An Examination of the Potential of Porcine Mitral Valvular Interstitial Cell Dedifferention by Alpha Smooth Muscle Actin Expression and Caspase activity

A Major Qualifying Project submitted
to the faculty of
WORCESTER POLYTECHNIC INSTITUTE
in partial fulfillment of the requirements for the
Degree of Bachelor of Science in
Biology and Biotechnology

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Date: December 12, 2006

Approved:

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Abstract

The aim of this study was to determine whether the apparent reduction in cell number and α-SMA expression of porcine mitral valvular interstitial cells plated on substrates of varying stiffness was due to apoptosis or dedifferentiation. This decrease was determined to be a result of apoptosis as indicated by caspase levels comparable to the positive control. A more thorough investigation into cellular communication and signaling will be invaluable to the development of next generation valve replacements.

Acknowledgments

First and foremost, we would like to thank our advisor Jill Rulfs for generously allowing us the space to work in her lab and for her invaluable help with everything from experimental design to writing. Next, we would like to thank Heather Hinds for offering her expertise and time in developing our project, for teaching us numerous research techniques and for assisting us with questions and everyday tasks. Jamie Grouf was always a resource in the lab and deserves our thanks. We also owe a thank you to Blood Farm for providing us with an unending supply of freshly excised hog hearts. Finally we are thankful for both our friends and family during our experience at WPI.

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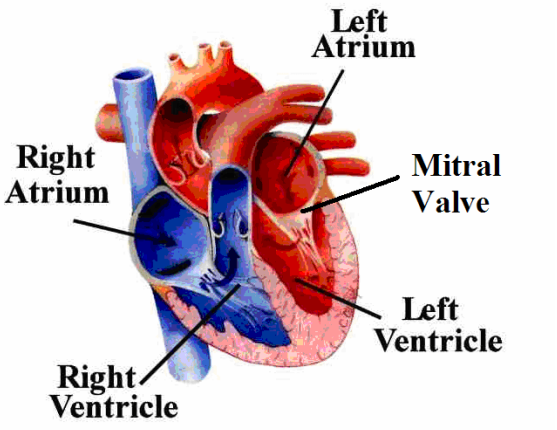
Introduction

The Circulatory System

Within three weeks of conception the heart of the developing embryo begins to beat. The heart of the embryo develops so early because it is part of a crucial transport system of the body, the circulatory system. The human embryo develops a circulatory system to interact with the maternal circulatory system to ensure food supply during development. The circulatory system continues to be imperative throughout life to transport materials to the cells of the body which are necessary for survival. The circulatory system is comprised of three parts: the heart, blood vessels, and the blood.

The main organ of the circulatory system, the heart, is located in the thoracic cavity between the sternum and the vertebrae. It is a hollow, yet muscular organ about the size of the fist. The main responsibility of the heart is to pump blood to and from the tissues of the body. Even though the heart is a single organ, the right and left sides of the heart function as two separate pumps. The heart has four chambers as seen in Figure 1. The upper chambers include the atria and the lower chambers are comprised of the

Figure 1. The heart's chambers (Hartzog 2005)



ventricles. The atria are responsible for receiving blood from circulation and transferring it to the lower chambers. The ventricles function to pump blood out of the heart.

Heart Valves

Blood flows through the heart in one direction--from the veins to the atria to the ventricles and to arteries. The one-way heart valves ensure that blood circulates in this direction every time through the heart. Two of the heart valves are positioned between the atrium and ventricle on the right and left sides and are called the right and left atrioventricular (AV) valves. The right AV valve is called a tricuspid valve because it consists of three cusps and the left AV valve is named a bicuspid valve because it consists of two cusps. Another name for the left AV valve is the mitral valve.

Anatomically, the mitral valve is located on the left side of the heart between the left atrium and the left ventricle as seen in Figure 1. The mitral valve has two leaflets called the anteromedial leaflet and the posterolateral leaflet which guard the opening. The opening is surrounded by the annulus, a tough fibrous ring. These two leaflets are prevented from prolapsing into the left atrium by the fibrous cords called chordae tendinae shown in Figure 2.

