2.103	0.598	No
Ln vs. Col I	125.376	1.907	0.755	No
Neonatal vs. Col III	105.814	1.885	0.758	No
Col III vs. Fn	86.945	1.822	0.791	No
Col III vs. Brain	102.940	1.721	0.847	No
Col I vs. Brain	77.459	1.669	0.866	No
Col V vs. Brain	68.290	1.606	0.888	No
Col III vs. Kidney	154.829	1.589	0.883	No
Fn vs. Adult	94.099	1.566	0.881	No
Col I vs. Kidney	129.348	1.440	0.926	No
Col V vs. Kidney	120.179	1.367	0.941	No
Ln vs. Col III	99.895	1.318	0.946	No
Brain vs. Adult	78.104	1.115	0.982	No
Fn vs. Fetal	37.263	1.094	0.979	No
Fetal vs. Adult	56.835	0.876	0.995	No
Fn vs. Kidney	67.884	0.770	0.997	No
Col III vs. Col V	34.650	0.734	0.996	No
Brain vs. Kidney	51.889	0.545	0.999	No
Col III vs. Col I	25.481	0.502	0.999	No
Brain vs. Fetal	21.269	0.429	0.999	No
Fn vs. Brain	15.995	0.371	0.998	No

Fetal vs. Kidney	30.621	0.335	0.995	No
Col I vs. Col V	9.169	0.323	0.984	No
Kidney vs. Adult	26.215	0.252	0.960	No
Neonatal vs. Ln	5.920	0.0846	0.933	No

Comparisons for factor: **ECM Protein within 40**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Col I vs. Fetal	306.038	10.030	<0.001	Yes
Col I vs. Neonatal	346.040	8.767	<0.001	Yes
Col I vs. Col III	353.042	7.845	<0.001	Yes
Col I vs. Adult	347.489	5.653	<0.001	Yes
Col V vs. Neonatal	227.678	4.831	<0.001	Yes
Col V vs. Fetal	187.676	4.701	<0.001	Yes
Col V vs. Col III	234.679	4.526	<0.001	Yes
Col I vs. Kidney	361.408	4.502	<0.001	Yes
Col I vs. Fn	285.737	4.412	<0.001	Yes
Col I vs. Ln	239.510	3.482	0.019	Yes
Col V vs. Adult	229.126	3.438	0.021	Yes
Col I vs. Col V	118.363	2.999	0.091	No
Col V vs. Kidney	243.045	2.883	0.125	No
Col V vs. Fn	167.374	2.401	0.414	No
Col V vs. Ln	121.147	1.650	0.961	No
Ln vs. Col III	113.532	1.483	0.989	No
Ln vs. Neonatal	106.531	1.451	0.990	No
Ln vs. Adult	107.980	1.237	0.999	No
Ln vs. Kidney	121.898	1.202	0.999	No
Col I vs. Brain	190.015	1.089	1.000	No
Fetal vs. Col III	47.003	1.035	1.000	No
Fetal vs. Neonatal	40.002	1.002	1.000	No
Ln vs. Fetal	66.529	0.964	1.000	No
Fn vs. Col III	67.305	0.922	1.000	No
Brain vs. Col III	163.026	0.918	1.000	No
Brain vs. Kidney	171.392	0.904	1.000	No
Brain vs. Neonatal	156.025	0.885	1.000	No
Fn vs. Neonatal	60.304	0.865	1.000	No
Brain vs. Adult	157.474	0.863	1.000	No
Fn vs. Kidney	75.671	0.767	1.000	No
Fn vs. Adult	61.753	0.734	1.000	No
Fetal vs. Kidney	55.369	0.688	1.000	No
Fetal vs. Adult	41.451	0.671	1.000	No
Brain vs. Fetal	116.023	0.665	1.000	No
Brain vs. Fn	95.721	0.521	1.000	No
Ln vs. Fn	46.227	0.516	1.000	No
Col V vs. Brain	71.653	0.406	1.000	No
Fn vs. Fetal	20.302	0.312	1.000	No
Brain vs. Ln	49.494	0.267	1.000	No
Neonatal vs. Kidney	15.367	0.182	1.000	No
Adult vs. Kidney	13.919	0.144	1.000	No
Neonatal vs. Col III	7.002	0.135	1.000	No
Col III vs. Kidney	8.366	0.0961	1.000	No
Adult vs. Col III	5.553	0.0793	0.996	No
Neonatal vs. Adult	1.449	0.0217	0.983	No

GATA4

Two Way Analysis of Variance Thursday, April 12, 2012, 12:31:54 PM

Data source: GATA data in Anovas

General Linear Model

Dependent Variable: Intensity

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
ECM condition	9	1197330.052	133036.672	3.129	0.001
Stiffness	2	38431.983	19215.991	0.452	0.637
ECM condition x Stiffness	18	2588103.358	143783.520	3.382	<0.001
Residual	842	35799317.871	42517.005		
Total	871	40158076.760	46105.714		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of ECM condition depends on what level of Stiffness is present. There is a statistically significant interaction between ECM condition and Stiffness. (P = <0.001)

Power of performed test with alpha = 0.0500: for ECM condition : 0.889
Power of performed test with alpha = 0.0500: for Stiffness : 0.0500
Power of performed test with alpha = 0.0500: for ECM condition x Stiffness : 0.996

Least square means for ECM condition :

Group	Mean	SEM
Adult	25.048	37.246
Brain	176.112	72.450
Col I	14.896	21.274
Col III	91.705	31.301
Col V	20.634	26.159
Fetal	1.853	16.275
Fn	15.753	30.398
Kidney	13.463	52.922
Ln	10.403	40.358
Neonatal	123.609	23.079

Least square means for Stiffness :

Group	Mean	SEM
8.000	60.864	16.937
25.000	56.371	17.579
40.000	30.808	27.303

Least square means for ECM condition x Stiffness :

Group	Mean	SEM
Adult x 8.000	67.527	55.108
Adult x 25.000	6.966	68.732

Adult x 40.000	0.651	68.732
Brain x 8.000	256.344	48.601
Brain x 25.000	21.459	48.601
Brain x 40.000	250.533	206.197
Col I x 8.000	11.298	51.549
Col I x 25.000	15.831	28.873
Col I x 40.000	17.558	24.133
Col III x 8.000	167.124	55.108
Col III x 25.000	105.749	59.524
Col III x 40.000	2.242	47.305
Col V x 8.000	9.395	65.205
Col V x 25.000	34.260	18.225
Col V x 40.000	18.246	39.683
Fetal x 8.000	2.644	21.155
Fetal x 25.000	1.000	35.362
Fetal x 40.000	1.916	26.187
Fn x 8.000	38.484	42.995
Fn x 25.000	7.159	19.841
Fn x 40.000	1.616	77.935
Kidney x 8.000	40.364	77.935
Kidney x 25.000	0.0255	103.098
Kidney x 40.000	6.040E-014	92.214
Ln x 8.000	2.646	57.189
Ln x 25.000	28.564	72.901
Ln x 40.000	3.375E-014	77.935
Neonatal x 8.000	12.816	41.239
Neonatal x 25.000	342.694	38.967
Neonatal x 40.000	15.318	39.683

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **ECM condition**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Neonatal vs. Fetal	121.756	4.311	<0.001	Yes
Neonatal vs. Col I	108.714	3.463	0.024	Yes
Neonatal vs. Col V	102.976	2.952	0.130	No
Neonatal vs. Fn	107.856	2.826	0.184	No
Col III vs. Fetal	89.852	2.547	0.366	No
Neonatal vs. Ln	113.206	2.435	0.456	No
Brain vs. Fetal	174.259	2.347	0.530	No
Neonatal vs. Adult	98.561	2.249	0.614	No
Brain vs. Col I	161.217	2.135	0.712	No
Brain vs. Fn	160.359	2.041	0.783	No
Col III vs. Col I	76.809	2.030	0.783	No
Brain vs. Col V	155.479	2.018	0.782	No
Brain vs. Ln	165.709	1.998	0.789	No
Neonatal vs. Kidney	110.146	1.908	0.846	No
Brain vs. Adult	151.064	1.854	0.871	No
Brain vs. Kidney	162.649	1.813	0.887	No
Col III vs. Col V	71.071	1.742	0.916	No
Col III vs. Fn	75.952	1.741	0.909	No
Col III vs. Ln	81.302	1.592	0.959	No
Col III vs. Adult	66.657	1.370	0.992	No

Col III vs. Kidney	78.242	1.273	0.997	No
Brain vs. Col III	84.407	1.069	1.000	No
Neonatal vs. Col III	31.904	0.820	1.000	No
Brain vs. Neonatal	52.503	0.690	1.000	No
Col V vs. Fetal	18.781	0.610	1.000	No
Adult vs. Fetal	23.195	0.571	1.000	No
Col I vs. Fetal	13.042	0.487	1.000	No
Fn vs. Fetal	13.900	0.403	1.000	No
Adult vs. Ln	14.645	0.267	1.000	No
Adult vs. Col I	10.153	0.237	1.000	No
Col V vs. Ln	10.230	0.213	1.000	No
Kidney vs. Fetal	11.610	0.210	1.000	No
Ln vs. Fetal	8.550	0.196	1.000	No
Adult vs. Fn	9.295	0.193	1.000	No
Adult vs. Kidney	11.585	0.179	1.000	No
Col V vs. Col I	5.738	0.170	1.000	No
Col V vs. Fn	4.881	0.122	1.000	No
Col V vs. Kidney	7.171	0.121	1.000	No
Fn vs. Ln	5.350	0.106	1.000	No
Col I vs. Ln	4.492	0.0985	1.000	No
Adult vs. Col V	4.414	0.0970	1.000	No
Kidney vs. Ln	3.060	0.0460	1.000	No
Fn vs. Kidney	2.290	0.0375	1.000	No
Col I vs. Kidney	1.433	0.0251	1.000	No
Fn vs. Col I	0.858	0.0231	0.982	No

Comparisons for factor: **Stiffness**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
8.000 vs. 40.000	30.056	0.935	0.725	No
25.000 vs. 40.000	25.563	0.787	0.677	No
8.000 vs. 25.000	4.493	0.184	0.854	No

Comparisons for factor: **Stiffness within Adult**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
8.000 vs. 40.000	66.875	0.759	0.832	No
8.000 vs. 25.000	60.561	0.687	0.742	No
25.000 vs. 40.000	6.315	0.0650	0.948	No

Comparisons for factor: **Stiffness within Brain**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
8.000 vs. 25.000	234.885	3.417	0.002	Yes
40.000 vs. 25.000	229.074	1.081	0.481	No
8.000 vs. 40.000	5.811	0.0274	0.978	No

Comparisons for factor: **Stiffness within Col I**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
40.000 vs. 8.000	6.260	0.110	0.999	No
25.000 vs. 8.000	4.533	0.0767	0.996	No
40.000 vs. 25.000	1.727	0.0459	0.963	No

Comparisons for factor: **Stiffness within Col III**

Comparison	Diff of Means	t	P	P<0.05
8.000 vs. 40.000	164.882	2.270	0.069	No
25.000 vs. 40.000	103.507	1.361	0.317	No
8.000 vs. 25.000	61.375	0.757	0.449	No

Comparisons for factor: **Stiffness within Col V**

Comparison	Diff of Means	t	P	P<0.05
25.000 vs. 8.000	24.865	0.367	0.976	No
25.000 vs. 40.000	16.015	0.367	0.918	No
40.000 vs. 8.000	8.850	0.116	0.908	No

Comparisons for factor: **Stiffness within Fetal**

Comparison	Diff of Means	t	P	P<0.05
8.000 vs. 25.000	1.643	0.0399	1.000	No
8.000 vs. 40.000	0.728	0.0216	1.000	No
40.000 vs. 25.000	0.916	0.0208	0.983	No

Comparisons for factor: **Stiffness within Fn**

Comparison	Diff of Means	t	P	P<0.05
8.000 vs. 25.000	31.325	0.662	0.881	No
8.000 vs. 40.000	36.868	0.414	0.897	No
25.000 vs. 40.000	5.543	0.0689	0.945	No

Comparisons for factor: **Stiffness within Kidney**

Comparison	Diff of Means	t	P	P<0.05
8.000 vs. 40.000	40.364	0.334	0.982	No
8.000 vs. 25.000	40.338	0.312	0.940	No
25.000 vs. 40.000	0.0255	0.000184	1.000	No