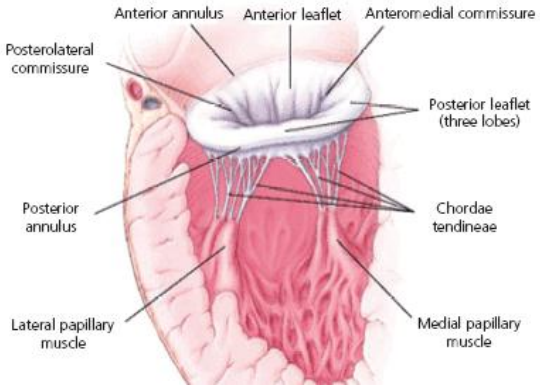


Figure 2. A close-up of the mitral valve (Focosi 2005)

The heart valves are made of connective tissue and therefore open and close passively. Heart valves open and close due to changes in pressure. The AV valves open during ventricular diastole when the pressure in the atria is greater than the pressure in the ventricles and close during ventricular systole when pressure in the ventricles is greater than pressure in the atria. Chordae tendinae function to prevent the valves from being opened in the opposite direction into the atria due to high pressure. Semilunar valves, on the other hand, open during ventricular systole when the pressure in the ventricles is greater than the pressure in the arteries and close during ventricular diastole when the pressure in the pulmonary trunk and aorta is greater than the pressure in the ventricles.

In addition to the external features of the valves, the AV valves are composed of several distinct layers of tissue: spongiosa, fibrosa, ventricularis, and the atrialis layer (Mulhollan and Gotlieb 1996). The spongiosa is composed of loose connective tissue, proteoglycans, elastin and collagen. The fibrosa layer contains several sheets of collagen bundles. The thinnest of the layers, the ventricularis, is comprised primarily of elastic

fibers. Lastly, the atrialis includes elastic fibers, smooth muscle cells, and valvular interstitial cells.

Heart Valve Disease

Mitral valve disease is one of many common heart problems that affect the population today. Mitral valve disease does not just affect the sick and elderly, it can affect anyone. Mitral valve disease can eventually lead to more serious problems such as heart failure and other serious complications. The most common mitral valve disorders include prolapse, regurgitation and stenosis.

Mitral valve prolapse is a disorder of the valve leaflets. It is more common in females, those with low body weight and low systolic blood pressure (Hayes 1997). The valve leaflets do not close properly because they are enlarged which cause them to prolapse into the left atrium. The main cause of this enlargement is an abnormal proliferation of the connective tissue in the valve. When the mitral valve leaflets do not close completely, regurgitation occurs. Regurgitation of the mitral valve allows blood from the left ventricle to go back into the left atrium during systole. This regurgitation increases the left atrial volume and pressure which can cause a progression of abnormalities.

Other less common causes of mitral valve prolapse include dysfunctional or ruptured chordae tendineae, papillary muscle or annulus (Horsham et al 1999). In these cases, other diseases such as rheumatic fever or myocardial infarction are the primary causes of dysfunction. The most common symptoms include palpitations and chest discomfort.

In mitral stenosis the orifice of the mitral valve becomes narrowed which obstructs blood flow from the left ventricle during diastole. Rheumatic fever remains the most common cause of mitral stenosis even though it is not that common in the United States (Hayes 1997). The signs and symptoms of mitral stenosis may not develop until years later. In the elderly population, mitral stenosis is caused by degenerative calcification of the valve annulus and leaflets. Rare but possible causes of mitral stenosis also include systemic lupus and rheumatoid arthritis.

In many cases of mitral valve disease, heart valve replacement has been an option. Heart valve replacement is becoming increasingly common in the United States with approximately 50,000 aortic replacements alone in the US and over 225,000 worldwide (Associated Press 2006). Valve surgery is quite effective in the fact that it alters the course of valvular disease for a period of time. However, there are several potential problems of valve replacement including thromboembolism, infections, immunological reactions, tissue deterioration, and the need for lifelong anticoagulation. Several of these shortcomings could be eliminated if we were to create a tissue engineered valve substitute.

Heart failure will become a challenge as life expectancy continues to rise. In order to address the issues of heart failure and other conditions such as mitral valve disease, the anatomy of the valve, the maintenance of these structures and their importance in overall function must be thoroughly understood.

Present Day Treatment for Valve Dysfunction

Numerous models of heart valve replacements have been designed since the 1960s. Their reliability has been investigated and several improvements have been made in the past four decades (Schoen and Levy 1999). When heart valve substitutes are made, several desirable characteristics must be taken into consideration to ensure mechanical dependability and patient satisfaction. The substitute must be: non-obstructive, and ensure prompt and complete closure. It must be infection resistant, non-thrombogenic, durable for extended intervals, chemically inert and non hemolytic. In addition it must be able to be easily and permanently inserted into the appropriate site, promote appropriate post surgical healing and function in a noise free manner. Current day valve replacements consist of prosthetic valves that are either manufactured of non biological materials or tissue valves that are made with at least a percentage of either human or animal tissue (Schoen and Levy 1999).

There are several advantages to having heart valve replacements, after all, one needs a valve for a properly functioning heart and subsequently a healthy circulatory system. Sometimes substitute heart valve implantation is necessary for survival. Thus one is more often than not willing to accept some of the disadvantages that may arise post operation. However there are many complications associated with heart valve replacement. Prosthesis related problems usually arise within 10 years leading to a further operations or even death in 50-60% of patients with substitute valves (Schoen and Levy 1999). Some of the most common complications that may arise include: thrombo-embolism, thrombosis, anticoagulation-related hemorrhages, prosthetic valve endocarditis, and structural and non structural dysfunction.

Potential Treatments for Valve Dysfunction

Since an ideal valve replacement model has not yet been achieved, there has been extensive research in the development of substitute heart valves particularly tissue engineered valves. Tissue engineering is a technique that allows an anatomically appropriate construct containing cells seeded on a scaffold to be fabricated *in vitro* in a bioreactor and then implanted into a patient. Thus far there have been attempts to use tissue engineering for heart valves in animals such as lambs (Schoen and Levy 1999). These recent efforts have shown great potential, however, the mechanisms of valvular morphogenesis, especially during embryological development, are extremely complex and not fully understood, limiting the ability of tissue engineers to mimic this process *in vitro*.

Valve Cells

It is important to look at the mitral valve on a cellular level because insufficient valve function can result from a disorder of normal cell function. Four types of cells are present in or around the valve including endocardial, cardiac muscle, smooth muscle, and cardiac valvular interstitial cells. The surface of the valve is covered with endocardial cells. The base of the valve has an extensive amount of cardiac muscle cells which are also known as cardiomyocytes. Smooth muscle cells are found in arterioles and venules surrounding the valve. Lastly, valvular interstitial cells form a complex cellular framework spanning the entire valve.

Because of their complexity, and their involvement in the process of valve pathogenesis, the valvular interstitial cells are of most importance to this study.

Knowledge of cardiac valvular interstitial cells' contractile, secretory, and proliferative functions is imperative to understanding how these cells respond to valvular injury and essentially how they repair themselves.

VIC differentiation

Rabkin-Aikawa *et al*. (2004) identified two distinct phenotypes of valvular interstitial cells: a normal or quiescent fibroblast-like cell type characterized by expression of vimentin. The other type expressed α- smooth muscle actin (SMA) and has myofibroblast-like qualities. This second, activated type is found in developing or injured valve tissue which is suggestive of a role in extracellular matrix remodeling. After a damaged valve has undergone remodeling, the myofibroblast-like cell is proposed to revert back to the quiescent fibroblast-like phenotype (Rabkin-Aikawa *et al* 2004). This plasticity is likely to be highly regulated, although little is known about the mechanism.