Comparisons for factor: **Stiffness within Ln**

Comparison	Diff of Means	t	P	P<0.05
25.000 vs. 8.000	25.917	0.280	0.989	No
25.000 vs. 40.000	28.564	0.268	0.955	No
8.000 vs. 40.000	2.646	0.0274	0.978	No

Comparisons for factor: **Stiffness within Neonatal**

Comparison	Diff of Means	t	P	P<0.05
25.000 vs. 40.000	327.376	5.886	<0.001	Yes
25.000 vs. 8.000	329.877	5.814	<0.001	Yes
40.000 vs. 8.000	2.502	0.0437	0.965	No

Comparisons for factor: **ECM condition within 8**

Comparison	Diff of Means	t	P	P<0.05
Brain vs. Fetal	253.701	4.786	<0.001	Yes
Brain vs. Neonatal	243.528	3.821	0.006	Yes
Brain vs. Col I	245.046	3.459	0.024	Yes
Brain vs. Ln	253.698	3.380	0.031	Yes
Brain vs. Fn	217.860	3.357	0.033	Yes
Brain vs. Col V	246.949	3.037	0.094	No

Col III vs. Fetal	164.481	2.786	0.192	No
Brain vs. Adult	188.817	2.570	0.327	No
Brain vs. Kidney	215.981	2.352	0.507	No
Col III vs. Neonatal	154.308	2.242	0.601	No
Col III vs. Ln	164.478	2.071	0.748	No
Col III vs. Col I	155.826	2.065	0.743	No
Col III vs. Col V	157.729	1.848	0.891	No
Col III vs. Fn	128.640	1.840	0.888	No
Col III vs. Kidney	126.761	1.328	0.998	No
Col III vs. Adult	99.597	1.278	0.999	No
Brain vs. Col III	89.220	1.214	0.999	No
Adult vs. Fetal	64.883	1.099	1.000	No
Adult vs. Ln	64.880	0.817	1.000	No
Adult vs. Neonatal	54.711	0.795	1.000	No
Fn vs. Fetal	35.840	0.748	1.000	No
Adult vs. Col I	56.229	0.745	1.000	No
Adult vs. Col V	58.132	0.681	1.000	No
Fn vs. Ln	35.837	0.501	1.000	No
Kidney vs. Fetal	37.720	0.467	1.000	No
Fn vs. Neonatal	25.668	0.431	1.000	No
Adult vs. Fn	29.043	0.416	1.000	No
Fn vs. Col I	27.186	0.405	1.000	No
Kidney vs. Ln	37.717	0.390	1.000	No
Fn vs. Col V	29.089	0.372	1.000	No
Kidney vs. Neonatal	27.547	0.312	1.000	No
Kidney vs. Col I	29.066	0.311	1.000	No
Kidney vs. Col V	30.968	0.305	1.000	No
Adult vs. Kidney	27.163	0.285	1.000	No
Neonatal vs. Fetal	10.173	0.219	1.000	No
Col I vs. Fetal	8.655	0.155	1.000	No
Neonatal vs. Ln	10.170	0.144	1.000	No
Col I vs. Ln	8.652	0.112	1.000	No
Col V vs. Fetal	6.752	0.0985	1.000	No
Col V vs. Ln	6.749	0.0778	1.000	No
Neonatal vs. Col V	3.421	0.0443	1.000	No
Neonatal vs. Col I	1.518	0.0230	1.000	No
Col I vs. Col V	1.903	0.0229	1.000	No
Kidney vs. Fn	1.880	0.0211	1.000	No
Ln vs. Fetal	0.00295	0.0000483	1.000	No

Comparisons for factor: **ECM condition within 25**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Neonatal vs. Fn	335.534	7.673	<0.001	Yes
Neonatal vs. Col V	308.434	7.170	<0.001	Yes
Neonatal vs. Col I	326.863	6.740	<0.001	Yes
Neonatal vs. Fetal	341.694	6.493	<0.001	Yes
Neonatal vs. Brain	321.234	5.157	<0.001	Yes
Neonatal vs. Adult	335.728	4.249	<0.001	Yes
Neonatal vs. Ln	314.130	3.800	0.006	Yes
Neonatal vs. Col III	236.945	3.330	0.034	Yes
Neonatal vs. Kidney	342.668	3.109	0.069	No
Col III vs. Fn	98.590	1.571	0.988	No
Col III vs. Fetal	104.749	1.513	0.993	No
Col III vs. Col I	89.918	1.359	0.999	No
Col III vs. Col V	71.489	1.148	1.000	No

Col III vs. Brain	84.290	1.097	1.000	No
Col III vs. Adult	98.783	1.086	1.000	No
Col V vs. Fn	27.101	1.006	1.000	No
Col III vs. Kidney	105.724	0.888	1.000	No
Col V vs. Fetal	33.260	0.836	1.000	No
Col III vs. Ln	77.185	0.820	1.000	No
Col V vs. Col I	18.430	0.540	1.000	No
Col V vs. Adult	27.294	0.384	1.000	No
Brain vs. Fetal	20.459	0.340	1.000	No
Ln vs. Fetal	27.564	0.340	1.000	No
Col V vs. Kidney	34.235	0.327	1.000	No
Col I vs. Fetal	14.830	0.325	1.000	No
Ln vs. Fn	21.405	0.283	1.000	No
Brain vs. Fn	14.300	0.272	1.000	No
Col I vs. Fn	8.671	0.248	1.000	No
Col V vs. Brain	12.801	0.247	1.000	No
Ln vs. Kidney	28.538	0.226	1.000	No
Ln vs. Adult	21.598	0.216	1.000	No
Brain vs. Kidney	21.434	0.188	1.000	No
Brain vs. Adult	14.493	0.172	1.000	No
Ln vs. Col I	12.733	0.162	1.000	No
Fn vs. Fetal	6.159	0.152	1.000	No
Col I vs. Kidney	15.805	0.148	1.000	No
Col I vs. Adult	8.865	0.119	1.000	No
Brain vs. Col I	5.629	0.0996	1.000	No
Ln vs. Brain	7.104	0.0811	1.000	No
Adult vs. Fetal	5.966	0.0772	1.000	No
Col V vs. Ln	5.696	0.0758	1.000	No
Fn vs. Kidney	7.134	0.0679	1.000	No
Adult vs. Kidney	6.941	0.0560	1.000	No
Fetal vs. Kidney	0.975	0.00894	1.000	No
Fn vs. Adult	0.193	0.00270	0.998	No

Comparisons for factor: **ECM condition within 40**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Brain vs. Fetal	248.618	1.196	1.000	No
Brain vs. Col III	248.291	1.174	1.000	No
Brain vs. Adult	249.882	1.150	1.000	No
Brain vs. Ln	250.533	1.137	1.000	No
Brain vs. Fn	248.917	1.129	1.000	No
Brain vs. Col I	232.975	1.122	1.000	No
Brain vs. Neonatal	235.215	1.120	1.000	No
Brain vs. Kidney	250.533	1.109	1.000	No
Brain vs. Col V	232.288	1.106	1.000	No
Col I vs. Fetal	15.642	0.439	1.000	No
Col V vs. Fetal	16.330	0.343	1.000	No
Col I vs. Col III	15.316	0.288	1.000	No
Neonatal vs. Fetal	13.402	0.282	1.000	No
Col V vs. Col III	16.004	0.259	1.000	No
Col I vs. Adult	16.907	0.232	1.000	No
Col V vs. Adult	17.594	0.222	1.000	No
Col I vs. Ln	17.558	0.215	1.000	No
Neonatal vs. Col III	13.076	0.212	1.000	No
Col V vs. Ln	18.246	0.209	1.000	No
Col I vs. Fn	15.942	0.195	1.000	No

Col V vs. Fn	16.629	0.190	1.000	No
Neonatal vs. Adult	14.667	0.185	1.000	No
Col I vs. Kidney	17.558	0.184	1.000	No
Col V vs. Kidney	18.246	0.182	1.000	No
Neonatal vs. Ln	15.318	0.175	1.000	No
Neonatal vs. Fn	13.702	0.157	1.000	No
Neonatal vs. Kidney	15.318	0.153	1.000	No
Col V vs. Neonatal	2.928	0.0522	1.000	No
Col I vs. Neonatal	2.240	0.0482	1.000	No
Col III vs. Ln	2.242	0.0246	1.000	No
Fetal vs. Ln	1.916	0.0233	1.000	No
Col III vs. Kidney	2.242	0.0216	1.000	No
Fetal vs. Kidney	1.916	0.0200	1.000	No
Col III vs. Adult	1.590	0.0191	1.000	No
Fetal vs. Adult	1.264	0.0172	1.000	No
Col V vs. Col I	0.688	0.0148	1.000	No
Fn vs. Ln	1.616	0.0147	1.000	No
Fn vs. Kidney	1.616	0.0134	1.000	No
Fn vs. Adult	0.965	0.00928	1.000	No
Col III vs. Fn	0.626	0.00686	1.000	No
Adult vs. Ln	0.651	0.00627	1.000	No
Col III vs. Fetal	0.326	0.00603	1.000	No
Adult vs. Kidney	0.651	0.00566	1.000	No
Fetal vs. Fn	0.299	0.00364	1.000	No
Kidney vs. Ln	2.665E-014	2.207E-016	1.000	No

MEF2C

Two Way Analysis of Variance Thursday, April 12, 2012, 12:35:33 PM

Data source: Mef data in Anovas

General Linear Model

Dependent Variable: Intensity

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
ECM Protein	9	2786875.241	309652.805	0.985	0.451
Stiffness	2	2143158.128	1071579.064	3.408	0.033
ECM Protein x Stiffness	18	19350528.964	1075029.387	3.419	<0.001
Residual	1522	478590676.553	314448.539		
Total	1551	520752844.426	335752.962		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of ECM Protein depends on what level of Stiffness is present. There is a statistically significant interaction between ECM Protein and Stiffness. (P = <0.001)

Power of performed test with alpha = 0.0500: for ECM Protein : 0.0500
Power of performed test with alpha = 0.0500: for Stiffness : 0.476
Power of performed test with alpha = 0.0500: for ECM Protein x Stiffness : 0.997

Least square means for ECM Protein :

Group	Mean	SEM
Adult	82.450	45.948
Brain	269.251	77.064
Col I	250.127	123.381
Col III	100.488	96.723
Col V	72.046	51.626
Fetal	129.576	84.632
Fn	65.709	42.922
Kidney	68.436	123.557
Ln	24.509	81.726
Neonatal	114.653	31.606

Least square means for Stiffness :

Group	Mean	SEM
8.000	102.168	49.038
40.000	49.342	39.394
25.000	201.664	46.930

Least square means for ECM Protein x Stiffness :

Group	Mean	SEM
Adult x 8.000	88.022	49.122
Adult x 40.000	6.228	64.865
Adult x 25.000	153.099	109.853
Brain x 8.000	57.306	111.272
Brain x 40.000	69.701	185.886
Brain x 25.000	680.747	76.281
Col I x 8.000	187.725	146.767
Col I x 40.000	179.897	37.463
Col I x 25.000	382.760	344.606
Col III x 8.000	-75.317	278.352
Col III x 40.000	-28.503	64.051
Col III x 25.000	405.283	54.907
Col V x 8.000	95.168	96.927
Col V x 40.000	90.195	82.163
Col V x 25.000	30.776	88.729
Fetal x 8.000	263.591	60.651
Fetal x 40.000	14.619	82.468
Fetal x 25.000	110.517	230.153
Fn x 8.000	41.977	78.643
Fn x 40.000	114.637	70.775
Fn x 25.000	40.512	73.385
Kidney x 8.000	36.606	241.429
Kidney x 40.000	4.868	221.583
Kidney x 25.000	163.834	213.036
Ln x 8.000	54.099	140.711
Ln x 40.000	2.813	149.025
Ln x 25.000	16.616	127.605
Neonatal x 8.000	272.500	58.548