Stiffness/Mechanical Properties

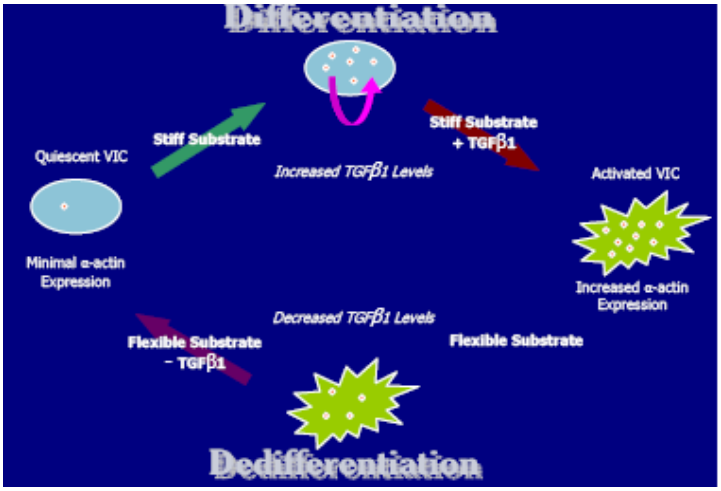
Mechanical stress on the normal fibroblast-like VICs induces changes that can lead to activation marked by α-SMA expression and matrix remodeling as a consequence of the activation (Tomasek *et al.* 2002). Cultured VICs plated on standard plastic flasks can not be expected to exhibit the same morphological features as cells *in vivo* due to the drastic change in surrounding, especially the surface to which they are attached. To provide a more *in vivo*-like setting in an *in vitro* environment, polyacrylamide substrates have been developed by Wang *et al*. (1998) to mimic mechanical properties of *in vitro*

substrate. A benefit to using polyacrylamide is the ability to prepare substrates of varying stiffness by changing the ratio of bis-acrylamide to acrylamide thus allowing examination of the effects of substrate stiffness on cell structure and function. Adding a coating of fibronectin enhances the model in that the cells may interact directly with the surface protein as is the case *in vivo*. This allows *in vitro* manipulation to more closely mimic pathological or physiological situations.

The Model

A model was created by Heather Hinds as shown in Figure 3 to describe the relationship between differentiation and dedifferentiation of porcine mitral valvular interstitial cells *in vitro.* When VICs are "activated" or differentiated from fibroblast to myofibroblast-like cells, an increase in alpha smooth muscle actin expression occurs. This occurs due to an increase in substrate stiffness and/or transforming growth factor- β (TGF- β1) levels. In this model, dedifferentiation which is characterized by a decrease in alpha smooth muscle actin expression, is a result of decreased substrate stiffness and a decrease in TGF-- β1 levels (Hinds 2006). Studies carried out by Hinds showed a decrease in α-SMA expression in valvular interstitial cells that were initially plated on plastic and then on two substrates of various stiffness, 7kPa and 75 kPa. The 7kPa

Figure 3. Model for VIC differentiation and dedifferentiation (Hinds 2006)



substrate was used to mimic a normal healthy valve whereas the 75kPa substrate was used to mimic a stiff pathological heart valve. According to the data obtained from Hinds, α-SMA expression changed from an average of 80% to 50% when plated on the 75 kPa substrates and from 80% on plastic to 45% when plated on the 7 kPa substrates. Although the number of cells seeded on each substrate was initially the same it appeared as though fewer cells were surviving. Our results will determine if the reason for this decrease was actually dedifferentiation as concluded by Hinds or apoptosis of the differentiated cells.