Neonatal x 40.000	38.963	52.736
Neonatal x 25.000	32.494	50.493

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **ECM Protein**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Brain vs. Fn	203.542	2.307	0.618	No
Brain vs. Ln	244.742	2.179	0.732	No
Brain vs. Col V	197.205	2.126	0.771	No
Brain vs. Adult	186.802	2.082	0.799	No
Brain vs. Neonatal	154.599	1.856	0.933	No
Col I vs. Ln	225.618	1.525	0.996	No
Col I vs. Fn	184.418	1.412	0.999	No
Brain vs. Kidney	200.815	1.379	0.999	No
Brain vs. Col III	168.764	1.365	0.999	No
Col I vs. Col V	178.081	1.331	0.999	No
Col I vs. Adult	167.678	1.274	1.000	No
Brain vs. Fetal	139.675	1.220	1.000	No
Col I vs. Neonatal	135.475	1.064	1.000	No
Col I vs. Kidney	181.691	1.041	1.000	No
Neonatal vs. Ln	90.143	1.029	1.000	No
Col I vs. Col III	149.639	0.954	1.000	No
Neonatal vs. Fn	48.944	0.918	1.000	No
Fetal vs. Ln	105.067	0.893	1.000	No
Col I vs. Fetal	120.551	0.806	1.000	No
Neonatal vs. Col V	42.606	0.704	1.000	No
Fetal vs. Fn	63.867	0.673	1.000	No
Adult vs. Ln	57.940	0.618	1.000	No
Col III vs. Ln	75.978	0.600	1.000	No
Fetal vs. Col V	57.530	0.580	1.000	No
Neonatal vs. Adult	32.203	0.577	1.000	No
Col V vs. Ln	47.537	0.492	1.000	No
Fetal vs. Adult	47.126	0.489	1.000	No
Fn vs. Ln	41.200	0.446	1.000	No
Fetal vs. Kidney	61.140	0.408	1.000	No
Neonatal vs. Kidney	46.216	0.362	1.000	No
Col III vs. Fn	34.779	0.329	1.000	No
Kidney vs. Ln	43.927	0.297	1.000	No
Adult vs. Fn	16.741	0.266	1.000	No
Col III vs. Col V	28.442	0.259	1.000	No
Fetal vs. Col III	29.088	0.226	1.000	No
Col III vs. Kidney	32.052	0.204	1.000	No
Col III vs. Adult	18.038	0.168	1.000	No
Fetal vs. Neonatal	14.923	0.165	1.000	No
Adult vs. Col V	10.403	0.151	1.000	No
Neonatal vs. Col III	14.165	0.139	1.000	No
Brain vs. Col I	19.124	0.131	1.000	No
Adult vs. Kidney	14.014	0.106	1.000	No
Col V vs. Fn	6.337	0.0944	1.000	No
Col V vs. Kidney	3.610	0.0270	1.000	No
Kidney vs. Fn	2.727	0.0208	0.983	No

Comparisons for factor: **Stiffness**

Comparison	Diff of Means	t	P	P<0.050
25.000 vs. 40.000	152.322	2.486	0.039	Yes
25.000 vs. 8.000	99.496	1.466	0.265	No
8.000 vs. 40.000	52.826	0.840	0.401	No

Comparisons for factor: **Stiffness within Adult**

Comparison	Diff of Means	t	P	P<0.05
25.000 vs. 40.000	146.870	1.151	0.578	No
8.000 vs. 40.000	81.793	1.005	0.531	No
25.000 vs. 8.000	65.077	0.541	0.589	No

Comparisons for factor: **Stiffness within Brain**

Comparison	Diff of Means	t	P	P<0.05
25.000 vs. 8.000	623.441	4.621	<0.001	Yes
25.000 vs. 40.000	611.046	3.041	0.005	Yes
40.000 vs. 8.000	12.396	0.0572	0.954	No

Comparisons for factor: **Stiffness within Col I**

Comparison	Diff of Means	t	P	P<0.05
25.000 vs. 40.000	202.863	0.585	0.914	No
25.000 vs. 8.000	195.035	0.521	0.842	No
8.000 vs. 40.000	7.828	0.0517	0.959	No

Comparisons for factor: **Stiffness within Col III**

Comparison	Diff of Means	t	P	P<0.05
25.000 vs. 40.000	433.786	5.142	<0.001	Yes
25.000 vs. 8.000	480.600	1.694	0.173	No
40.000 vs. 8.000	46.814	0.164	0.870	No

Comparisons for factor: **Stiffness within Col V**

Comparison	Diff of Means	t	P	P<0.05
40.000 vs. 25.000	59.419	0.491	0.947	No
8.000 vs. 25.000	64.392	0.490	0.859	No
8.000 vs. 40.000	4.973	0.0391	0.969	No

Comparisons for factor: **Stiffness within Fetal**

Comparison	Diff of Means	t	P	P<0.05
8.000 vs. 40.000	248.972	2.432	0.045	Yes
8.000 vs. 25.000	153.074	0.643	0.770	No
25.000 vs. 40.000	95.898	0.392	0.695	No

Comparisons for factor: **Stiffness within Fn**

Comparison	Diff of Means	t	P	P<0.05
40.000 vs. 25.000	74.125	0.727	0.849	No
40.000 vs. 8.000	72.660	0.687	0.742	No
8.000 vs. 25.000	1.465	0.0136	0.989	No

Comparisons for factor: **Stiffness within Kidney**

Comparison	Diff of Means	t	P	P<0.05
25.000 vs. 40.000	158.966	0.517	0.938	No
25.000 vs. 8.000	127.228	0.395	0.906	No
8.000 vs. 40.000	31.737	0.0968	0.923	No

Comparisons for factor: **Stiffness within Ln**

Comparison	Diff of Means	t	P	P<0.05
8.000 vs. 40.000	51.287	0.250	0.992	No
8.000 vs. 25.000	37.484	0.197	0.976	No
25.000 vs. 40.000	13.803	0.0704	0.944	No

Comparisons for factor: **Stiffness within Neonatal**

Comparison	Diff of Means	t	P	P<0.05
8.000 vs. 25.000	240.006	3.104	0.006	Yes
8.000 vs. 40.000	233.537	2.964	0.006	Yes
40.000 vs. 25.000	6.469	0.0886	0.929	No

Comparisons for factor: **ECM Protein within 8**

Comparison	Diff of Means	t	P	P<0.05
Neonatal vs. Adult	184.479	2.414	0.514	No
Neonatal vs. Fn	230.523	2.351	0.567	No
Fetal vs. Adult	175.570	2.249	0.658	No
Fetal vs. Fn	221.614	2.231	0.666	No
Neonatal vs. Brain	215.195	1.711	0.976	No
Fetal vs. Brain	206.286	1.628	0.988	No
Neonatal vs. Col V	177.332	1.566	0.992	No
Fetal vs. Col V	168.423	1.473	0.997	No
Neonatal vs. Ln	218.401	1.433	0.998	No
Fetal vs. Ln	209.492	1.367	0.999	No
Neonatal vs. Col III	347.818	1.223	1.000	No
Fetal vs. Col III	338.909	1.190	1.000	No
Neonatal vs. Kidney	235.895	0.950	1.000	No
Fetal vs. Kidney	226.986	0.912	1.000	No
Col I vs. Fn	145.748	0.875	1.000	No
Col I vs. Col III	263.042	0.836	1.000	No
Col I vs. Brain	130.419	0.708	1.000	No
Col I vs. Ln	133.625	0.657	1.000	No
Col I vs. Adult	99.703	0.644	1.000	No
Col V vs. Col III	170.485	0.578	1.000	No
Adult vs. Col III	163.339	0.578	1.000	No
Neonatal vs. Col I	84.775	0.537	1.000	No
Col I vs. Kidney	151.119	0.535	1.000	No
Col I vs. Col V	92.557	0.526	1.000	No
Adult vs. Fn	46.045	0.497	1.000	No
Fetal vs. Col I	75.866	0.478	1.000	No
Brain vs. Col III	132.623	0.442	1.000	No
Col V vs. Fn	53.191	0.426	1.000	No
Ln vs. Col III	129.417	0.415	1.000	No
Fn vs. Col III	117.294	0.406	1.000	No
Kidney vs. Col III	111.923	0.304	1.000	No
Col V vs. Brain	37.862	0.257	1.000	No

Adult vs. Brain	30.716	0.253	1.000	No
Col V vs. Ln	41.068	0.240	1.000	No
Adult vs. Ln	33.922	0.228	1.000	No
Col V vs. Kidney	58.562	0.225	1.000	No
Adult vs. Kidney	51.416	0.209	1.000	No
Brain vs. Fn	15.329	0.112	1.000	No
Neonatal vs. Fetal	8.909	0.106	1.000	No
Brain vs. Kidney	20.700	0.0779	1.000	No
Ln vs. Fn	12.122	0.0752	1.000	No
Col V vs. Adult	7.146	0.0658	1.000	No
Ln vs. Kidney	17.494	0.0626	1.000	No
Fn vs. Kidney	5.371	0.0212	1.000	No
Brain vs. Ln	3.206	0.0179	0.986	No

Comparisons for factor: **ECM Protein within 40**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Col I vs. Col III	208.400	2.809	0.203	No
Col I vs. Adult	173.668	2.318	0.599	No
Col I vs. Neonatal	140.934	2.179	0.724	No
Col I vs. Fetal	165.277	1.825	0.949	No
Fn vs. Col III	143.140	1.500	0.997	No
Col I vs. Ln	177.084	1.152	1.000	No
Col V vs. Col III	118.698	1.139	1.000	No
Fn vs. Adult	108.409	1.129	1.000	No
Col I vs. Col V	89.702	0.993	1.000	No
Fn vs. Fetal	100.018	0.920	1.000	No
Fn vs. Neonatal	75.674	0.857	1.000	No
Col I vs. Fn	65.259	0.815	1.000	No
Neonatal vs. Col III	67.466	0.813	1.000	No
Col V vs. Adult	83.966	0.802	1.000	No
Col I vs. Kidney	175.028	0.779	1.000	No
Fn vs. Ln	111.825	0.678	1.000	No
Col V vs. Fetal	75.575	0.649	1.000	No
Col I vs. Brain	110.195	0.581	1.000	No
Col V vs. Neonatal	51.232	0.525	1.000	No
Col V vs. Ln	87.382	0.513	1.000	No
Brain vs. Col III	98.204	0.499	1.000	No
Fn vs. Kidney	109.769	0.472	1.000	No
Fetal vs. Col III	43.122	0.413	1.000	No
Neonatal vs. Adult	32.735	0.392	1.000	No
Adult vs. Col III	34.731	0.381	1.000	No
Col V vs. Kidney	85.327	0.361	1.000	No
Brain vs. Adult	63.473	0.322	1.000	No
Brain vs. Ln	66.889	0.281	1.000	No
Brain vs. Fetal	55.082	0.271	1.000	No
Neonatal vs. Fetal	24.344	0.249	1.000	No
Neonatal vs. Ln	36.151	0.229	1.000	No
Fn vs. Brain	44.936	0.226	1.000	No
Fn vs. Col V	24.443	0.225	1.000	No
Brain vs. Kidney	64.833	0.224	1.000	No
Ln vs. Col III	31.315	0.193	1.000	No
Brain vs. Neonatal	30.738	0.159	1.000	No
Neonatal vs. Kidney	34.095	0.150	1.000	No
Kidney vs. Col III	33.371	0.145	1.000	No
Col V vs. Brain	20.494	0.101	1.000	No

Fetal vs. Adult	8.391	0.0800	1.000	No
Fetal vs. Ln	11.807	0.0693	1.000	No
Fetal vs. Kidney	9.751	0.0412	1.000	No
Adult vs. Ln	3.416	0.0210	1.000	No
Kidney vs. Ln	2.056	0.00770	1.000	No
Adult vs. Kidney	1.360	0.00589	0.995	No

Comparisons for factor: **ECM Protein within 25**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Brain vs. Neonatal	648.253	7.086	<0.001	Yes
Brain vs. Fn	640.235	6.049	<0.001	Yes
Brain vs. Col V	649.971	5.555	<0.001	Yes
Col III vs. Neonatal	372.789	4.998	<0.001	Yes
Brain vs. Ln	664.131	4.467	<0.001	Yes
Col III vs. Fn	364.771	3.980	0.003	Yes
Brain vs. Adult	527.648	3.945	0.003	Yes
Col III vs. Col V	374.507	3.589	0.013	Yes
Brain vs. Col III	275.464	2.931	0.119	No
Col III vs. Ln	388.668	2.798	0.171	No
Brain vs. Fetal	570.230	2.352	0.486	No
Brain vs. Kidney	516.913	2.284	0.538	No
Col III vs. Adult	252.184	2.053	0.742	No
Col III vs. Fetal	294.766	1.246	1.000	No
Col III vs. Kidney	241.449	1.098	1.000	No
Col I vs. Neonatal	350.266	1.006	1.000	No
Adult vs. Neonatal	120.605	0.998	1.000	No
Col I vs. Ln	366.144	0.996	1.000	No
Col I vs. Col V	351.984	0.989	1.000	No
Col I vs. Fn	342.248	0.971	1.000	No
Adult vs. Col V	122.323	0.866	1.000	No
Adult vs. Fn	112.587	0.852	1.000	No
Brain vs. Col I	297.987	0.844	1.000	No
Adult vs. Ln	136.483	0.811	1.000	No
Col I vs. Fetal	272.243	0.657	1.000	No
Col I vs. Adult	229.661	0.635	1.000	No
Kidney vs. Neonatal	131.340	0.600	1.000	No
Kidney vs. Ln	147.218	0.593	1.000	No
Kidney vs. Col V	133.058	0.577	1.000	No
Kidney vs. Fn	123.322	0.547	1.000	No
Col I vs. Kidney	218.926	0.540	1.000	No
Fetal vs. Ln	93.901	0.357	1.000	No
Fetal vs. Neonatal	78.023	0.331	1.000	No
Fetal vs. Col V	79.741	0.323	1.000	No
Fetal vs. Fn	70.005	0.290	1.000	No
Kidney vs. Fetal	53.317	0.170	1.000	No
Adult vs. Fetal	42.582	0.167	1.000	No
Fn vs. Ln	23.897	0.162	1.000	No
Neonatal vs. Ln	15.878	0.116	1.000	No
Col V vs. Ln	14.160	0.0911	1.000	No
Fn vs. Neonatal	8.018	0.0900	1.000	No
Fn vs. Col V	9.736	0.0846	1.000	No
Col III vs. Col I	22.523	0.0645	1.000	No
Kidney vs. Adult	10.735	0.0448	0.999	No
Neonatal vs. Col V	1.718	0.0168	0.987	No

Alpha Actin

Two Way Analysis of Variance Thursday, April 12, 2012, 12:36:43 PM

Data source: Alpha data in Anovas

General Linear Model

Dependent Variable: Intensity

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
ECM Protein	9	1996577.622	221841.958	2.708	0.004
Stiffness	2	176517.127	88258.564	1.077	0.341
ECM Protein x Stiffness	18	5116569.108	284253.839	3.470	<0.001
Residual	1547	126735382.798	81923.324		
Total	1576	136260835.685	86459.921		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of ECM Protein depends on what level of Stiffness is present. There is a statistically significant interaction between ECM Protein and Stiffness. (P = <0.001)

Power of performed test with alpha = 0.0500: for ECM Protein : 0.790
Power of performed test with alpha = 0.0500: for Stiffness : 0.0600
Power of performed test with alpha = 0.0500: for ECM Protein x Stiffness : 0.997

Least square means for ECM Protein :

Group	Mean	SEM
Adult	17.593	23.591
Brain	194.520	38.213
Col I	-13.064	58.304
Col III	16.251	52.036
Col V	-4.934	24.851
Fetal	1.569	39.071
Fn	11.658	22.256
Kidney	21.062	65.751
Ln	-0.816	42.771
Neonatal	12.607	15.806

Least square means for Stiffness :

Group	Mean	SEM
8.000	5.258	22.228
40.000	19.761	18.764
25.000	51.916	24.216

Least square means for ECM Protein x Stiffness :

Group	Mean	SEM
Adult x 8.000	66.600	23.681
Adult x 40.000	60.319	31.894
Adult x 25.000	-74.141	46.145
Brain x 8.000	-59.256	60.602
Brain x 40.000	105.649	93.372
Brain x 25.000	537.167	43.898
Col I x 8.000	84.356	72.695
Col I x 40.000	-21.113	19.448
Col I x 25.000	-102.434	153.312
Col III x 8.000	-53.433	149.418
Col III x 40.000	48.870	33.048
Col III x 25.000	53.317	26.921
Col V x 8.000	-7.606	42.239
Col V x 40.000	-1.285	42.226
Col V x 25.000	-5.909	44.635
Fetal x 8.000	-11.292	31.241
Fetal x 40.000	-16.557	42.933
Fetal x 25.000	32.558	112.597
Fn x 8.000	4.157	40.065
Fn x 40.000	16.602	37.219
Fn x 25.000	14.214	38.306
Kidney x 8.000	5.988	124.980
Kidney x 40.000	2.887	117.587
Kidney x 25.000	54.312	92.540
Ln x 8.000	1.205	76.305
Ln x 40.000	-4.677	78.732
Ln x 25.000	1.025	60.736
Neonatal x 8.000	21.859	28.131
Neonatal x 40.000	6.915	26.056
Neonatal x 25.000	9.046	28.985

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **ECM Protein**

Comparison	Diff of Means	t	P	P<0.050
Brain vs. Neonatal	181.913	4.399	<0.001	Yes
Brain vs. Col V	199.454	4.376	<0.001	Yes
Brain vs. Fn	182.862	4.135	0.002	Yes
Brain vs. Adult	176.927	3.940	0.004	Yes
Brain vs. Fetal	192.951	3.531	0.017	Yes
Brain vs. Ln	195.336	3.406	0.027	Yes
Brain vs. Col I	207.584	2.978	0.109	No
Brain vs. Col III	178.269	2.761	0.199	No
Brain vs. Kidney	173.458	2.281	0.572	No
Adult vs. Col V	22.526	0.657	1.000	No
Neonatal vs. Col V	17.540	0.596	1.000	No
Fn vs. Col V	16.592	0.497	1.000	No
Adult vs. Col I	30.657	0.487	1.000	No
Neonatal vs. Col I	25.670	0.425	1.000	No
Fn vs. Col I	24.722	0.396	1.000	No
Kidney vs. Col I	34.126	0.388	1.000	No
Adult vs. Ln	18.409	0.377	1.000	No

Col III vs. Col I	29.315	0.375	1.000	No
Kidney vs. Col V	25.996	0.370	1.000	No
Col III vs. Col V	21.185	0.367	1.000	No
Adult vs. Fetal	16.023	0.351	1.000	No
Neonatal vs. Ln	13.422	0.294	1.000	No
Kidney vs. Ln	21.878	0.279	1.000	No
Neonatal vs. Fetal	11.037	0.262	1.000	No
Fn vs. Ln	12.474	0.259	1.000	No
Kidney vs. Fetal	19.493	0.255	1.000	No
Col III vs. Ln	17.067	0.253	1.000	No
Col III vs. Fetal	14.682	0.226	1.000	No
Fn vs. Fetal	10.089	0.224	1.000	No
Fetal vs. Col I	14.633	0.208	1.000	No
Adult vs. Fn	5.935	0.183	1.000	No
Adult vs. Neonatal	4.986	0.176	1.000	No
Ln vs. Col I	12.248	0.169	1.000	No
Fetal vs. Col V	6.503	0.140	1.000	No
Kidney vs. Fn	9.404	0.135	1.000	No
Col V vs. Col I	8.130	0.128	1.000	No
Kidney vs. Neonatal	8.456	0.125	1.000	No
Ln vs. Col V	4.118	0.0832	1.000	No
Col III vs. Fn	4.593	0.0812	1.000	No
Col III vs. Neonatal	3.645	0.0670	1.000	No
Kidney vs. Col III	4.811	0.0574	1.000	No
Kidney vs. Adult	3.469	0.0497	1.000	No
Fetal vs. Ln	2.385	0.0412	1.000	No
Neonatal vs. Fn	0.949	0.0348	0.999	No
Adult vs. Col III	1.341	0.0235	0.981	No

Comparisons for factor: **Stiffness**

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
25.000 vs. 8.000	46.658	1.419	0.399	No
25.000 vs. 40.000	32.155	1.050	0.502	No
40.000 vs. 8.000	14.503	0.499	0.618	No

Comparisons for factor: **Stiffness within Adult**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
8.000 vs. 25.000	140.741	2.713	0.020	Yes
40.000 vs. 25.000	134.460	2.397	0.033	Yes
8.000 vs. 40.000	6.281	0.158	0.874	No

Comparisons for factor: **Stiffness within Brain**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
25.000 vs. 8.000	596.423	7.970	<0.001	Yes
25.000 vs. 40.000	431.517	4.182	<0.001	Yes
40.000 vs. 8.000	164.905	1.481	0.139	No

Comparisons for factor: **Stiffness within Col I**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
8.000 vs. 40.000	105.470	1.402	0.410	No
8.000 vs. 25.000	186.790	1.101	0.469	No
40.000 vs. 25.000	81.320	0.526	0.599	No

Comparisons for factor: **Stiffness within Col III**

Comparison	Diff of Means	t	P	P<0.05
25.000 vs. 8.000	106.750	0.703	0.861	No
40.000 vs. 8.000	102.303	0.669	0.754	No
25.000 vs. 40.000	4.447	0.104	0.917	No

Comparisons for factor: **Stiffness within Col V**

Comparison	Diff of Means	t	P	P<0.05
40.000 vs. 8.000	6.320	0.106	0.999	No
40.000 vs. 25.000	4.624	0.0753	0.996	No
25.000 vs. 8.000	1.696	0.0276	0.978	No

Comparisons for factor: **Stiffness within Fetal**

Comparison	Diff of Means	t	P	P<0.05
25.000 vs. 40.000	49.116	0.408	0.968	No
25.000 vs. 8.000	43.851	0.375	0.914	No
8.000 vs. 40.000	5.265	0.0992	0.921	No

Comparisons for factor: **Stiffness within Fn**

Comparison	Diff of Means	t	P	P<0.05
40.000 vs. 8.000	12.445	0.228	0.994	No
25.000 vs. 8.000	10.057	0.181	0.979	No
40.000 vs. 25.000	2.388	0.0447	0.964	No

Comparisons for factor: **Stiffness within Kidney**

Comparison	Diff of Means	t	P	P<0.05
25.000 vs. 40.000	51.425	0.344	0.981	No
25.000 vs. 8.000	48.324	0.311	0.940	No
8.000 vs. 40.000	3.101	0.0181	0.986	No

Comparisons for factor: **Stiffness within Ln**

Comparison	Diff of Means	t	P	P<0.05
25.000 vs. 40.000	5.702	0.0573	1.000	No
8.000 vs. 40.000	5.882	0.0536	0.998	No
8.000 vs. 25.000	0.180	0.00184	0.999	No

Comparisons for factor: **Stiffness within Neonatal**

Comparison	Diff of Means	t	P	P<0.05
8.000 vs. 40.000	14.944	0.390	0.972	No
8.000 vs. 25.000	12.813	0.317	0.938	No
25.000 vs. 40.000	2.131	0.0547	0.956	No

Comparisons for factor: **ECM Protein within 8**

Comparison	Diff of Means	t	P	P<0.05
Adult vs. Fetal	77.893	1.987	0.886	No
Adult vs. Brain	125.856	1.934	0.910	No
Adult vs. Col V	74.206	1.532	0.997	No

Col I vs. Brain	143.613	1.517	0.997	No
Adult vs. Fn	62.443	1.342	1.000	No
Adult vs. Neonatal	44.741	1.217	1.000	No
Neonatal vs. Brain	81.115	1.214	1.000	No
Col I vs. Fetal	95.649	1.209	1.000	No
Col I vs. Col V	91.962	1.094	1.000	No
Col I vs. Fn	80.199	0.966	1.000	No
Fn vs. Brain	63.413	0.873	1.000	No
Col I vs. Col III	137.789	0.829	1.000	No
Adult vs. Ln	65.395	0.819	1.000	No
Col I vs. Neonatal	62.498	0.802	1.000	No
Adult vs. Col III	120.033	0.793	1.000	No
Col I vs. Ln	83.151	0.789	1.000	No
Neonatal vs. Fetal	33.151	0.789	1.000	No
Fetal vs. Brain	47.964	0.703	1.000	No
Col V vs. Brain	51.650	0.699	1.000	No
Ln vs. Brain	60.461	0.620	1.000	No
Neonatal vs. Col V	29.465	0.581	1.000	No
Col I vs. Kidney	78.368	0.542	1.000	No
Neonatal vs. Col III	75.292	0.495	1.000	No
Adult vs. Kidney	60.612	0.476	1.000	No
Kidney vs. Brain	65.244	0.470	1.000	No
Fn vs. Col III	57.590	0.372	1.000	No
Neonatal vs. Fn	17.702	0.362	1.000	No
Ln vs. Col III	54.638	0.326	1.000	No
Kidney vs. Col III	59.421	0.305	1.000	No
Fn vs. Fetal	15.450	0.304	1.000	No
Col V vs. Col III	45.827	0.295	1.000	No
Fetal vs. Col III	42.141	0.276	1.000	No
Neonatal vs. Ln	20.654	0.254	1.000	No
Col I vs. Adult	17.756	0.232	1.000	No
Fn vs. Col V	11.763	0.202	1.000	No
Ln vs. Fetal	12.497	0.152	1.000	No
Kidney vs. Fetal	17.281	0.134	1.000	No
Neonatal vs. Kidney	15.871	0.124	1.000	No
Kidney vs. Col V	13.594	0.103	1.000	No
Ln vs. Col V	8.811	0.101	1.000	No
Col V vs. Fetal	3.687	0.0702	1.000	No
Col III vs. Brain	5.823	0.0361	1.000	No
Fn vs. Ln	2.952	0.0343	1.000	No
Kidney vs. Ln	4.783	0.0327	0.999	No
Kidney vs. Fn	1.831	0.0140	0.989	No