Materials and Methods

MITRAL VALVULAR INTERSTITIAL CELL ISOLATION

Porcine hearts were obtained from Blood Farm in Groton, MA. Within four hours postmortem the heart valves were excised. Two incisions were made in the heart, the first adjacent to the coronary artery and the second perpendicular to the first incision. The heart was opened and the distal leaflet of the mitral valve was trimmed approximately 5 mm from the myocardium. The dissected portions of the mitral valve were placed in a beaker containing phosphate-buffered saline (PBS 150mM sodium chloride and 10mM sodium phosphate pH 7.4). Both surfaces of the leaflet pieces were firmly scraped with a sterile cell scraper to remove endocardial cells and then rinsed in sterile PBS. Leaflets were minced to 0.5cm^2 pieces and digested on a rocking platform for 3 hours in 3 mL digestion media containing 600U/ml collagenase, 1X antibiotic/antimycotic (Sigma) in Dulbecco's Modified Eagle's Medium (DMEM) (Cambrex). Undigested tissue was removed by filtering though a nylon mesh. Medium (see CELL CULTURE MEDIUM AND CONDITIONS) was added to the filtrate and centrifuged for 5 minutes at 1500G. The supernatant was aspirated off and cells were re-suspended in fresh media. Media was changed every 3 days. Cells were plated at 1 x 10^5 cells/100mm dish and passaged once a week.

CELL CULTURE MEDIUM AND CONDITIONS

Medium consisted of (DMEM) supplemented with 2mM L-glutamine, 10% heat inactivated fetal bovine serum, and 1X antibiotic/antimycotic solution (Sigma). Cells were cultured at 37°C in 5% CO_2 and 95% air.

SUBSTRATE PREPARATION

Polyacrylamide preparation and subsequent fibronectin treatment of the substrate surface were all completed according to the Wang *et al* (1998) protocol. The following modifications were made to this protocol: 51mm x 75mm glass slides were activated to promote adhesion of polyacrylamide to the glass surface (Electron Microscopy Science) and 45mm round cover slips were used to spread polyacrylamide on the glass slide surface. Polyacylamide substrates were prepared by varying the ratio of acrylamide and bis-acrylamide to a desired stiffness of 7 kPa and 75 kPa. The final ratio of acrylamide to bis-acrylamide for the 7 kPa construct was 5%/0.025% and for the 75 kPa construct, 8%/0/8% (Engler, Griffin *et al*. 2004). To achieve a desired thickness of 75µm, 84µl of the polyacrylamide solution was placed on the activated glass slide and covered with a 45mm #1 round cover slip. Following polymerization of the polyacrylamide, substrates were rinsed in 50mM HEPES buffer pH 8.5, and the #1 cover slip was removed to expose the polyacrylamide surface. Fibronectin coating of the polyacrylamide substrate was then completed according to the Wang *et al* (1998) protocol. Substrates were UV sterilized for 15 minutes in a sterile hood and placed in a sterile 150mm culture dish (Corning). The substrates were equilibrated overnight at 37°C in 20ml of the appropriate culture media and checked for contamination.

IMMUNOFLUORESCENCE

Mitral vavlular interstitial cells grown on plastic and 7 kPa and 75 kPa substrates were fixed and permeabilized with one drop of 10X fixation permeabilization buffer (BD Biosciences). Cells were stained for alpha smooth muscle actin using a monoclonal

antibody. FITC conjugated anti-alpha smooth actin (Sigma) was diluted 1:1000 in permeabilization and wash buffer (BD Biosciences) and incubated for one hour at 4°C. Substrates were washed overnight in 0.1% Tween-20 in phosphate-buffered saline to reduce background fluorescence due to residual antibody remaining within the polyacrylamide surface. The cells were then incubated with Hoescht 33342 (Molecular Probes) at a dilution of 1:2000 in reagent grade de-ionized H_2O for 5 minutes at room temperature. After the nuclear stain, the substrates were washed three times in 0.1% tween-20 in PBS and then prepared for imaging.