Comparisons for factor: **ECM Protein within 40**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Adult vs. Col I	81.432	2.180	0.739	No
Col III vs. Col I	69.984	1.825	0.955	No
Adult vs. Fetal	76.876	1.437	0.999	No
Brain vs. Col I	126.763	1.329	1.000	No
Adult vs. Neonatal	53.404	1.297	1.000	No
Col III vs. Fetal	65.428	1.208	1.000	No
Brain vs. Fetal	122.207	1.189	1.000	No
Adult vs. Col V	61.605	1.164	1.000	No
Brain vs. Col V	106.935	1.044	1.000	No
Brain vs. Neonatal	98.734	1.019	1.000	No

Col III vs. Neonatal	41.955	0.997	1.000	No
Col III vs. Col V	50.156	0.935	1.000	No
Brain vs. Ln	110.327	0.903	1.000	No
Fn vs. Col I	37.716	0.898	1.000	No
Adult vs. Fn	43.717	0.892	1.000	No
Brain vs. Fn	89.047	0.886	1.000	No
Neonatal vs. Col I	28.028	0.862	1.000	No
Adult vs. Ln	64.996	0.765	1.000	No
Brain vs. Kidney	102.763	0.684	1.000	No
Col III vs. Fn	32.268	0.648	1.000	No
Col III vs. Ln	53.547	0.627	1.000	No
Fn vs. Fetal	33.160	0.584	1.000	No
Brain vs. Col III	56.779	0.573	1.000	No
Adult vs. Kidney	57.433	0.471	1.000	No
Neonatal vs. Fetal	23.472	0.467	1.000	No
Brain vs. Adult	45.330	0.459	1.000	No
Col V vs. Col I	19.828	0.427	1.000	No
Col III vs. Kidney	45.984	0.376	1.000	No
Fn vs. Col V	17.888	0.318	1.000	No
Col V vs. Fetal	15.272	0.254	1.000	No
Adult vs. Col III	11.449	0.249	1.000	No
Fn vs. Ln	21.280	0.244	1.000	No
Fn vs. Neonatal	9.687	0.213	1.000	No
Ln vs. Col I	16.436	0.203	1.000	No
Kidney vs. Col I	24.000	0.201	1.000	No
Neonatal vs. Col V	8.200	0.165	1.000	No
Kidney vs. Fetal	19.444	0.155	1.000	No
Neonatal vs. Ln	11.592	0.140	1.000	No
Ln vs. Fetal	11.880	0.132	1.000	No
Fn vs. Kidney	13.716	0.111	1.000	No
Fetal vs. Col I	4.556	0.0967	1.000	No
Kidney vs. Ln	7.564	0.0534	1.000	No
Col V vs. Ln	3.392	0.0380	1.000	No
Neonatal vs. Kidney	4.028	0.0334	0.999	No
Kidney vs. Col V	4.172	0.0334	0.973	No

Comparisons for factor: **ECM Protein within 25**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Brain vs. Neonatal	528.120	10.040	<0.001	Yes
Brain vs. Adult	611.307	9.598	<0.001	Yes
Brain vs. Col III	483.850	9.396	<0.001	Yes
Brain vs. Fn	522.952	8.976	<0.001	Yes
Brain vs. Col V	543.076	8.675	<0.001	Yes
Brain vs. Ln	536.141	7.154	<0.001	Yes
Brain vs. Kidney	482.855	4.714	<0.001	Yes
Brain vs. Fetal	504.608	4.175	0.001	Yes
Brain vs. Col I	639.601	4.011	0.002	Yes
Col III vs. Adult	127.458	2.386	0.464	No
Neonatal vs. Adult	83.187	1.527	0.991	No
Fn vs. Adult	88.355	1.473	0.994	No
Kidney vs. Adult	128.453	1.242	1.000	No
Col III vs. Col V	59.227	1.136	1.000	No
Col III vs. Neonatal	44.271	1.119	1.000	No
Col V vs. Adult	68.231	1.063	1.000	No
Col III vs. Col I	155.751	1.001	1.000	No

Ln vs. Adult	75.166	0.985	1.000	No
Fetal vs. Adult	106.699	0.877	1.000	No
Kidney vs. Col I	156.746	0.875	1.000	No
Col III vs. Fn	39.103	0.835	1.000	No
Col III vs. Ln	52.292	0.787	1.000	No
Fn vs. Col I	116.648	0.738	1.000	No
Neonatal vs. Col I	111.480	0.714	1.000	No
Fetal vs. Col I	134.992	0.710	1.000	No
Ln vs. Col I	103.459	0.627	1.000	No
Col V vs. Col I	96.524	0.604	1.000	No
Kidney vs. Col V	60.222	0.586	1.000	No
Kidney vs. Ln	53.287	0.481	1.000	No
Kidney vs. Neonatal	45.266	0.467	1.000	No
Kidney vs. Fn	40.098	0.400	1.000	No
Fn vs. Col V	20.124	0.342	1.000	No
Fetal vs. Col V	38.468	0.318	1.000	No
Neonatal vs. Col V	14.956	0.281	1.000	No
Fetal vs. Ln	31.533	0.246	1.000	No
Fetal vs. Neonatal	23.512	0.202	1.000	No
Fn vs. Ln	13.189	0.184	1.000	No
Col III vs. Fetal	20.759	0.179	1.000	No
Adult vs. Col I	28.293	0.177	1.000	No
Fetal vs. Fn	18.344	0.154	1.000	No
Kidney vs. Fetal	21.754	0.149	1.000	No
Neonatal vs. Ln	8.021	0.119	1.000	No
Fn vs. Neonatal	5.168	0.108	0.999	No
Ln vs. Col V	6.935	0.0920	0.995	No
Kidney vs. Col III	0.995	0.0103	0.992	No

7.2 Statistical Analysis for Infarct Experiments

Two way ANOVAs were run on SigmaPlot in both trials of the infarct experiment for the data obtained from quantifying histology. The ANOVAs compared the intensity of Nkx2.5 or GATA4 while one of the three variables, ECM composition, stiffness, or oxygen content, was held constant. The condition held constant is listed under Data source for each ANOVA. A p value less than 0.05 was determined a statistically significant difference of means. If significance was found, the Holm-Sidak method was used to perform pairwise multiple comparisons within groups. The conditions for stiffness are listed as healthy or infarct, for ECM composition the conditions are described as doped or normal, and for oxygen content, they are listed as normoxia and hypoxia.

Trial 1

Two Way Analysis of Variance Monday, April 16, 2012, 5:22:51 PM

Data source: Nkx2.5 expression in hypoxic conditions

General Linear Model

Dependent Variable: nkx

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
stiffness	1	2448778.573	2448778.573	51.208	<0.001
ecm	1	102987.286	102987.286	2.154	0.143
stiffness x ecm	1	622033.751	622033.751	13.008	<0.001
Residual	665	31800159.662	47819.789		
Total	668	34411983.749	51514.946		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of stiffness depends on what level of ecm is present. There is a statistically significant interaction between stiffness and ecm. (P = <0.001)

Power of performed test with alpha = 0.0500: for stiffness : 1.000
Power of performed test with alpha = 0.0500: for ecm : 0.175
Power of performed test with alpha = 0.0500: for stiffness x ecm : 0.951

Least square means for stiffness :

Group	Mean	SEM
Healthy	300.949	11.644
Infarct	164.437	15.111

Least square means for ecm :

Group	Mean	SEM
Doped	218.695	15.528
Normal	246.691	11.081

Least square means for stiffness x ecm :

Group	Mean	SEM
Healthy x Doped	321.352	19.880
Healthy x Normal	280.546	12.130
Infarct x Doped	116.039	23.860
Infarct x Normal	212.836	18.548

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **ecm within Healthy**

Comparison	Diff of Means	t	P	P<0.05

Doped vs. Normal	40.807	1.752	0.080	No

Comparisons for factor: **ecm within Infarct**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Normal vs. Doped	96.798	3.203	0.001	Yes

Comparisons for factor: **stiffness within Doped**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Healthy vs. Infarct	205.314	6.611	<0.001	Yes

Comparisons for factor: **stiffness within Normal**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Healthy vs. Infarct	67.710	3.055	0.002	Yes

Two Way Analysis of Variance Monday, April 16, 2012, 5:23:15 PM

Data source: GATA4 intensity in hypoxia

General Linear Model

Dependent Variable: gata

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
stiffness	1	179251.683	179251.683	2.046	0.153
ecm	1	11736.405	11736.405	0.134	0.714
stiffness x ecm	1	27971.232	27971.232	0.319	0.572
Residual	665	58256792.141	87604.199		
Total	668	58540193.065	87635.020		

The difference in the mean values among the different levels of stiffness is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in ecm. There is not a statistically significant difference (P = 0.153).

The difference in the mean values among the different levels of ecm is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in stiffness. There is not a statistically significant difference (P = 0.714).

The effect of different levels of stiffness does not depend on what level of ecm is present. There is not a statistically significant interaction between stiffness and ecm. (P = 0.572)

Power of performed test with alpha = 0.0500: for stiffness : 0.163
Power of performed test with alpha = 0.0500: for ecm : 0.0500
Power of performed test with alpha = 0.0500: for stiffness x ecm : 0.0500

Least square means for stiffness :

Group	Mean	SEM
Healthy	376.335	15.760
Infarct	339.401	20.452

Least square means for ecm :

Group	Mean	SEM
Doped	362.593	21.017
Normal	353.142	14.998

Least square means for stiffness x ecm :

Group	Mean	SEM
Healthy x Doped	373.765	26.907
Healthy x Normal	378.904	16.418
Infarct x Doped	351.421	32.294
Infarct x Normal	327.380	25.105

Two Way Analysis of Variance Monday, April 16, 2012, 5:24:09 PM

Data source: Nkx2.5 Intensity in Normoxia

General Linear Model (No Interactions)

Dependent Variable: nkx

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
stiffness	1	10750945.264	10750945.264	33.776	<0.001
ecm	2	6196677.847	3098338.924	9.734	<0.001
Residual	313	99628708.622	318302.583		
Total	316	111474436.295	352767.203		

The difference in the mean values among the different levels of stiffness is greater than would be expected by chance after allowing for effects of differences in ecm. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of ecm is greater than would be expected by chance after allowing for effects of differences in stiffness. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

Power of performed test with alpha = 0.0500: for stiffness : 1.000
Power of performed test with alpha = 0.0500: for ecm : 0.979

Least square means for stiffness :

Group	Mean	SEM
Healthy	396.192	61.904
Infarct	-121.553	53.094

Least square means for ecm :

Group	Mean	SEM
Doped	-184.699	88.156
Normal	281.883	44.543
Doped	314.775	79.281

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **stiffness**

Comparison	Diff of Means	t	P	P<0.050
Healthy vs. Infarct	517.746	6.348	<0.001	Yes

Comparisons for factor: **ecm**

Comparison	Diff of Means	t	P	P<0.050
Normal vs. Doped	466.582	4.724	<0.001	Yes
Doped vs. Doped	499.475	4.213	<0.001	Yes
Doped vs. Normal	32.892	0.362	0.718	No

Two Way Analysis of Variance Monday, April 16, 2012, 5:24:34 PM

Data source: GATA4 intensity in Normoxia

General Linear Model (No Interactions)

Dependent Variable: gata

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
stiffness	1	2418094.682	2418094.682	98.302	<0.001
ecm	2	1757373.777	878686.889	35.721	<0.001
Residual	313	7699362.374	24598.602		
Total	316	10314721.974	32641.525		