CASPASE ASSAY

The Caspase-8 Assay protocol (Biovision) was followed. SHSY-neuronal cells were plated at a concentration of 3×10^5 to be used as a positive control. They were incubated with 20µM Yankner peptide which induces apoptosis in neuronal cell cultures (Yanker 1990). Normal, healthy valvular interstitial cells grown on plastic were used as a negative control. Three million cells were removed from 7 kPa and 75 kPa substrates trypsin digestion. The caspase assay was performed in triplicate in a 96-well plate. Reaction buffer containing 10 mM DTT was added to each sample. 5µl of the 1mM IETD-AFC substrate was added to each sample and incubated at 37°C for 1-2 hours. Samples were read in a spectrophotometer at 405nm and relative caspase activity was determined by comparison with the negative and positive controls.

Results

Validating the Model System – Alpha Smooth Muscle Actin Expression and Substrate Stiffness

As an indicator of valvular interstitial cell differentiation to a myofibroblast phenotype, expression of alpha smooth muscle actin was examined by staining VICs with FITC conjugated anti-alpha smooth muscle actin. DAPI staining allowed concurrent visualization of nuclei. There was a greater degree of alpha smooth muscle actin expression in 75 kPa substrates than in 7 kPa. This result is consistent with previous data collected by Hinds (2006) and validates the model for VIC differentiation and dedifferentiation by showing a decrease in α-SMA expression after replating, especially on the 7 kPa substrates.