The difference in the mean values among the different levels of stiffness is greater than would be expected by chance after allowing for effects of differences in ecm. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of ecm is greater than would be expected by chance after allowing for effects of differences in stiffness. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

Power of performed test with alpha = 0.0500: for stiffness : 1.000
Power of performed test with alpha = 0.0500: for ecm : 1.000

Least square means for stiffness :

Group	Mean	SEM
Healthy	217.207	17.209
Infarct	-28.338	14.760

Least square means for ecm :

Group	Mean	SEM
Doped	-83.570	24.507
Normal	154.269	12.383
Doped	212.604	22.040

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **stiffness**

Comparison	Diff of Means	t	P	P<0.050
Healthy vs. Infarct	245.544	10.831	<0.001	Yes

Comparisons for factor: **ecm**

Comparison	Diff of Means	t	P	P<0.050
Doped vs. Doped	296.174	8.986	<0.001	Yes
Normal vs. Doped	237.839	8.662	<0.001	Yes
Doped vs. Normal	58.335	2.308	0.022	Yes

Two Way Analysis of Variance Monday, April 16, 2012, 5:26:43 PM

Data source: Nkx2.5 intensity in infarct stiffness

General Linear Model

Dependent Variable: nkx

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
ecm	1	101324.424	101324.424	7.223	0.007
oxygen	1	1550217.719	1550217.719	110.510	<0.001
ecm x oxygen	1	417307.551	417307.551	29.749	<0.001
Residual	423	5933775.750	14027.839		
Total	426	8606503.540	20203.060		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of ecm depends on what level of oxygen is present. There is a statistically significant interaction between ecm and oxygen. (P = <0.001)

Power of performed test with alpha = 0.0500: for ecm : 0.708
Power of performed test with alpha = 0.0500: for oxygen : 1.000
Power of performed test with alpha = 0.0500: for ecm x oxygen : 1.000

Least square means for ecm :

Group	Mean	SEM
Doped	85.970	9.441
Normal	117.923	7.225

Least square means for oxygen :

Group	Mean	SEM
Hypoxic	164.437	8.184
Normoxic	39.456	8.624

Least square means for ecm x oxygen :

Group	Mean	SEM
Doped x Hypoxic	116.039	12.923
Doped x Normoxic	55.902	13.768
Normal x Hypoxic	212.836	10.046
Normal x Normoxic	23.010	10.388

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **oxygen within Doped**

Comparison	Diff of Means	t	P	P<0.05
Hypoxic vs. Normoxic	60.136	3.185	0.002	Yes

Comparisons for factor: **oxygen within Normal**

Comparison	Diff of Means	t	P	P<0.05
Hypoxic vs. Normoxic	189.826	13.136	<0.001	Yes

Comparisons for factor: **ecm within Hypoxic**

Comparison	Diff of Means	t	P	P<0.05
Normal vs. Doped	96.798	5.914	<0.001	Yes

Comparisons for factor: **ecm within Normoxic**

Comparison	Diff of Means	t	P	P<0.05
Doped vs. Normal	32.892	1.907	0.057	No

Two Way Analysis of Variance Monday, April 16, 2012, 5:28:59 PM

Data source: GATA4 intensity in infarct stiffness

General Linear Model

Dependent Variable: gata

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
ecm	1	168358.318	168358.318	3.775	0.053
oxygen	1	7710626.112	7710626.112	172.880	<0.001
ecm x oxygen	1	29179.636	29179.636	0.654	0.419
Residual	423	18866269.591	44601.110		
Total	426	27636568.469	64874.574		

The difference in the mean values among the different levels of ecm is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in oxygen. There is not a statistically significant difference (P = 0.053).

The difference in the mean values among the different levels of oxygen is greater than would be expected by chance after allowing for effects of differences in ecm. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of ecm does not depend on what level of oxygen is present. There is not a statistically significant interaction between ecm and oxygen. (P = 0.419)

Power of performed test with alpha = 0.0500: for ecm : 0.366
Power of performed test with alpha = 0.0500: for oxygen : 1.000
Power of performed test with alpha = 0.0500: for ecm x oxygen : 0.0500

Least square means for ecm :

Group	Mean	SEM
Doped	220.626	16.835
Normal	179.439	12.884

Least square means for oxygen :

Group	Mean	SEM
Hypoxic	339.401	14.593
Normoxic	60.664	15.377

Least square means for ecm x oxygen :

Group	Mean	SEM
Doped x Hypoxic	351.421	23.043
Doped x Normoxic	89.832	24.550
Normal x Hypoxic	327.380	17.913
Normal x Normoxic	31.497	18.523

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **oxygen**

Comparison	Diff of Means	t	P	P<0.050
Hypoxic vs. Normoxic	278.736	13.148	<0.001	Yes

Data source: Nkx2.5 intensity in normal stiffness

General Linear Model (No Interactions)

Dependent Variable: nkx

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
ecm	2	6292482.424	3146241.212	13.914	<0.001
oxygen	1	3332425.498	3332425.498	14.738	<0.001
Residual	555	125495092.533	226117.284		
Total	558	131831376.316	236256.947		

The difference in the mean values among the different levels of ecm is greater than would be expected by chance after allowing for effects of differences in oxygen. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of oxygen is greater than would be expected by chance after allowing for effects of differences in ecm. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

Power of performed test with alpha = 0.0500: for ecm : 0.999
Power of performed test with alpha = 0.0500: for oxygen : 0.974

Least square means for ecm :

Group	Mean	SEM
Doped	451.457	54.930
Normal	410.651	33.891
Doped	-55.931	72.525

Least square means for oxygen :

Group	Mean	SEM
Hypoxic	138.621	37.508
Normoxic	398.831	49.744

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **ecm**

Comparison	Diff of Means	t	P	P<0.050
Normal vs. Doped	466.582	5.828	<0.001	Yes
Doped vs. Doped	507.389	5.577	<0.001	Yes
Doped vs. Normal	40.807	0.632	0.527	No

Comparisons for factor: **oxygen**

Comparison	Diff of Means	t	P	P<0.050
Normoxic vs. Hypoxic	260.210	4.177	<0.001	Yes

Two Way Analysis of Variance Monday, April 16, 2012, 5:29:56 PM

Data source: GATA4 intensity in normal stiffness

General Linear Model (No Interactions)

Dependent Variable: gata

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
ecm	2	1599231.500	799615.750	9.424	<0.001
oxygen	1	510673.720	510673.720	6.019	0.014
Residual	555	47089884.923	84846.640		
Total	558	52904497.566	94810.928		

The difference in the mean values among the different levels of ecm is greater than would be expected by chance after allowing for effects of differences in oxygen. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of oxygen is greater than would be expected by chance after allowing for effects of differences in ecm. There is a statistically significant difference (P = 0.014). To isolate which group(s) differ from the others use a multiple comparison procedure.

Power of performed test with alpha = 0.0500: for ecm : 0.975
Power of performed test with alpha = 0.0500: for oxygen : 0.606

Least square means for ecm :

Group	Mean	SEM
Doped	322.834	33.648
Normal	327.973	20.760
Doped	90.134	44.426

Least square means for oxygen :

Group	Mean	SEM
Hypoxic	297.912	22.976
Normoxic	196.049	30.471

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **ecm**

Comparison	Diff of Means	t	P	P<0.050
Normal vs. Doped	237.839	4.850	<0.001	Yes
Doped vs. Doped	232.700	4.175	<0.001	Yes
Normal vs. Doped	5.139	0.130	0.897	No

Comparisons for factor: **oxygen**

Comparison	Diff of Means	t	P	P<0.050
Hypoxic vs. Normoxic	101.863	2.669	0.008	Yes

Two Way Analysis of Variance Monday, April 16, 2012, 5:32:24 PM

Data source: Nkx2.5 intensity in infarct ECM

General Linear Model

Dependent Variable: nkx

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
stiffness	1	963862.283	963862.283	20.748	<0.001
oxygen	1	1820946.464	1820946.464	39.198	<0.001
stiffness x oxygen	1	674544.548	674544.548	14.521	<0.001
Residual	330	15330025.658	46454.623		
Total	333	19814822.561	59503.972		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of stiffness depends on what level of oxygen is present. There is a statistically significant interaction between stiffness and oxygen. (P = <0.001)

Power of performed test with alpha = 0.0500: for stiffness : 0.998
Power of performed test with alpha = 0.0500: for oxygen : 1.000
Power of performed test with alpha = 0.0500: for stiffness x oxygen : 0.971

Least square means for stiffness :

Group	Mean	SEM
Healthy	197.763	17.525
Infarct	85.970	17.181

Least square means for oxygen :

Group	Mean	SEM
Hypoxic	218.695	15.305
Normoxic	65.038	19.186

Least square means for stiffness x oxygen :

Group	Mean	SEM
Healthy x Hypoxic	321.352	19.594
Healthy x Normoxic	74.174	29.063
Infarct x Hypoxic	116.039	23.517
Infarct x Normoxic	55.902	25.055

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **oxygen within Healthy**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Hypoxic vs. Normoxic	247.179	7.052	<0.001	Yes

Comparisons for factor: **oxygen within Infarct**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Hypoxic vs. Normoxic	60.136	1.750	0.081	No

Comparisons for factor: **stiffness within Hypoxic**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Healthy vs. Infarct	205.314	6.707	<0.001	Yes

Comparisons for factor: **stiffness within Normoxic**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Healthy vs. Infarct	18.271	0.476	0.634	No

Two Way Analysis of Variance Monday, April 16, 2012, 5:32:47 PM

Data source: GATA4 intensity in infarct ECM

General Linear Model

Dependent Variable: gata

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
stiffness	1	15425.661	15425.661	0.208	0.649
oxygen	1	6852405.143	6852405.143	92.408	<0.001
stiffness x oxygen	1	102673.617	102673.617	1.385	0.240
Residual	330	24470873.993	74154.164		
Total	333	31530705.656	94686.804		

The difference in the mean values among the different levels of stiffness is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in oxygen. There is not a statistically significant difference (P = 0.649).

The difference in the mean values among the different levels of oxygen is greater than would be expected by chance after allowing for effects of differences in stiffness. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of stiffness does not depend on what level of oxygen is present. There is not a statistically significant interaction between stiffness and oxygen. (P = 0.240)

Power of performed test with alpha = 0.0500: for stiffness : 0.0500
Power of performed test with alpha = 0.0500: for oxygen : 1.000

Power of performed test with alpha = 0.0500: for stiffness x oxygen : 0.0887

Least square means for stiffness :

Group	Mean	SEM
Healthy	206.484	22.142
Infarct	220.626	21.708

Least square means for oxygen :

Group	Mean	SEM
Hypoxic	362.593	19.337
Normoxic	64.517	24.240

Least square means for stiffness x oxygen :

Group	Mean	SEM
Healthy x Hypoxic	373.765	24.756
Healthy x Normoxic	39.202	36.719
Infarct x Hypoxic	351.421	29.712
Infarct x Normoxic	89.832	31.656

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **oxygen**

Comparison	Diff of Means	t	P	P<0.050
Hypoxic vs. Normoxic	298.076	9.613	<0.001	Yes

Two Way Analysis of Variance Monday, April 16, 2012, 5:33:12 PM

Data source: Nkx2.5 intensity in normal ECM

General Linear Model

Dependent Variable: nkx

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
stiffness	1	9736098.793	9736098.793	54.342	<0.001
oxygen	1	140715.358	140715.358	0.785	0.376
stiffness x oxygen	1	5752974.420	5752974.420	32.110	<0.001
Residual	648	116098842.626	179164.881		
Total	651	128100184.547	196774.477		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of stiffness depends on what level of oxygen is present. There is a statistically significant interaction between stiffness and oxygen. (P = <0.001)

Power of performed test with alpha = 0.0500: for stiffness : 1.000
Power of performed test with alpha = 0.0500: for oxygen : 0.0500
Power of performed test with alpha = 0.0500: for stiffness x oxygen : 1.000

Least square means for stiffness :

Group	Mean	SEM
Healthy	410.651	30.168
Infarct	117.923	25.822

Least square means for oxygen :

Group	Mean	SEM
Hypoxic	246.691	21.449
Normoxic	281.883	33.419

Least square means for stiffness x oxygen :

Group	Mean	SEM
Healthy x Hypoxic	280.546	23.479
Healthy x Normoxic	540.756	55.579
Infarct x Hypoxic	212.836	35.902
Infarct x Normoxic	23.010	37.124