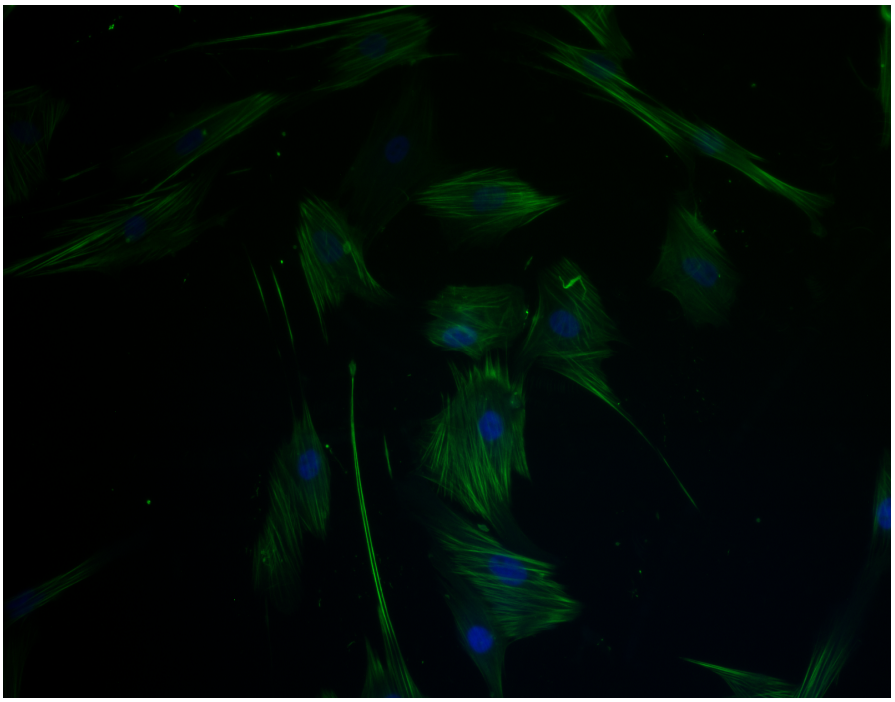


Figure 4. α-SMA expression in VICs grown on 75 kPa substrate

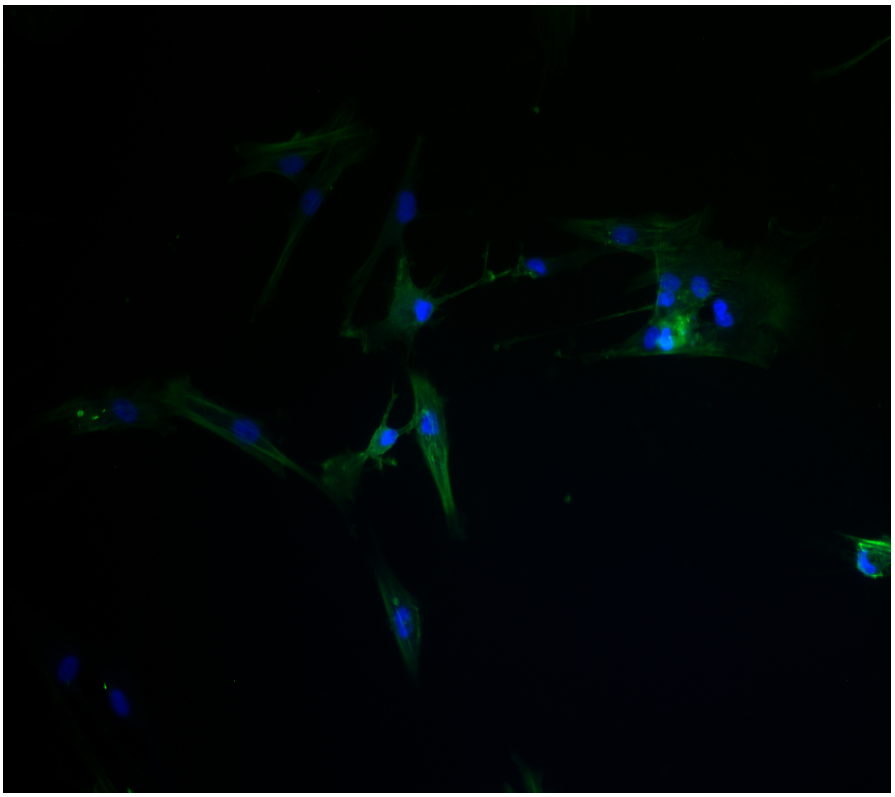


Figure 5. α-SMA expression in VICs grown on 7 kPa substrate

VICs grown for 96 hours on plastic show significant α-SMA expression (Hinds, 2006), while cells transferred to substrates of 7 kPa and 75 kPa for an additional 96 hours showed a relative decrease in α-SMA expression. The relative intensity of α-SMA staining was greater in cells grown on the 75 kPa substrates (Figure 4) than those grown on the 7 kPa (Figure 5). Thus α-SMA expression appears to increase with increasing substrate stiffness.

Assessing Levels of Apoptosis by Caspase Activity

Dedifferentiation may offer an explanation for decreased α-SMA expression in VICs plated on substrates of decreasing stiffness. However, it is also possible that the differentiated cells may be dying off and new less differentiated cells are repopulating the substrates. An assay to determine expression levels of caspases was performed in order to determine whether the VICs were demonstrating increased levels of apoptosis. As shown by the caspase assay, cells grown on both the 7 and 75 kPa substrates expressed caspase levels comparable to those of the positive control with cells grown on 7 kPa showing slightly more activity than those grown on the 75 kPa substrate. Figure 6 displays the results from the assay, which was performed on two separate cell preparations.

	VICs			Apoptotic control
	7 kPa	75 kPa	Plastic	SHSY cells
Run 1	0.158	0.156	-	0.156
Run 2	0.179	0.134	0.104	-
Averages	0.169	0.145	0.104	0.156

Figure 6. The relative caspase activities of VICs plated on three substrates: 7 kPa, 75 kPa and plastic. The numbers represent absorbance at the 405 nm wavelength. Increasing absorbance corresponds to increasing levels of caspase activity. Neuronal SHSY cells treated with 20 μM Yankner peptide served as a positive control for apoptosis.

Discussion

As demonstrated in this paper, as well as by Heather Hinds, levels of alpha smooth muscle actin expression were highest in cells plated on plastic suggesting that nearly all the cells were differentiated myofibroblasts. Micrographs taken showed that after plating VICs on plastic for 72 hours, α-SMA expression was high. When these fully differentiated cells were then plated on softer substrates (7 and 75 kPa), expression of α-SMA per cell appears reduced relative to those on plastic. Additionally, although the numbers of cells initially plated on the constructs was consistent, over time those cultured on the 7 and 75 kPa substrates had fewer cells than expected. The combination of fewer cells and an apparent reduction of expression in α-SMA provided us with two possible hypotheses. First, when grown on a softer substrate, cells show a reduction in α-SMA expression, suggesting they may be reverting toward a less differentiated state, or dedifferentiating. The other possibility is that, since fewer cells were viable, cells expressing α-SMA had undergone apoptosis and the apparent reversal of expression is the result of proliferation by a small subset of the original cells which did not express α-SMA, and remained less or un-differentiated on the 7 and 75 kPa substrates.