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **oxygen within Healthy**

Comparison	Diff of Means	t	P	P<0.05
Normoxic vs. Hypoxic	260.210	4.313	<0.001	Yes

Comparisons for factor: **oxygen within Infarct**

Comparison	Diff of Means	t	P	P<0.05
Hypoxic vs. Normoxic	189.826	3.676	<0.001	Yes

Comparisons for factor: **stiffness within Hypoxic**

Comparison	Diff of Means	t	P	P<0.05
Healthy vs. Infarct	67.710	1.578	0.115	No

Comparisons for factor: **stiffness within Normoxic**

Comparison	Diff of Means	t	P	P<0.05
Healthy vs. Infarct	517.746	7.746	<0.001	Yes

Two Way Analysis of Variance Monday, April 16, 2012, 5:33:50 PM

Data source: GATA4 intensity in normal ECM

General Linear Model

Dependent Variable: gata

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
stiffness	1	2506743.010	2506743.010	39.155	<0.001
oxygen	1	4493751.162	4493751.162	70.192	<0.001
stiffness x oxygen	1	1069282.371	1069282.371	16.702	<0.001
Residual	648	41485280.522	64020.495		
Total	651	52945006.403	81328.735		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of stiffness depends on what level of oxygen is present. There is a statistically significant interaction between stiffness and oxygen. (P = <0.001)

Power of performed test with alpha = 0.0500: for stiffness : 1.000
Power of performed test with alpha = 0.0500: for oxygen : 1.000
Power of performed test with alpha = 0.0500: for stiffness x oxygen : 0.987

Least square means for stiffness :

Group	Mean	SEM
Healthy	327.973	18.033
Infarct	179.439	15.436

Least square means for oxygen :

Group	Mean	SEM
Hypoxic	353.142	12.822
Normoxic	154.269	19.977

Least square means for stiffness x oxygen :

Group	Mean	SEM
Healthy x Hypoxic	378.904	14.035
Healthy x Normoxic	277.042	33.224
Infarct x Hypoxic	327.380	21.461
Infarct x Normoxic	31.497	22.192

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **oxygen within Healthy**

Comparison	Diff of Means	t	P	P<0.05
Hypoxic vs. Normoxic	101.863	2.824	0.005	Yes

Comparisons for factor: **oxygen within Infarct**

Comparison	Diff of Means	t	P	P<0.05
Hypoxic vs. Normoxic	295.883	9.584	<0.001	Yes

Comparisons for factor: **stiffness within Hypoxic**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Healthy vs. Infarct	51.524	2.009	0.045	Yes

Comparisons for factor: **stiffness within Normoxic**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Healthy vs. Infarct	245.544	6.146	<0.001	Yes

Trial 2

Two Way Analysis of Variance Monday, April 16, 2012, 5:53:26 PM

Data source: Nkx2.5 intensity in hypoxia

General Linear Model

Dependent Variable: nkx

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
stiffness	1	56642.250	56642.250	4.343	0.038
ecm comp	1	296199.293	296199.293	22.713	<0.001
stiffness x ecm comp	1	48891.787	48891.787	3.749	0.053
Residual	516	6729191.393	13041.069		
Total	519	7074758.301	13631.519		

The difference in the mean values among the different levels of stiffness is greater than would be expected by chance after allowing for effects of differences in ecm comp. There is a statistically significant difference (P = 0.038). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of ecm comp is greater than would be expected by chance after allowing for effects of differences in stiffness. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of stiffness does not depend on what level of ecm comp is present. There is not a statistically significant interaction between stiffness and ecm comp. (P = 0.053)

Power of performed test with alpha = 0.0500: for stiffness : 0.432
Power of performed test with alpha = 0.0500: for ecm comp : 0.999
Power of performed test with alpha = 0.0500: for stiffness x ecm comp : 0.364

Least square means for stiffness :

Group	**Mean**	**SEM**
Healthy	118.117	8.350
Infarct	88.262	11.641

Least square means for ecm comp :

Group	Mean	SEM
Doped	69.053	11.667
Normal	137.326	8.312

Least square means for stiffness x ecm comp :

Group	Mean	SEM
Healthy x Doped	97.850	7.843
Healthy x Normal	138.385	14.743
Infarct x Doped	40.257	21.977
Infarct x Normal	136.267	7.682

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **stiffness**

Comparison	Diff of Means	t	P	P<0.050
Healthy vs. Infarct	29.855	2.084	0.038	Yes

Comparisons for factor: **ecm comp**

Comparison	Diff of Means	t	P	P<0.050
Normal vs. Doped	68.272	4.766	<0.001	Yes

Two Way Analysis of Variance Monday, April 16, 2012, 5:53:49 PM

Data source: GATA4 intensity in hypoxia

General Linear Model

Dependent Variable: gata

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
stiffness	1	41575.721	41575.721	0.321	0.571
ecm comp	1	73815.721	73815.721	0.569	0.451
stiffness x ecm comp	1	3179126.579	3179126.579	24.525	<0.001
Residual	516	66887680.186	129627.287		
Total	519	70866300.398	136543.931		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of stiffness depends on what level of ecm comp is present. There is a statistically significant interaction between stiffness and ecm comp. (P = <0.001)

Power of performed test with alpha = 0.0500: for stiffness : 0.0500
Power of performed test with alpha = 0.0500: for ecm comp : 0.0500
Power of performed test with alpha = 0.0500: for stiffness x ecm comp : 1.000

Least square means for stiffness :

Group	**Mean**	**SEM**
Healthy	323.369	26.324
Infarct	348.948	36.700

Least square means for ecm comp :

Group	**Mean**	**SEM**
Doped	319.117	36.785
Normal	353.199	26.206

Least square means for stiffness x ecm comp :

Group	**Mean**	**SEM**
Healthy x Doped	418.163	24.728
Healthy x Normal	228.576	46.481
Infarct x Doped	220.072	69.289
Infarct x Normal	477.823	24.219

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **ecm comp within Healthy**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Doped vs. Normal	189.587	3.601	<0.001	Yes

Comparisons for factor: **ecm comp within Infarct**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Normal vs. Doped	257.752	3.512	<0.001	Yes

Comparisons for factor: **stiffness within Doped**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Healthy vs. Infarct	198.091	2.693	0.007	Yes

Comparisons for factor: **stiffness within Normal**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Infarct vs. Healthy	249.248	4.756	<0.001	Yes

Two Way Analysis of Variance Monday, April 16, 2012, 5:54:10 PM

Data source: Nkx2.5 intensity in normoxia

General Linear Model

Dependent Variable: nkx

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
stiffness	1	18788.267	18788.267	3.545	0.062
ecm	1	21087.514	21087.514	3.979	0.048
stiffness x ecm	1	57202.953	57202.953	10.793	0.001
Residual	132	699615.497	5300.117		
Total	135	1183096.659	8763.679		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of stiffness depends on what level of ecm is present. There is a statistically significant interaction between stiffness and ecm. (P = 0.001)

Power of performed test with alpha = 0.0500: for stiffness : 0.337
Power of performed test with alpha = 0.0500: for ecm : 0.387
Power of performed test with alpha = 0.0500: for stiffness x ecm : 0.893

Least square means for stiffness :

Group	Mean	SEM
Healthy	88.153	7.007
Infarct	44.248	22.241

Least square means for ecm :

Group	Mean	SEM
Doped	42.944	21.603
Normal	89.457	8.780

Least square means for stiffness x ecm :

Group	Mean	SEM
Healthy x Doped	26.592	10.000
Healthy x Normal	149.713	9.817
Infarct x Doped	59.295	42.032
Infarct x Normal	29.201	14.560

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **ecm within Healthy**

Comparison	Diff of Means	t	P	P<0.05
Normal vs. Doped	123.121	8.786	<0.001	Yes

Comparisons for factor: **ecm within Infarct**

Comparison	Diff of Means	t	P	P<0.05
Doped vs. Normal	30.095	0.677	0.500	No

Comparisons for factor: **stiffness within Doped**

Comparison	Diff of Means	t	P	P<0.05
Infarct vs. Healthy	32.704	0.757	0.450	No

Comparisons for factor: **stiffness within Normal**

Comparison	Diff of Means	t	P	P<0.05
Healthy vs. Infarct	120.512	6.863	<0.001	Yes

Two Way Analysis of Variance Monday, April 16, 2012, 5:54:44 PM

Data source: GATA4 intensity in normoxia

General Linear Model

Dependent Variable: gata

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
stiffness	1	1863142.169	1863142.169	9.481	0.003
ecm	1	913538.940	913538.940	4.649	0.033
stiffness x ecm	1	561845.241	561845.241	2.859	0.093
Residual	132	25939596.560	196512.095		
Total	135	37858364.639	280432.331		

The difference in the mean values among the different levels of stiffness is greater than would be expected by chance after allowing for effects of differences in ecm. There is a statistically significant difference (P = 0.003). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of ecm is greater than would be expected by chance after allowing for effects of differences in stiffness. There is a statistically significant difference (P = 0.033). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of stiffness does not depend on what level of ecm is present. There is not a statistically significant interaction between stiffness and ecm. (P = 0.093)

Power of performed test with alpha = 0.0500: for stiffness : 0.841
Power of performed test with alpha = 0.0500: for ecm : 0.462
Power of performed test with alpha = 0.0500: for stiffness x ecm : 0.256

Least square means for stiffness :

Group	Mean	SEM
Healthy	763.169	42.664
Infarct	325.961	135.429

Least square means for ecm :

Group	Mean	SEM
Doped	391.492	131.541
Normal	697.638	53.464

Least square means for stiffness x ecm :

Group	Mean	SEM
Healthy x Doped	490.052	60.892
Healthy x Normal	1036.287	59.774
Infarct x Doped	292.933	255.938
Infarct x Normal	358.990	88.659

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **stiffness**

Comparison	Diff of Means	t	P	P<0.050
Healthy vs. Infarct	437.208	3.079	0.003	Yes

Comparisons for factor: **ecm**

Comparison	Diff of Means	t	P	P<0.050
Normal vs. Doped	306.146	2.156	0.033	Yes

Two Way Analysis of Variance Monday, April 16, 2012, 5:44:59 PM

Data source: Nkx2.5 intensity in infarct stiffness

General Linear Model

Dependent Variable: nkx

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
ecm	1	10472.129	10472.129	0.601	0.439
oxygen	1	18676.695	18676.695	1.072	0.302
ecm x oxygen	1	38328.674	38328.674	2.199	0.139
Residual	272	4740931.019	17429.893		
Total	275	5184572.872	18852.992		

The difference in the mean values among the different levels of ecm is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in oxygen. There is not a statistically significant difference (P = 0.439).

The difference in the mean values among the different levels of oxygen is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in ecm. There is not a statistically significant difference (P = 0.302).

The effect of different levels of ecm does not depend on what level of oxygen is present. There is not a statistically significant interaction between ecm and oxygen. (P = 0.139)

Power of performed test with alpha = 0.0500: for ecm : 0.0500

Power of performed test with alpha = 0.0500: for oxygen : 0.0550
Power of performed test with alpha = 0.0500: for ecm x oxygen : 0.180

Least square means for ecm :

Group	Mean	SEM
Doped	49.776	40.173
Normal	82.734	13.929

Least square means for oxygen :

Group	Mean	SEM
Hypoxic	88.262	13.458
Normoxic	44.248	40.333

Least square means for ecm x oxygen :

Group	Mean	SEM
Doped x Hypoxic	40.257	25.408
Doped x Normoxic	59.295	76.223
Normal x Hypoxic	136.267	8.881
Normal x Normoxic	29.201	26.404

Two Way Analysis of Variance Monday, April 16, 2012, 5:45:59 PM

Data source: GATA4 intensity in infarct stiffness

General Linear Model

Dependent Variable: gata

Normality Test (Shapiro-Wilk) Failed ($P < 0.050$)

Equal Variance Test: Failed ($P < 0.050$)

Source of Variation	DF	SS	MS	F	P
ecm	1	252718.033	252718.033	1.848	0.175
oxygen	1	5093.883	5093.883	0.0372	0.847
ecm x oxygen	1	88569.466	88569.466	0.647	0.422
Residual	272	37206179.955	136787.426		
Total	275	39057235.437	142026.311		

The difference in the mean values among the different levels of ecm is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in oxygen. There is not a statistically significant difference ($P = 0.175$).

The difference in the mean values among the different levels of oxygen is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in ecm. There is not a statistically significant difference ($P = 0.847$).

The effect of different levels of ecm does not depend on what level of oxygen is present. There is not a statistically significant interaction between ecm and oxygen. ($P = 0.422$)

Power of performed test with alpha = 0.0500: for ecm : 0.140
Power of performed test with alpha = 0.0500: for oxygen : 0.0500

Power of performed test with alpha = 0.0500: for ecm x oxygen : 0.0500

Least square means for ecm :

Group	Mean	SEM
Doped	256.503	112.541
Normal	418.406	39.021

Least square means for oxygen :

Group	Mean	SEM
Hypoxic	348.948	37.700
Normoxic	325.961	112.990

Least square means for ecm x oxygen :

Group	Mean	SEM
Doped x Hypoxic	220.072	71.177
Doped x Normoxic	292.933	213.532
Normal x Hypoxic	477.823	24.879
Normal x Normoxic	358.990	73.970

Two Way Analysis of Variance Monday, April 16, 2012, 5:46:30 PM

Data source: Nkx2.5 intensity in normal stiffness

General Linear Model

Dependent Variable: nkx

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
ecm	1	458355.298	458355.298	64.118	<0.001
oxygen	1	61464.249	61464.249	8.598	0.004
ecm x oxygen	1	116723.705	116723.705	16.328	<0.001
Residual	376	2687875.870	7148.606		
Total	379	3197533.468	8436.764		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of ecm depends on what level of oxygen is present. There is a statistically significant interaction between ecm and oxygen. (P = <0.001)

Power of performed test with alpha = 0.0500: for ecm : 1.000
Power of performed test with alpha = 0.0500: for oxygen : 0.799
Power of performed test with alpha = 0.0500: for ecm x oxygen : 0.985

Least square means for ecm :

Group	Mean	SEM
Doped	62.221	6.492
Normal	144.049	7.892

Least square means for oxygen :

Group	Mean	SEM
Hypoxic	118.117	6.182
Normoxic	88.153	8.137

Least square means for ecm x oxygen :

Group	Mean	SEM
Doped x Hypoxic	97.850	5.807
Doped x Normoxic	26.592	11.614
Normal x Hypoxic	138.385	10.915
Normal x Normoxic	149.713	11.401

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **oxygen within Doped**

Comparison	Diff of Means	t	P	P<0.05
Hypoxic vs. Normoxic	71.258	5.488	<0.001	Yes

Comparisons for factor: **oxygen within Normal**

Comparison	Diff of Means	t	P	P<0.05
Normoxic vs. Hypoxic	11.329	0.718	0.473	No

Comparisons for factor: **ecm within Hypoxic**

Comparison	Diff of Means	t	P	P<0.05
Normal vs. Doped	40.535	3.278	0.001	Yes

Comparisons for factor: **ecm within Normoxic**

Comparison	Diff of Means	t	P	P<0.05
Normal vs. Doped	123.121	7.565	<0.001	Yes

Two Way Analysis of Variance Monday, April 16, 2012, 5:47:00 PM

Data source: GATA4 intensity in normal stiffness

General Linear Model

Dependent Variable: gata

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
ecm	1	2176799.919	2176799.919	14.715	<0.001
oxygen	1	13240648.596	13240648.596	89.507	<0.001

ecm x oxygen	1	9265843.421	9265843.421	62.637	<0.001
Residual	376	55621096.791	147928.449		
Total	379	77227335.356	203766.056		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of ecm depends on what level of oxygen is present. There is a statistically significant interaction between ecm and oxygen. (P = <0.001)

Power of performed test with alpha = 0.0500: for ecm : 0.973
Power of performed test with alpha = 0.0500: for oxygen : 1.000
Power of performed test with alpha = 0.0500: for ecm x oxygen : 1.000

Least square means for ecm :

Group	**Mean**	**SEM**
Doped	454.107	29.533
Normal	632.431	35.899

Least square means for oxygen :

Group	**Mean**	**SEM**
Hypoxic	323.369	28.121
Normoxic	763.169	37.016

Least square means for ecm x oxygen :

Group	**Mean**	**SEM**
Doped x Hypoxic	418.163	26.415
Doped x Normoxic	490.052	52.831
Normal x Hypoxic	228.576	49.654
Normal x Normoxic	1036.287	51.861

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **oxygen within Doped**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Normoxic vs. Hypoxic	71.889	1.217	0.224	No

Comparisons for factor: **oxygen within Normal**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Normoxic vs. Hypoxic	807.711	11.250	<0.001	Yes

Comparisons for factor: **ecm within Hypoxic**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Doped vs. Normal	189.587	3.371	<0.001	Yes

Comparisons for factor: **ecm within Normoxic**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**

Normal vs. Doped	546.235	7.378	<0.001	Yes

Two Way Analysis of Variance Monday, April 16, 2012, 5:49:30 PM

Data source: Nkx2.5 intensity in infarct ECM

General Linear Model

Dependent Variable: nkx

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
stiffness	1	1572.486	1572.486	0.312	0.577
oxygen	1	6921.841	6921.841	1.375	0.242
stiffness x oxygen	1	20696.482	20696.482	4.112	0.043
Residual	291	1464723.608	5033.414		
Total	294	1727271.673	5875.074		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of stiffness depends on what level of oxygen is present. There is a statistically significant interaction between stiffness and oxygen. (P = 0.043)

Power of performed test with alpha = 0.0500: for stiffness : 0.0500
Power of performed test with alpha = 0.0500: for oxygen : 0.0877
Power of performed test with alpha = 0.0500: for stiffness x oxygen : 0.405

Least square means for stiffness :

Group	Mean	SEM
Healthy	62.221	5.448
Infarct	49.776	21.588

Least square means for oxygen :

Group	Mean	SEM
Hypoxic	69.053	7.249
Normoxic	42.944	21.052

Least square means for stiffness x oxygen :

Group	Mean	SEM
Healthy x Hypoxic	97.850	4.873
Healthy x Normoxic	26.592	9.745
Infarct x Hypoxic	40.257	13.654
Infarct x Normoxic	59.295	40.961

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **oxygen within Healthy**

Comparison	Diff of Means	t	P	P<0.05
Hypoxic vs. Normoxic	71.258	6.540	<0.001	Yes

Comparisons for factor: **oxygen within Infarct**

Comparison	Diff of Means	t	P	P<0.05
Normoxic vs. Hypoxic	19.038	0.441	0.660	No

Comparisons for factor: **stiffness within Hypoxic**

Comparison	Diff of Means	t	P	P<0.05
Healthy vs. Infarct	57.593	3.973	<0.001	Yes

Comparisons for factor: **stiffness within Normoxic**

Comparison	Diff of Means	t	P	P<0.05
Infarct vs. Healthy	32.704	0.777	0.438	No

Two Way Analysis of Variance Monday, April 16, 2012, 5:49:57 PM

Data source: GATA4 intensity in infarct ECM

General Linear Model

Dependent Variable: gata

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.085)

Source of Variation	DF	SS	MS	F	P
stiffness	1	396467.089	396467.089	3.657	0.057
oxygen	1	53185.367	53185.367	0.491	0.484
stiffness x oxygen	1	2.402	2.402	0.0000222	0.996
Residual	291	31546316.866	108406.587		
Total	294	32914330.133	111953.504		

The difference in the mean values among the different levels of stiffness is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in oxygen. There is not a statistically significant difference (P = 0.057).

The difference in the mean values among the different levels of oxygen is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in stiffness. There is not a statistically significant difference (P = 0.484).

The effect of different levels of stiffness does not depend on what level of oxygen is present. There is not a statistically significant interaction between stiffness and oxygen. (P = 0.996)

Power of performed test with alpha = 0.0500: for stiffness : 0.352
Power of performed test with alpha = 0.0500: for oxygen : 0.0500
Power of performed test with alpha = 0.0500: for stiffness x oxygen : 0.0500

Least square means for stiffness :

Group	Mean	SEM
Healthy	454.107	25.282
Infarct	256.503	100.188

Least square means for oxygen :

Group	Mean	SEM
Hypoxic	319.117	33.639
Normoxic	391.492	97.700

Least square means for stiffness x oxygen :

Group	Mean	SEM
Healthy x Hypoxic	418.163	22.613
Healthy x Normoxic	490.052	45.226
Infarct x Hypoxic	220.072	63.364
Infarct x Normoxic	292.933	190.093

Two Way Analysis of Variance Monday, April 16, 2012, 5:50:28 PM

Data source: Nkx2.5 intensity in normal ECM

General Linear Model

Dependent Variable: nkx

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.068)

Source of Variation	DF	SS	MS	F	P
stiffness	1	189460.674	189460.674	11.341	<0.001
oxygen	1	115475.734	115475.734	6.912	0.009
stiffness x oxygen	1	176599.544	176599.544	10.571	0.001
Residual	357	5964083.281	16706.116		
Total	360	6251799.909	17366.111		

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of stiffness depends on what level of oxygen is present. There is a statistically significant interaction between stiffness and oxygen. (P = 0.001)

Power of performed test with alpha = 0.0500: for stiffness : 0.913
Power of performed test with alpha = 0.0500: for oxygen : 0.683
Power of performed test with alpha = 0.0500: for stiffness x oxygen : 0.889

Least square means for stiffness :

Group	Mean	SEM
Healthy	144.049	12.064
Infarct	82.734	13.637

Least square means for oxygen :

Group	Mean	SEM
Hypoxic	137.326	9.408
Normoxic	89.457	15.588

Least square means for stiffness x oxygen :

Group	Mean	SEM
Healthy x Hypoxic	138.385	16.686
Healthy x Normoxic	149.713	17.428
Infarct x Hypoxic	136.267	8.694
Infarct x Normoxic	29.201	25.850

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **oxygen within Healthy**

Comparison	Diff of Means	t	P	P<0.05
Normoxic vs. Hypoxic	11.329	0.470	0.639	No

Comparisons for factor: **oxygen within Infarct**

Comparison	Diff of Means	t	P	P<0.05
Hypoxic vs. Normoxic	107.066	3.926	<0.001	Yes

Comparisons for factor: **stiffness within Hypoxic**

Comparison	Diff of Means	t	P	P<0.05
Healthy vs. Infarct	2.118	0.113	0.910	No

Comparisons for factor: **stiffness within Normoxic**

Comparison	Diff of Means	t	P	P<0.05
Healthy vs. Infarct	120.512	3.865	<0.001	Yes

Two Way Analysis of Variance Monday, April 16, 2012, 5:50:51 PM

Data source: GATA4 intensity in normal ECM

General Linear Model

Dependent Variable: gata

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
stiffness	1	2308409.599	2308409.599	13.448	<0.001
oxygen	1	5978733.985	5978733.985	34.830	<0.001
stiffness x oxygen	1	10815783.936	10815783.936	63.009	<0.001
Residual	357	61280959.880	171655.350		

Total	360	82061813.076	227949.481

Main effects cannot be properly interpreted if significant interaction is determined. This is because the size of a factor's effect depends upon the level of the other factor.

The effect of different levels of stiffness depends on what level of oxygen is present. There is a statistically significant interaction between stiffness and oxygen. (P = <0.001)

Power of performed test with alpha = 0.0500: for stiffness : 0.957
Power of performed test with alpha = 0.0500: for oxygen : 1.000
Power of performed test with alpha = 0.0500: for stiffness x oxygen : 1.000

Least square means for stiffness :

Group	**Mean**	**SEM**
Healthy	632.431	38.671
Infarct	418.406	43.712

Least square means for oxygen :

Group	**Mean**	**SEM**
Hypoxic	353.199	30.156
Normoxic	697.638	49.968

Least square means for stiffness x oxygen :

Group	**Mean**	**SEM**
Healthy x Hypoxic	228.576	53.488
Healthy x Normoxic	1036.287	55.866
Infarct x Hypoxic	477.823	27.870
Infarct x Normoxic	358.990	82.863

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **oxygen within Healthy**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Normoxic vs. Hypoxic	807.711	10.443	<0.001	Yes

Comparisons for factor: **oxygen within Infarct**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Hypoxic vs. Normoxic	118.834	1.359	0.175	No

Comparisons for factor: **stiffness within Hypoxic**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Infarct vs. Healthy	249.248	4.133	<0.001	Yes

Comparisons for factor: **stiffness within Normoxic**

Comparison	**Diff of Means**	**t**	**P**	**P<0.05**
Healthy vs. Infarct	677.297	6.777	<0.001	Yes

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