To test whether VICs plated on the 7 kPa and 75 kPa substrates were undergoing increased apoptosis, as opposed to dedifferentiation, a caspase assay was performed to examine if apoptosis was the cause of the apparent reduction in cell number and α-SMA expression. Our initial results indicated that compared to a positive control of cells actively undergoing apoptosis, the level of caspase activity in mitral valvular interstitial cells plated on the 7 and 75 kPa substrates was high, even exceeding the caspase activity

of the positive control. This result was confirmed in a second experiment where, again, the levels of caspase activity were greater in the 7 kPa and 75 kPa samples of mitral VICs than in the positive control. Thus it appears that instead of dedifferentiating, old cells which expressed α-SMA are dying off and new, undifferentiated cells are taking their place. Detection of caspases does not rule out dedifferentiation, though it does suggest strongly that the decrease in α-SMA expression is due at least in part to apoptosis of old cells and the formation of new, quiescent cells. If substrate stiffness was a factor in caspase activity, the two different stiffnesses should show different readings. Based on the hypothesis that the 7 kPA substrate induces VICs to dedifferentiate, a higher degree of apoptosis for cells grown on a stiffer substrate would be expected than on the 75 kPa substrate.

If changes in the stiffness of the microenvironment can influence the differentiation of a valvular interstitial cell, perhaps attributes other than stiffness could also promote the reversion of fully differentiated myofibroblast-like VICs to fibroblast-like VICs. Changes in cytokines, such as TGF-β, have been implicated in the differentiation process. Since these cells were cultures in 10% FBS, the levels of TGF- β are undefined, and may vary from lot to lot of serum. Future experiments should address a possible role for growth factors in both the differentiation and the potential dedifferentiation of mitral VICs *in vitro*– however, our results suggest that while substrate stiffness may play a role in VIC activation to the mitral VIC phenotype, simply changing the mechanical properties of the substrate is not sufficient to reverse this process.

In the absence of dedifferentiation, scaffolds for valve replacements would be more difficult to seed and grow than if dedifferentiation were possible. It may still be feasible to seed a scaffold with an individual's own cells, but they must be robust enough to overcome the high incidence of apoptosis and to repopulate the construct. Alternatively, cells could be expanded *in vitro* on substrates that encourage the dedifferentiated phenotype prior to seeding a scaffold. Cellular communication and control over differentiation is not limited to the VICs themselves. Nearby epithelial cells that directly interact with pumping blood and factors in the extracellular matrix likely provide signals to maintain or modify the state of the cell. These factors should also be examined in an *in vitro* model to help elucidate further possible mechanisms of control that could be used in designing scaffolds. Perhaps modulation of differentiation could be achieved by decorating scaffolds with growth factors or other signaling molecules.

Furthermore, it is known that dedifferentiation is not physiologically normal. Cancer is a pathological state where uncontrolled proliferation is often accompanied by dedifferentiation. Reversion to a more primitive developmental state may result in cells no longer able perform the VICs function of matrix remodeling. For the most part, the metabolic energy of a cell is put to use in driving the cell unidirectionally, with deviations resulting in malfunction of the cell. Additional in vitro studies to examine the ability of cells on softer substrates to remodel.

Future studies with more diverse materials will help to develop better models for growing a functional valve from a patient's own cells. New culture conditions could allow laboratory grown mitral VICs to remain in a physiological state, similar to fresh isolates, with minimal further differentiation. More studies into the mechanisms of

cellular communication are required to better understand the cell's relationship with its environment.

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